6 Paths of ERK5 signaling pathway regulate hepatocyte proliferation in rat liver regeneration

Zhanpeng Li1,2, Zhenchao Cheng1,2, Gaiping Wang1, Xioxia Hao2, Lijun Zhang1,2 and Cunshuan Xu1,2*

1 College of Life Sciences, Henan Normal University, Xinxiang, Henan Province, China
2 Key Laboratory for Cell Differentiation Regulation, Xinxiang, Henan Province, China

Received 14 October 2011; revised 26 April 2012

Generally, extra-cellular-signal-regulated kinase 5 (ERK5) signaling pathway regulates many physiological activities, such as cell proliferation and cell differentiation. However, little is known about how ERK5 signaling pathway composed of 15 paths participates in regulating hepatocyte proliferation during liver regeneration (LR). In this study, to explore the influence ERK5 signaling pathway upon hepatocytes at gene transcription level, rat genome 230 2.0 array was used to detect expression changes of 75 related genes in isolated hepatocytes from rat regenerating liver. Bioinformatics and systems biology methods were applied to analyze the precise role of ERK5 signaling pathway in regulating hepatocyte proliferation during LR. Results showed that 62 genes were contained in the array and 22 genes were significantly changed. It was found that 6 paths were related to hepatocyte proliferation during rat LR. Among them, paths 3, 6 and 13 of ERK5 signaling pathway modulated cell cycle progression by decreasing the negative influence on ERK5 and paths 3, 4, 8 and 9 by reinforcing the positive influence on ERK5. In summary, the study shows that 22 genes and 6 paths of ERK5 signaling pathway participate in regulating proliferation of hepatocytes in rat LR.

Keywords: ERK5 signaling pathway; Rat liver regeneration; Hepatocyte proliferation; Rat genome 230 2.0 array; Gene expression profiles

The liver has important physiological functions and a strong ability to regenerate1. After injury or partial hepatectomy (PH), it can rapidly recover its original size and weight through cell proliferation and growth, which is called liver regeneration (LR)2,3. Rat LR involves many physiological activities, including activation of growth factors and cytokines, synthesis and activation of transcription factors and enzymes and synthesis and reconstruction of extra-cellular matrix4. PH is a fundamental parameter of liver response to damage, thus is widely applied in understanding the mechanisms involved in LR5. It is well-known that hepatocytes account for 70~80% of hepatic mass and 65% of total hepatic cells and have physiological functions, including material storage, substance metabolism, bile secretion, oxidation protection, detoxification, biotransformation, immune response, etc6. Normally, only 0.0012~0.01% hepatocytes of adult rat liver are involved in mitosis5. But a large amount of the residual hepatocytes rapidly enter into cell cycle to compensate for the lost liver tissue after PH, which is regulated by many signaling pathways, including extra-cellular signal regulated kinase 5 (ERK5) signaling pathway7.

Generally, ERK5 signaling pathway is a complex network composed of many paths, which plays a vital role in cell survival, cell proliferation and cell differentiation8. ERK5 signaling pathway can be classified into 15 paths based on the ERK5 activating factors. Among them, G-protein activates ERK5 by activating MEK5 (Paths 1-2)9. Extra-cellular stimulations promote phosphorylation of cascade (MEKK2/3→MEK5→ERK5) through activating c-Src (Path 3)10. Growth factors or mitogens (EGF/G-CSF) combine with growth factor receptors, then the latter activate c-Src, WNK1 or Ras and promote phosphorylation of cascade (Paths 4-6)11. TSAd activates ERK5 signaling pathway by phosphorylation of cascade (Path 7). Brain-derived neurotrophic factors (BDNF) and neurotrophic factors (eg. NT3 or NT4) combine with the Grb2/Sos complex and promote

*Corresponding author
Tel.: +86-0373-3326001
Fax: +86-0373-3326524
E-mail: xucs@x263.net, cellkeylab@126.com

Abbreviations: BDNF, brain-derived neurotrophic factors; ERK5, extra-cellular signal regulated kinase 5; LR, liver regeneration; PH, partial hepatectomy.
phosphorylation of cascade through activating Ras protein (Paths 8-13)\textsuperscript{12,13}. Cytokines activate ERK5 by inducing the phosphorylation of Gab1 and Shp-2 (Path 14)\textsuperscript{14}. Nerve growth factors and receptor tyrosine kinases could also activate ERK5 directly (Path 15). However, it is unclear which paths participate in the regulation of rat LR, especially hepatocyte proliferation during rat LR.

In this study, rat genome 230 2.0 array has been used to detect expression changes of genes related to ERK5 signaling pathway in isolated hepatocytes during rat LR. Bioinformatics and systems biology methods have been used to analyze the regulatory role of these paths in hepatocyte proliferation, in order to understand the relevance of the ERK5 signaling pathway to rat LR at the gene transcriptional level.

**Materials and Methods**

**Isolation and identification of hepatocytes of rat regenerating liver**

A total of 114 healthy Sprague-Dawley (SD) rats, 12-weeks old and each weighting 230 ± 20 g were provided by the Animal Center of Henan Normal University. They were randomly divided into 9 2/3 hepatectomy (PH) groups, 9 sham operation (SO) groups and 1 control group. Each group contained 6 rats. PH was performed following the method of Higgins and Anderson\textsuperscript{2} and then hepatocytes were taken from regenerating liver at 0, 2, 6, 12, 24, 30, 36, 72, 120 and 168 h after PH, respectively. Briefly, the rats were anesthetized by ether and sterilized with 75% alcohol before opening the abdominal cavity.

After liver was exposed, vena cava below and above the liver was ligated and a tube was inserted into liver portal vein. Then, liver was perfused with calcium-free perfusate at 37°C till the liver surface turned slightly yellow, then with 15 ml of 0.05% collagenase IV solution to disperse liver cells. The cells were collected and washed with PBS at 37°C for three-times and the cell concentration was adjusted to 1 × 10^6 cells/ml. 6 ml of mixed cell suspension was spread on to the surface of 4 ml 60% percoll in a 10 ml tube for a single 200 g centrifugation at 4°C for 5 min. Pellet was harvested and hepatocytes were obtained. Finally, the hepatocytes marker proteins albumin (ALB) and glucose-6-phosphatase (G6P) were used to identify hepatocytes by immunocytochemistry\textsuperscript{15,16}. All the handling procedures were carried out in compliance with the current Animal Protection Law of China.

**Rat genome 230 2.0 microarray detection and data analysis**

The total RNA of 1 × 10^6 hepatocytes was extracted according to the manual of Trizol reagent (Invitrogen Corporation, Carlsbad, California, USA) and purified following the RNeasy mini protocol (Qiagen, Inc, Valencia, CA, USA). The quality of total RNA was assessed by optical density measurement at 260/280nm and agarose electrophoresis (180 V, 0.5 h). It was regarded as qualified sample, when the ratio of 28S RNA to 18S RNA was 2:1. The first strand of cDNA was synthesized using T7-oligo dT(24) (Keeck Foundation, New Haven, CT), SuperScript II RT (Invitrogen Corporation, Carlsbad, CA) and 5 µg of total RNA. The second strand was synthesized using the Affymetrix cDNA single-stranded cDNA synthesis kit. The cDNA product was purified following the cDNA purification protocol. The 12 µl purified cDNA and the reagents in the GeneChip In Vitro Transcript Labeling Kit (ENZO Biochemical, New York, USA) were used to synthesize biotin-labeled cRNA. The labeled cRNA was purified using the RNeasy Mini Kit columns (Qiagen, Valencia, CA). The concentration, purity and quality of cDNA and cRNA's were assessed based on the detection method of total RNA as described above. 15 µl cRNA (1 µg/µl) was incubated with 6 µl 5x fragmentation buffer and 9 µl RNase-free water for 35 min at 94°C and digested into 35-200 bp cRNA fragments.

The pre-hybridized rat genome 230 2.0 microarray was put into a hybridization buffer which was prepared following the Affymetrix protocol and hybridized in a rotating chamber (60 rpm for 16 h at 45°C). The hybridized arrays were washed by wash buffer to remove the hybridization buffer and stained in GeneChip® Fluidics Station 450 (Affymetrix Inc., Santa Clara, USA). Then the arrays were scanned and imaged with a GeneChip® Scanner 3000 (Affymetrix). The images showing gene expression abundance were converted into signal values, signal detection values (P, A, M) and experiment/control values (Ri) using Affymetrix GCOS 2.0.

The data of each array was initially normalized by scaling all signals to a target intensity of 200. When P value was < 0.05, it represented that the gene was present (P), while P<0.065 indicated
marginal (M) and P>0.065 as absent (A). On the other hand, the normalized signal values in PH to that in control were used to calculate the relative values, i.e., ratio values of gene expression abundance. The ratio values of ≥3, ≤0.33 and 0.33-2.99 indicated that gene expression was significantly up-regulated, significantly down-regulated and biologically insignificant respectively. To minimize the technical error from array analysis, each sample was tested at least for three times using rat genome 230 2.0 microarray and the average value of three assessments was calculated as the corrective value. Finally, these values were analyzed with GeneMath, GeneSpring, Microsoft Excel Software and Pathway Studio 7.0.

**Quantitative real-time RT-PCR**

To verify the chip data, six genes were selected for quantitative real-time polymerase chain reaction (RT-PCR) analysis. Primer sequences were designed by Primer Express 2.0 software according to GenBank number of six target genes. For *alb* (NM_134326), *g6pc* (NM_013098), *apo*e (NM_138828), *jun* (NM_021835), *trim24* (NM_001044266) and *myc* (NM_012603) and synthesized by Shanghai Generay Biotech Co. Ltd. The gene-specific primers were as follows: forward primer 5'-AC AAGAGCCCGAAGAAACGA-3' and reverse primer 5'-TCTTGGAACCTCTGCAAAAT-3' for *alb*, forward primer 5'-CTCAAGGAACGCC TATG-3' and reverse primer 5'-ACGGAGCTGT TTGCTTAAT-3' for *g6pc*, forward primer 5'-CCTGAACCGCTTCTGGGATT-3' and reverse primer 5'-GCTCTTCCTGGACCTGGTCA-3' for *apo*e, forward primer 5'-TGCAAAGA TGGAAACGACCTT-3' and reverse primer 5'-CTCAGGAACGCCTT-3' for *jun*, forward primer 5'-GAAGAGCCCGAAGAAACGA-3' and reverse primer 5'-CTGCTTCTGGAACCTTGGTCA-3' for *myc*.

Prior to RT, contaminating genomic DNA was removed by Dnase I (Promega). Total RNA (2 μg) was reverse-transcribed using random primers and reverse transcription kit (Promega). First-strand cDNA samples were subjected to quantitative PCR amplification by using SYBR® Green I on the Rotor-Gene 3000A (Corbett Robotics, Brisbane, Australia). All of the PCR cycling conditions were modified to 95°C for 2 min, followed by 40 cycles of 95°C for 15s, 60°C for 15s, and 72°C for 30s. Every sample was analyzed in triplicate. Standard curves were generated from five repeated ten-fold serial dilutions of cDNA and the copy numbers of target genes in every milliliter of the sample were calculated according to their corresponding standard curves.

**Identification of genes associated with ERK5 signaling pathway in hepatocytes during liver regeneration**

To identify rat hepatocytes associated with ERK5 signaling pathway, the phrase "ERK5 signaling pathway" was input into NCBI (www.ncbi.nlm.nih.gov) and RGD (rgd.mcw.edu) to collect genes related to ERK5 signaling pathway in rat, mouse and human. Then, these genes were further obtained according to the biological pathway maps at the databases, including BIOCARATA (www.biocarta.com) and KEGG (www.genome.jp/kegg/pathway.html) and GENMAPP (www.genmapp.org) etc. and reconfirmed with related reports. To confirm the genes related to LR, genes were considered as the significantly up-regulated when expression changes were ≥3 as compared with control, as significantly down-regulated when expression changes were ≤0.33 and as significantly up/down-regulated when gene's expression changes were ≥3 at one time point and ≤0.33 at another time point in rat LR.

**Synergy analysis of genes related to ERK5 signaling pathway**

According to the classifications of physiological activities supported from GO (www.geneontology.org), gene expression abundance detected by rat genome 230 2.0 chip in rat model of NASH, and functions and interactions of genes curated from ResnetCore1.2 database which is built-in Pathway Studio 7.0 software, the multi-variate statistical-supporting mathematical model was applied to measure the synergy role of related genes with time series analysis and correlation analysis:

\[
E_t = \frac{\sum_{i=1}^{n} \sum_{k=1}^{n} [X_i(t) + X_k(t)] * r_{ik}}{n(n+1)}
\]

where \( E \) (equation) describes the synergistic value of genes participating in a physiological process and \( t \) represents a certain time point. \( r_{ik} \) represents correlation coefficient of genes \( i \) and \( k \).
expression abundance of genes $i$ and $k$ at a certain time point and $n$ shows the total number of genes in a certain physiological process. The synergistic value of genes participating in a physiological activity at a certain time point in PH group ($E_t$) was significantly greater than that in control group ($E_c$), suggesting that the physiological activity in PH at this time point was more elevated than in control. $E_t$ less than $E_c$ indicated that the physiological activity at this time point was weaker than in control and $E_t$ having no remarkable difference to $E_c$, it indicated that the physiological activities in both PH and control group were comparable.

**Results**

Expression profiles of genes related to ERK5 signaling pathway in hepatocytes during LR

The data from NCBI, RGD, etc. and biological pathway maps in QIAGEN, KEGG and GENMAPP etc. showed that 75 genes were involved in ERK5 signaling pathway. Of these, 62 genes were in rat genome 230 2.0 array. The detection of expression trends of 6 genes, including *alb*, *g6pc*, *apoE*, *jun*, *trim24* and *myc* by the RT-PCR and rat genome 230 2.0 array methods was generally consistent, suggesting that the array check results were reliable (Fig. 1). Moreover, the array analysis showed that 22 genes associated with ERK5 signaling pathway were significantly changed in the hepatocytes of regenerating liver, which possibly belonged to rat LR related genes. The genes that activated ERK5 signaling pathway included 7 up-regulated genes (*Src*, *Pdgfra*, *Pdgfrb*, *Bdnf*, *Sh2b2*, *Shc2*, *Nf3*) and 2 down-regulated genes (*Egf*, *Mras*), and genes that inhibited ERK5 signaling pathway included 12 up-regulated genes (*Gna15*, *Gnat1*, *Gnat2*, *Gnaz*, *Gnb3*, *Gnb5*, *Gng11*, *Gng2*, *Gng3*, *Gng8*, *Ngf*, *Ntrk1*) and 1 down-regulated gene (*Gna14*) (Table 1, Fig. 2).

Physiological activities governed by ERK5 signaling pathway in hepatocytes during LR

The gene synergy and the physiological activities in ERK5 signaling pathway of hepatocytes during LR analyzed using the mathematical model ($E_t$) showed that 6 paths of the pathway were related to hepatocyte proliferation during rat LR. In early phase of LR, $E_t$ of genes related to path 13 which inhibited the activity of ERK5$^{12}$ was lower and the genes related to path 3 which promoted ERK5$^{10}$ was higher in PH than in control. In middle phase, $E_t$ of genes related to paths 4, 8 and 9 which activated ERK5$^{13}$ was higher and of the genes related to paths 3 and 6 which inhibited the activity of ERK5$^{10,11}$ was lower than in control. In late phase, none of these paths were found to be involved in LR (Fig. 3).

---

**Fig. 1**—Comparison of results relative to the level of control sample at 10 time points after PH [Comparison of results obtained with microarray and RT-PCR analysis for changes in mRNA levels in hepatocytes during LR at 10 time points after PH relative to the level of control sample in microarray analysis (solid lines) and RT-PCR analysis (dotted lines) are shown for the following genes related to hepatocyte: *alb*, *g6pc*, *apoE*, *jun*, *trim24* and *myc*]
### Pathways of ERK5 Signaling Pathway Regulate Hepatocyte Proliferation

**Pathways 1-2: GPCR → MEK5 → ERK5**

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Recovery time (h) after partial hepatectomy (PH)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2</strong> Ras</td>
<td>0.31 0.51 1.25 0.48 0.82 0.37 1.28 0.72 0.68</td>
</tr>
<tr>
<td>/Gna14</td>
<td>1.00 2.85 3.09 3.66 3.48 1.55 5.96 2.00 4.21</td>
</tr>
<tr>
<td>/Gna15</td>
<td>4.17 3.95 6.12 5.96 3.12 2.03 2.82 3.04 1.26</td>
</tr>
<tr>
<td>/Gnat1</td>
<td>1.24 0.98 5.30 5.64 1.58 1.39 1.54 1.33 2.33</td>
</tr>
<tr>
<td>/Gnat2</td>
<td>3.90 3.04 2.85 3.78 1.92 5.79 5.05 3.71 4.91</td>
</tr>
</tbody>
</table>

**Pathway 3: Stress → Src → ERK5**

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Recovery time (h) after partial hepatectomy (PH)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>7</strong> Src</td>
<td>1.33 1.77 2.40 2.06 3.11 1.89 4.78 2.04 1.48</td>
</tr>
</tbody>
</table>

**Pathways 4-6: Mitogens → MEK5 → ERK5**

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Recovery time (h) after partial hepatectomy (PH)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>9</strong> Src</td>
<td>1.33 1.77 2.40 2.06 3.11 1.89 4.78 2.04 1.48</td>
</tr>
<tr>
<td><strong>10</strong> Mitogens</td>
<td>0.71 0.51 0.18 0.96 0.29 0.38 0.65 1.09 1.28</td>
</tr>
<tr>
<td><strong>12</strong> GFR</td>
<td>2.39 3.70 5.12 6.44 2.30 2.15 32.45 8.41 0.85</td>
</tr>
<tr>
<td><strong>10</strong> GFR</td>
<td>1.10 0.97 0.84 2.02 1.07 1.32 5.41 1.48 1.33</td>
</tr>
<tr>
<td><strong>13</strong> Ras</td>
<td>0.32 0.88 0.76 0.71 0.66 0.50 0.80 0.66 0.63</td>
</tr>
</tbody>
</table>

**Pathways 8-9: BDNF → Ras → ERK5**

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Recovery time (h) after partial hepatectomy (PH)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>17</strong> BDNF</td>
<td>3.22 3.41 4.32 6.46 3.46 1.92 3.08 3.34 3.99</td>
</tr>
<tr>
<td><strong>17</strong> rAPS</td>
<td>4.27 2.76 1.94 2.03 2.23 1.84 3.85 2.98 3.41</td>
</tr>
<tr>
<td><strong>17</strong> Shc</td>
<td>1.87 3.14 4.55 5.08 4.19 4.79 6.84 2.05 1.42</td>
</tr>
<tr>
<td><strong>17</strong> Ras</td>
<td>0.32 0.88 0.76 0.71 0.66 0.50 0.80 0.66 0.63</td>
</tr>
</tbody>
</table>

**Pathways 10-11: NT3 → Ras → ERK5**

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Recovery time (h) after partial hepatectomy (PH)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>18</strong> NT3</td>
<td>3.34 3.92 2.60 2.05 0.55 1.09 3.79 1.80 2.84</td>
</tr>
<tr>
<td><strong>18</strong> rAPS</td>
<td>4.27 2.76 1.94 2.03 2.23 1.84 3.85 2.98 3.41</td>
</tr>
<tr>
<td><strong>18</strong> Shc</td>
<td>1.87 3.14 4.55 5.08 4.19 4.79 6.84 2.05 1.42</td>
</tr>
<tr>
<td><strong>18</strong> Ras</td>
<td>0.32 0.88 0.76 0.71 0.66 0.50 0.80 0.66 0.63</td>
</tr>
</tbody>
</table>

**Pathways 12-13: NT4 → Ras → ERK5**

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Recovery time (h) after partial hepatectomy (PH)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>19</strong> Shc</td>
<td>1.87 3.14 4.55 5.08 4.19 4.79 6.84 2.05 1.42</td>
</tr>
</tbody>
</table>

**Pathway 15: NGF/TRKA → ERK5**

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Recovery time (h) after partial hepatectomy (PH)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>20</strong> NGF</td>
<td>4.81 1.11 2.62 0.99 1.26 2.17 2.38 3.42 4.68</td>
</tr>
<tr>
<td><strong>20</strong> TRKA</td>
<td>1.30 3.76 2.52 2.37 2.42 1.73 3.45 2.26 2.13</td>
</tr>
</tbody>
</table>

The values in red represent the expression abundance of up-regulated genes, those in green that of the down-regulated and those in black that of the insignificantly changed.
Discussion

Previous study has shown that LR involves a number of physiological activities, which is regulated by many signaling pathways, including ERK5 signaling pathway. Generally, ERK5 signaling pathway regulates a variety of physiological activities, including cell proliferation, cell differentiation and cell survival. In our study, we found that 22 genes and 6 paths of ERK5 signaling pathway regulated proliferation of hepatocytes in the whole process of LR.

During LR, the first cell cycle of hepatocytes is divided into G0 phase (0-6 h after PH), G1 phase (6-12 h after PH), S phase (12-24 h after PH), G2 phase (24-30 h after PH) and M phase (30-36 h after PH) and the second occurs during 36-66 h after PH. Cell differentiation and tissue reconstruction occur during 120-168 h after PH. In this study, the expression profiles of genes related to the 15 paths of ERK5 signaling pathway were analyzed using \[ E_t \]. We found that paths 3 and 13 in PH group exhibited the earliest remarkable changes (\[ E_t \] of genes related to path 3 was higher and the genes related to path 13 was lower in PH than in control) at 12 h after PH
compared with those in the control, suggesting their possible involvement in regulating G1/S progression of hepatocyte proliferation. This result was consistent with the previous study. It is reported that extra-cellular stimulations could regulate the secretion of TNF by activating ERK5, thus promote liver cell proliferation. E, of genes related to path 13 restraining hepatocyte proliferation was lower in PH than in control, which confirmed the stimulatory role of path 13 in hepatocyte proliferation at the transcriptional level.

The proliferation of hepatocytes is reported to occur mainly within 72 h after PH. Systems biology methods indicated that paths 3 and 6 of ERK5 signaling pathway modulated hepatocyte proliferation by decreasing the negative influence on ERK5 and paths 4, 8 and 9 regulated hepatocyte proliferation through reinforcing the positive influence on ERK5 during 24-72 h after PH, which suggested that ERK5 signaling pathway played a critical role in promoting hepatocyte proliferation during rat LR. It is also found that the combination of EGF and their receptors induce ERK5 phosphorylation through activating c-Src. Moreover, the phosphorylated ERK5 could promote hepatocyte proliferation.

The previous researches have shown that the neurotrophic factors (BDNF, NTs) could activate ERK5 and promote cell proliferation and cell differentiation through Rap1-MEKK2 signaling cascade. It is also reported that ERK5 signaling pathway is of significant importance in G2/M progression. Taken together, the above results have demonstrated that EGF, c-Src, BDNF and NTs are involved in promoting hepatocytes proliferation during 24-72 h after PH of rat LR. Moreover, we also found that EGF-activated path 4 and BDNF-mediated paths 8 and 9 were novel and important to the process of rat LR, which were not reported previously.

In conclusion, this study analyzed gene expression profiles of ERK5 signaling pathway of hepatocytes using rat genome 230 2.0 array and the regulation of rat LR by ERK5 signaling pathway using systems biology methods. It was found that many kinds of stimulating factors could activate ERK5, and 6 paths of ERK5 signaling pathway regulated hepatocyte proliferation in rat LR. Further studies are in progress to explore the role of She2 (path 8-9) and Egf (path 4) in regulation of hepatocyte proliferation in rat LR using RNA interference and chemical or physical stimulation in vivo and in vitro.

Acknowledgements

The work was supported by the National Basic Research 973 Pre-research Program of China (Grant no. 2010CB534905), the Henan Scientific and Technological Research Projects (Grant no. 122300410339), the Doctoral Scientific Research Start-up Foundation of Henan Normal University (Grant no. 11128), and the National Undergraduate Innovative Experiment Program (Grant No. 101047611).

References

2. Higgins G M & Anderson R M (1931) Arch Pathol 12, 186-202