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Expression of Granulocyte Colony-Stimulating Factor Receptor in Rat
Probe Development, Detection of Anatomic Localization and Regulation of Receptor Expression

Inaugural-Dissertation
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Presented by

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Introduction

I. Background

Granulocyte colony-stimulating factor (G-CSF) is in broad clinical use. As a major hematopoietic growth factor, G-CSF activates a receptor of the hematopoietic receptor superfamily, the G-CSF receptor (G-CSFR). Although the biological and clinical effects of G-CSF are relatively well studied, little is known about the G-CSF receptor expression in nonhematopoietic tissues. However, such knowledge is essential to determine which cells may respond to the administration of G-CSF.

G-CSFR on Neutrophils

G-CSF is the principal growth factor regulating the maturation, proliferation and differentiation of the precursor cells of neutrophilic granulocytes all of which express G-SCFR. In addition, some hematopoietic stem and progenitor cells express G-CSFR in a stage-specific manner (McKinstry et al., 1997).

G-CSF is routinely used for stem cell mobilization (Thomas et al., 2002). Since G-CSF administration reduces the duration of neutropenia, enhance hematopoietic reconstitution and increase the progenitor cell yields, it has been used to treat idiopathic and iatrogenic neutropenia, as well as postoperative and post-traumatic patients at risk of sepsis, even in immunosuppression related neutropenia in organ graft recipients (Schmaldienst et al., 2000; Turgeon et al., 2000).

G-CSFR on Monocytes and Lymphocytes

G-CSF effects as a growth factor in hematopoiesis exceed by far the role of a neutrophil recruitment signal, since it also induces the proliferation and mobilization of other cells of leukocyte lineage. G-CSF treatment leads to the rapid increase of bone marrow cellularity and is also involved in the release of cells from BM into the circulation. Furthermore, monocytes and macrophages were also identified to express G-CSFR (Boneberg et al., 2000), mediating a suppressing effect on inflammatory responses by G-CSF. G-CSF attenuates inflammatory responses directly by reducing

proinflammatory cytokine formation (interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), IL-12 and IL-1β) in activated monocytes and macrophages (Attalah et al., 2002). G-CSF also has an indirect modulatory effect by increasing the number of peripheral lymphocytes, and attenuating the release of interferon-gamma (IFN- γ). These effects suggest a shift towards the *Th2*-type response of the specific immune system favoring humoral defense (Boneberg, 2000) and stimulation of host immunity. G-CSF treatment has shown encouraging results in a broad variety of animal infection models in terms of improved survival, reduced bacterial load, enhanced neutrophil action and immigration into infected sites (reviewed by Hareng, 2002). In human infections, treatment with G-CSF was associated with a reduced risk for postoperative infections, accelerated eradication of pathogens from the infected site and reduced infection related mortality (Lyman, 2002).

G-CSFR on Endothelial Cells

In addition to hematopoietic cells, G-CSFR has been detected on vascular endothelial cells, suggesting a role in endothelial cell growth and migration. Bussolino reported that G-CSF could induce endothelial cells to express G-CSFR and induce proliferation and migration leading to angiogenesis in vitro and in vivo (Bussolino et al., 1991). Dogs treated with G-CSF have increased endothelialization of synthetic vascular grafts. This was attributed to increased number of circulating bone marrow progenitor cells (Shi et al., 2002). Moreover, Kocher demonstrated that G-CSF mobilized bone marrow from adult humans contains endothelial precursors with phenotypic and functional characteristics of embryonic hemangioblast. Thus, it can be used to directly induce blood vessel formation in the infarct-bed and proliferation of preexisting vessels after experimental myocardial infarction (Kocher et al., 2001). Likewise, Norol reported in nonhuman primates submitted to coronary artery ligation, that mobilization of stem cells by G-CSF could promote angiogenesis in the infarcted myocardium, without detectable myocardial repair (Norol et al., 2003). A less favorable property of G-CSF is its potential to promote tumor growth, at least in part, by stimulating angiogenesis in which bone marrow-derived endothelial progenitor cells play a role (Natori et al., 2002).

Taken together, various actions of G-CSF are triggered by binding to its receptor, thereby activating intracellular signaling cascades. However, the broad clinical use of G-CSF stands in strong contrast to the relatively poor understanding of the pattern of its receptor expression. Moreover, evaluation of G-CSFR expression may be of importance for understanding the reported effect on liver regeneration. This project is dedicated to determine the patterns of G-CSFR expression in parenchymal organs of rats under different experimental conditions.

II. Aim

As a step toward better understanding the effects of G-CSF, especially the effect on liver, the study was designed to investigate the expression of the receptor for G-CSF on the mRNA and protein level in rats. The experimental aims were summarized as follows:

- To develop a probe for the detection of G-CSFR transcription
- To determine the anatomic location of G-CSFR in nonhematopoietic tissues of adult rats and thereby elucidate which cells, especially in the liver, express the G-CSFR, and therefore may respond to the administration of G-CSF.
- To study the regulation of G-CSFR expression under different experimental conditions

Materials and Methods

Investigating the expression and regulation of the expression of G-CSFR requires a multistep approach. Reagents have to be generated that allow the demonstration of G-CSFR mRNA and protein expression. The cellular distribution of this receptor must be pinpointed and kinetic changes of the receptor expression must be demonstrated.

Strategy

I. Develop a probe for G-CSFR

1. Design primers for G-CSFR;
2. DNA and RNA extraction;
3. Amplify G-CSFR from DNA by polymerase cycle reactions (PCR) and from RNA by Reverse transcription polymerase cycle reactions (RT-PCR);
4. Sequence;
    i. Sequence PCR products directly
    ii. Clone PCR products
    iii. Sequence insert of plasmid
5. Design, synthesize and label a single stranded probe.
    i. Design probe
    ii. Generate probe by asymmetric PCR
    iii. Label aminoallyl-dUTP incorporated probe

II. Investigate the G-CSFR mRNA distribution in normal rats.

1. Isolate RNA and amplify G-CSFR by RT-PCR;
    i. Isolate total RNA
    ii. Perform RT-PCR
2. Detect the G-CSFR mRNA expression by Northern blot analysis;
3. Detect the anatomic localization of G-CSFR mRNA by Fluorescence *in situ* hybridization (FISH);
4. Determine the anatomic localization of G-CSFR protein by immunohisto-chemistry (IHC).

III. Observe the G-CSFR expression in nonhematopoietic organs of G-CSF treated and/or 90% hepatectomized (PH) rats

1. Examine the histological changes after G-CSF treatment and/or 90%PH;
2. Determine the protein expression of G-CSFR by IHC after G-CSF treatment and/or 90% PH.

Materials

Reagents and equipments for this project were listed in table 1and 2.

Methods

I. Probe development for G-CSFR.

I.1 Primer design for rat G-CSFR
I.2 DNA and RNA extraction
I.3 Amplification of G-CSFR from genomic DNA by PCR and from and RNA by RT-PCR
I.4 Sequencing
I.5 Designing, synthesizing and labeling single stranded probe

I.1 Primer design for rat G-CSFR

In order to detect G-CSFR expression, a specific probe is required to screen the mRNA distribution. Since there is no commercially probe available and the full-length sequence is unavailable in the Genbank database, a specific probe had to be designed. The known sequences of the human and mouse G-CSFR genes were taken fom the Genbank database (http://www.ncbi.nlm.nih.gov/). Alignment was done using the NBRF program ALIGN. Conserved regions were pinpointed and primers were designed according to the guidelines for primer design (table 3). The Tm

of pairs of primers was estimated using the Primer Express software (PE Applied Biosystems) (list of primers see table 4).
All primers were generated by MWG Biotech (Ebersberg, Germany).

I.2 DNA and RNA extraction

I.2.1 Animals and Samples

Inbred male Lewis rats (Charels River Wiga GmbH, Germany) were used in the experiment. Animals were employed when they reached a body weight of 250~350g. They were housed under standard animal care conditions and were fed with standard rat chow before and after the operation. Animal housing and procedures were carried out according to the German Animal Welfare Legislation.
After sacrifice, samples of heart, lung, spleen, kidney, liver, small bowel, pancreas and BM were snap frozen and stored in liquid nitrogen. Additional tissue samples were fixed in 10% buffered formalin. Embedding in paraffin was done using a Tissue Processor TPC15 (Medite Inc. Germany). Paraffin blocks were stored at room temperature for routine histologic processing and immunohistochemical staining.

I.2.2 DNA and RNA purification

Genomic DNA was extracted from normal rat liver tissue, obtained during liver resection. DNA was extracted by tissue homogenization, proteinase K digestion, phenol extraction and ethanol precipitation (for protocol see table 5). RNA was extracted from liver of normal rat using a QIAGEN RNeasy RNA purification kit (table 6)
Purified products were separated electrophoretically on 2% agarose gels and stained with ethidium bromide. The results were documented by photographs under UV-light (table 7).

I.3 Amplification of G-CSFR from DNA by PCR and from RNA by RT-PCR

Genomic DNA and RNA were used to generate PCR fragments of the G-CSFR gene and its transcript. Comparing them revealed the intron and exon structure of the gene.

I.3.1 Touch-down PCR

Touch-down PCR was performed to facilitate the amplification of the specific G-CSFR gene sequence according to the following protocol (table 8). Since Touch-down PCR uses a cycling program with varying annealing temperatures, it enhances the specificity of the initial primer-template duplex formation and hence the specificity of the final PCR product. Hotstar Taq DNA Polymerase was used in the PCR reaction. It was activated by a 10-15 minute incubation at 95ºC which can minimize nonspecific amplification products, primer-dimers and background.
Amplified bands were electrophoresed on 2% agarose gels (table 7).

I.3.2 RT-PCR

Two step RT-PCR was conducted on total RNA. (table 9) M-MuLV reverse transcriptase (MBI, Fermentas, Germany) was used to reverse transcribe the RNA to cDNA, followed by Hotstar PCR.

I.3.3 Purification of PCR products

PCR products were purified using the QIAquick PCR purification kit (table 10). In this process additional primers, nucleotides, polymerases, and salts after DNA synthesis were removed.

I.4 Sequencing

I.4.1 Direct sequencing of PCR products

Direct sequencing of PCR products was achieved by *cycle sequencing*. Sequencing was performed using an ABI PRISM 310 sequencing Analyzer system (table 11).

I.4.2 Cloning

Double-stranded G-CSFR DNA was cloned into the plasmid pCR4-TOPO. This plasmid was transformed into *Escherichia coli* strain DH5α (table 12). The colonies were selected and analyzed by PCR and restriction analysis with *EcoR I*.
Plasmids were purified according to the protocols of the QIAGEN plasmid purification kit. The procedure included alkaline lysis of the bacteria, followed by binding of the plasmid DNA to an Anion-Exchange Resin under appropriate low-salt and pH conditions. Plasmid DNA was eluted in a high-salt buffer and then concentrated and desalted by isopropanol precipitation (table 13).

I.4.3 Sequencing of plasmid DNA

Plasmid DNA was sequenced using an ABI PRISM BigDye terminator cycle sequencing kit employing M13 primers (forward: 5'-GTA AAA CGA CGG CCA G-3'; reverse: 5'-CAG GAA ACA GCT ATG AC-3') in an ABI PRISM 377 automated sequencing system. Analysis of the results were performed with the ABI PRISM GS STR POP4, GeneScan Analysis Software. Alignment scores were generated using the NBRF program ALIGN using the MD data matrix with a bias of +6 and a gap penalty of 6. Sequencing results were confirmed by analyzing samples of five independent rats.

I.5 Probe generation and labeling

I.5.1 Probe design

The design of the probe was performed using a set of guidelines

Probe design and optimization criteria:

- Probe length of 200~300bp
- specificity of the selected sequence documented by using the BLAST
- Considering genomic information to pick an intron spacing region
- Normalizing GC content / T_m across the whole probe to ensure identical hybridization conditions for all parts of the probes

- Avoiding secondary structures, such as hairpins and palindromes, for minimal self-hybridization

Asymmetric PCR was employed to generate a single-stranded DNA (ssDNA) probe. Two different methods were compared to obtain labeled probes.

I.5.2 Asymmetric PCR

Plasmid DNA was used as a template. Asymmetric PCR was performed using the forward primer at 0.5µM,and the reverse primer at 0.005 µM.

I.5.2.1 Incorporation of fluorescin-12-dUTP

dTTP was partially replaced by Fluorescin-12-dUTP at a ratio of 3:1 in the PCR reaction mix (table 15). The PCR products were purified using the QIAquick PCR purification kit (table 10).

I.5.2.2 Incorporation of aminoallyl-dUTP

dTTP was partially replaced by aminoallyl-dUTP at ratio of 1:2 in the PCR reaction mix (table 15). The PCR products were purified before the labeling reaction using the QIAquick PCR purification kit (table 10).

I.5.3 Aminoallyl-UTP labeling procedure

Activated 5/6 Carboxyfluorescein (emp Biotech, Germany) and Cy3 monofunctional dye (Amersham Parmacia Biotech, UK) were used in the labeling reaction of the aminoallyl-dUTP containing amplicon (table 13). Excess dye was removed by gel filtration through G50 columns before hybridization (table 14).

Unlabeled nucleotides, Fluorescin-12-dUTP and aminoallyl-dUTP dilution (diluted to 1/10, $1/10^2$, $1/10^3$, $1/10^4$ and $1/10^5$, irrespectively) were used to compare the efficiency of the PCR reaction. One tenth of the amplicons were electrophoresed on 2% agarose gels (figure 1A).

II. Investigation of G-CSFR distribution in normal rats

II.1 Isolation of total RNA and amplification of G-CSFR by RT-PCR
II.2 Detection of G-CSFR mRNA expression by Northern blot analysis
II.3 Detection of the anatomic localization of G-CSFR mRNA by FISH
II.4 Detection of the anatomic localization of G-CSFR protein by IHC

G-CSFR mRNA expression in normal rats was tested by RT-PCR and Northern blotting. The anatomic localization of G-CSFR was demonstrated on the RNA level by *in situ* hybridization using the FISH technique and on the protein level by IHC.

II.1 Isolation of total RNA and amplification of G-CSFR by RT-PCR

II.1.1 RNA Isolation

Total RNA was extracted from 9 organs (liver, heart, lung, kidney, intestine, pancreas, lymph node, spleen and bone marrow) of six normal rats employing the QIAGEN RNeasy RNA purification kit (table 6). RNA samples were quantified using a spectrophotometer (table 7).

II.1.2 RT- PCR

Reverse transcription was performed using *M-MuLV* Reverse Transcriptase (table 9). The primers used to amplify the DNA fragment encoding part of extracellular region of receptor were the same as those used to generate the probe. The expected size of the amplicon was 280bp. Verification of equal template concentrations among samples was accomplished using primers that amplify a fragment of 28s rRNA. One of tenth of the PCR products were separated electrophoretically on 2% agarose gels and stained with ethidium bromide. The results were documented by photographs under UV-light (table 7).

II.2 Detection of G-CSFR mRNA expression by Northern blot analysis

Northern blot hybridization was performed to examine the mRNA levels of G-CSFR in nonhematopoietic tissues of normal rats. Total RNA was separated according to size in formamide/formaldehyde gels, transferred to positively charged nylon filters and hybridized using the labeled ssDNA probe. All aqueous solutions were prepared with 0.1% diethylprocarbonate (DEPC)-treated water.

The basic steps in northern analysis include:

- Isolation of total RNA (table 6)
- Separation of RNA according to size in a denaturing agarose gel (table 17)
- Transfer of the RNA to a nylon membrane (table 18)
- Fixation of the RNA to the membrane (table 18)
- Hybridization of probes (table 19)
- Documentation of the results (table 20)

II.3 Detection of the anatomic localization of G-CSFR mRNA by FISH

FISH procedures were performed on slides from paraffin embedded samples of six normal Lewis rats (table 21). Negative controls for FISH include hybridization of the same tissue with a sense probe.

Slides were observed using a fluorescent microscope (OLYMPUS BX60). The images were taken using a VYSIS CCD camera and transferred to a Macintosh computer using Smart Capture ® (Digital Scientific, Cambridge/ UK).

II. 4 Detection of the anatomic localization of G-CSFR protein by IHC

IHC was carried out on slides from paraffin embedded tissues (table 22). Anti-G-CSFR antibody was an affinity purified rabbit polyclonal antibody raised against a peptide mapping to the amino terminus of G-CSFR (Santa Cruz, CA) of mouse origin cross reacting with rat and human G-CSFR.

Purified antibodies from non-immunized rabbits were used as negative control.

Results were recorded according to the intensity of staining and the distribution in tissues. Images were taken using a a digital camera (Nikon CoolPix 995) mounted to a microscope (OLYMPUS BX50).

III. Regulation of G-CSFR protein expression in nonhematopoietic organs by G-CSF treatment and 90% partial hepatectomy in rats

III.1 G-CSF administration and partial hepatectomy

Five rats were treated G-CSF (rh-G-CSF, Neupogen, Germany) at doses of 100µg/kg/day subcutaneously for five days prior to sacrifice.

90% partial hepatectomies (PH) were performed in 30 rats according to the technique described by Higgin's (Higgins, 1931). Half of the animals in each group received G-CSF at doses of 100µg/kg/day subcutaneously starting five days prior to the liver resection until sacrifice. Animals were sacrificed at three time points (6h, 24h, 7d) after the operation (for group distribution see table 23).
A full autopsy was performed. Samples of heart, lung, spleen, kidney, liver, small bowel, pancreas, lymph node and BM were taken and fixed in 10% buffered formalin.

Results:

I. Probe development for G-CSFR

I.1 Primer design for rat G-CSFR
I.2 DNA and RNA extraction
I.3 Amplify G-CSFR from DNA by PCR and from RNA by RT-PCR
I.4 Sequencing
I.5 Probe designing, synthesizing and labeling

In the current study, a combined approach involving gene expression analysis of tissues and *in situ* Hybridization, as well as immunohistochemistry was carried out to study expression patterns of G-CSFR mRNA and protein, respectively.

I.1 Primer design

The sequence of murine and human G-CSFR is available in the Genbank database. Alignment of murine and human G-CSFR nucleotide sequence showed a 72.5% homology. Ten conserved regions were detected (table 4) Seven of them were used as templates for the primers. The remaining three were rejected due to their low GC content. Six pairs of primers were designed (list of primers see table 4).

I.2 DNA and RNA extraction

Genomic DNA was isolated and purified from rat liver and bone marrow. $OD_{260/280}$ was in the range of 1.73 to 1.82; RNA was isolated from rat liver tissue, $OD_{260/280}$ was in the range of 1.67 to 1.78.
No degradation of DNA or RNA could be detected on the agarose gels.

I.3 PCR and RT-PCR reaction

Six different primer sets were used to amplify parts of the G-CSFR by Touch-down PCR using DNA and by RT-PCR using total RNA.

When the length of 6 RT-PCR and 6 PCR products were compared, only the first pair of primers produced an amplicon that was close to the predicted length, and the product showed a single band on agarose gel. (table 24)
Gene structure was shown in figure 1A.
With the forward primer (5'- TGA GCT CTG CGGG CTCC), and the reverse primer (antisense 3'-GAC TCC ATG CCA TGG CCC), an amplicon with a length of 500 nucleotides was generated. Since this part maps to the putative signal binding region of the human G-CSFR and parts of this sequence are specific for the G-CSFR, as shown by sequential analysis of the Sequence using BLAST (NCBI).

I.4 Sequencing

The sequencing results of the amplicon showed a 350nt readable fragment, 150nt shorter than expected from the length of the PCR product. The full sequence of the amplicon was only readable after cloning the amplicon and transforming it into DH5α and then using plasmid DNA. The DNA sequences of G-CSFR are shown in figure 1B.
Sequencing results were compared with the database EMBL/Genbank/DDJB. This fragment was 72% homologous with the extracellular domain of human G-CSFR from 1058 to 1506, containing an additional 50 bases. Compared to mouse G-CSFR, a 75% homology was demonstrated. The sequence maps to the mouse gene starting at 1062 and ending at the 1511. (figure 1B)

I.5 Probe generation and labeling

According to the guidelines of probe design, a 300bp region was determined as probe. A new set of primer was designed for PCR.
With the forward primer (5'-ACG GGA TCC GTG AGA CGC), and the reverse primer (5'- CTT CTC AAT TAC CAG CGA TGG), a 280nt ssDNA amplicon was synthesized by asymmetric PCR. Comparing the efficiency of the PCR reaction using unlabeled nucleotides, Fluorescin-12-dUTP and aminoallyl-dUTP (figure 2A).
It showed an, at least, hundred-fold higher

efficiency of the PCR reaction using unlabeled nucleotides or aminoallyl-dUTP compared to Fluorescin-12-dUTP.

II. G-CSFR distribution in normal rats

II.1 Amplification of G-CSFR gene by RT-PCR
II.2 Detection of G-CSFR mRNA expression by Northern blot analysis
II.3 Detection of the anatomic localization of G-CSFR mRNA by FISH
II.4 Detection of the anatomic localization of G-CSFR protein by IHC

II.1 Amplification of the G-CSFR gene by RT-PCR

II.1.1 RNA Isolation

RNA isolated from rat tissues showed discrete bands of high molecular weight RNA between 7 kb and 15 kb in size, (composed of mRNA's and hnRNA's) two predominant ribosome RNA bands at ~5 kb (28s) and at ~2kb (18s), and low molecular weight RNA between 0.1 and 0.3 kb (tRNA, 5s). The isolated RNA had an A_{260}/A_{280} ratio range from 1.75 to 1.89 when diluted in TE. No signs of degradation could be detected. (figure 2B)

II.1.2 RT-PCR

Total RNA was reverse transcribed and amplified by PCR. Using agarose gels it could be demonstrated that the amplicon had a size of 280 bp.
G-CSFR mRNA was detected in all tested tissues (figure 2C).

II.2 Detection of G-CSFR mRNA expression by Northern blot analysis

In Northern blots a band of about 3kb was detected after hybridization in all tested samples (figure 2D). A high expression level could be found in the bone marrow and in the spleen.

II.3 Detection of the anatomic localization of G-CSFR mRNA by FISH

Fluorescent *in situ* hybridization was performed to examine the cellular distribution of mRNA encoding the receptor.

The mRNA for G-CSFR was detected in the cytoplasm of cells.

Endothelial cells of arteries and veins and neurons consistently expressed G-CSFR mRNA in every organ. Fibroblasts and smooth muscle cells showed G-CSFR mRNA only in a very low number of cells.

Parenchymal cells demonstrated various expression patterns (table 25):

Liver

G-CSFR mRNA was detected in single hepatocytes in zone 2 and 3 (figure 3A)

The biliary epithelial cells were negative for G-CSFR mRNA.

Heart

G-CSFR mRNA was detected in most cardiac myocytes (figure 3B)

Lung

Expression of G-CSFR mRNA was not evident in cylinder cells of the respiratory tract. However mRNA expression could be detected in most type II pneumocytes (figure 3C)

Kidney

G-CSFR mRNA was present in all epithelial cells of the proximal convoluted tubule. There was a significant decrease in the numbers of tubular cells that express G-CSF mRNA as the ducts descend. Only single urothelial cells of the renal pelvis showed a positive signal. Glomerular cells were negative. (figure 3D)

Intestine

G-CSFR mRNA was evident in single mucosal epithelial cells, mostly located at the base of crypts (figure 3E).

Pancreas

Most acinar cells expressed G-CSFR mRNA. Pancreatic ductular epithelial cells and islet cells were negative (figure 3F).

Spleen

In the white pulp of the spleen a few cells showed G-CSFR mRNA expression (figure 3.G).

Lymph node

Single cells expressing G-CSFR mRNA were detected in lymphatic follicles (figure 3H).

BM

Myeloid precursor cells showed positive staining. Erythrocytes and megakaryocytes did not show G-CSFR mRNA expression, whereas most of the polymorpho-nucleocyte (PMN) population demonstrated a positive signal.

II. 4 Detection of the anatomic localization of G-CSFR protein by IHC

In immunohistochemistry, positive staining was also observed in the cytoplasm.
Endothelial cells of arteries, veins and neurons consistently showed a positive staining. Fibroblasts and smooth muscle cells did not show a positive staining.
Parenchymal cells demonstrated various staining patterns (table 26):

Liver

Rare hepatocytes in zone 2 and 3 showed an intense positive staining. Morphologically they were indistinguishable from the surrounding hepatocytes that were not stained. Only a small number of biliary epithelial cells showed a faint positive staining. Trace levels of G-CSFR staining could be observed in the majority of Kupffer cells. (figure 4A)

Heart

Myocytes showed slight and a few myocytes moderate anti-G-CSFR immunostaining (figure 5A).

Lung

Cylinder cells of the respiratory tract showed a faint positive staining. Type 2 pneumocytes were also weakly positive (figure 6A).

Kidney

The tubular epithelial cells, both of the convoluted portion and the straight portion of proximal tubules, showed slight staining. In collecting ducts, there was a decrease in the intensity of G-CSFR staining in the epithelial as the ducts descend (figure 7A).

Intestine

Single epithelial cells showed positive staining. The staining was found only in the apical parts of the cytoplasm. Positive cells were found more often at the base of the intestinal crypts (figure 8A).

Pancreas

Most acinar cells showed a moderate to low positive staining. Islet cells showed no pronounced staining (figure 9A)

Pancreatic ductular epithelial cells showed a slight positive staining in a low number of cells in small ducts and a slightly increasing number of cells, some of them also showing a little higher intensity of the staining in larger ducts.

III. Regulation of G-CSFR protein expression in nonhematopoietic organs of G-CSF treated and/or 90% hepatectomized rats

III.2 Immunohistochemical analysis

In IHC positive staining was observed in the cytoplasm.

Endothelial cells of arteries, veins and neurons consistently showed a positive staining. The intensity of the staining was increased in all experimental groups in comparison to normal rats. Fibroblasts and smooth muscle cells did not show a positive staining.

Parenchymal cells demonstrated various staining patterns (table 26)

Liver

After 90%PH, an increased number of mainly periportal and pericentral hepatocytes, some of them forming small groups showed a high positive staining at 6 hours, with slightly higher cell number at 24 hours. On day 7, the staining was approaching the

level observed in normal animals. Only a small number of biliary epithelial cells showed a faint positive staining. (figure 4B-D)

In G-CSF-treated rats, the number of hepatocytes showing a high level of cytoplasmic staining increased a little and the other hepatocytes showed a faint positivity. (figure 4E)

After 90%PH and G-CSF treatment nearly all hepatocytes showed a strong positive staining after 6 hours. The number hepatocytes demonstrating a strong positivity decreased at 24 hours and 7days. (figure 4.F-H)

The expression of G-CSFR on biliary epithelial cells was slightly induced by G-CSF administration. (figure 4G-insert)

Heart

In the 90% PH group, myocytes showed a intense staining at 6 hours, gradually decreasing to nearly the level found in normal animals until 7 days. (figure 5.B-D)

In the G-CSF treated group, myocytes showed a moderate and some of them a high intensity of the staining. (figure 5E).

After 90%PH and G-CSF treatment myocytes showed an intense staining at 6 hours gradually decreasing at 24 hours and 7 days, but showing a higher level than myocytes after PH only. (figure 5 F-H)

Lung

In the 90%PH group, slight G-CSFR staining was detected in the apical part of the cylinder cells of the respiratory tract and in type 2 pneumocytes at 6 hours, 24 hours and 7 days. (figure 6B-D)

In G-CSF treated animals, the apical part of the cylinder cells of the respiratory tract and in type 2 pneumocytes showed a moderate positivity for G-CSFR. (figure 6E)

In 90%PH with G-CSF treatment animals showed a slight increase in the intensity of the stain compared with G-CSF treatment only at 6 hours, decreasing at 24 hours and 7 days (figure 6F-H)

Kidney

In the 90%PH group an increased positivity of tubular epithelial cells could be observed. (figure 7B-D)

In the G-CSF treated group single cells in the mesangium showed a weak positivity. Tubular epithelial cells showed an increased positivity compared to normal animals. (figure 7E)

In 90%PH with G-CSF treatment animals the proximal tubular cells showed strong positivity until 7 days. The epithelial cells of distal convoluted tubules showed strong staining at 6 and 24 hours decreasing at 7 days (figure 7F - H).

Intestine

After 90% PH, medium intensity staining was found in nearly all of mucosal epithelial cells at 6 hours. The staining showed an apical orientation. The number of positive cells decreased at 24 hours and 7 days. (figure 8 B-D)

In G-CSF treated rats an increased number of mucosal epithelial cells showed a positive staining compared to normal animals. (figure 8E)

After 90% PH and G-CSF treatment, medium intensity staining was found in nearly all mucosal epithelial cells at 6 and 24 hours. The staining showed an apical orientation. The number of positive cells decreased at 7 days. (figure 8F-H)

Pancreas

In 90% PH group, the acinar cells showed a strong staining at all time points. Pancreatic ductular epithelial cells showed a strong staining at 6 and 24 hour, decreasing at 7 days. There was also a transient slight positivity in islet cells. (figure 9B-D)

In the G-CSF-treated animals, acinar cells stained intensely positive for G-CSFR. The islet cells showed low positivity. (figure 9E)

After 90%PH and G-CSF treatment, the acinar cells showed a strong staining at all time points. Pancreatic ductular epithelial cells showed a strong staining at 6 and 24 hour, decreasing at 7 days. There was also a transient slight positivity in islet cells. (figure 9F-H)

Discussion

Adult stem cells are not only a hot topic in science but they are entering generally available sources of information like CNN (2000). Therapeutic breakthroughs by the help of adult stem cells are reported in normal newspapers (Fagan, 2003). Stem cell mobilization by G-CSF treatment is an widely used procedure to mobilize stem cells prior to stem cell transplantation. Mobilizing stem cells by G-CSF also showed a positive effect on the myocardial regeneration after infarctions (Kuethe et al., 2004). In a rat model hepatocellular damage ($CdCl_2$-damage:(Theocharis et al., 1998), thioacetamid damage (Theocharis et al., 1999): showed a delayed onset of proliferation. Administration of G-CSF led to an accelerated onset of proliferation. There were no indications given in these papers whether this is due to a direct effect of G-CSF on liver cells or due to immigrated bone marrow derived stem cells. Irrespective the underlying reason for the effect of G-CSF administration in these experiments- G-CSF acts by way of its receptor: G-CSFR.

To reach a deeper understanding of G-CSFR, our first step was to demonstrate the distribution of the G-CSFR. We are working in the rat model. The first challenge was to obtain and whenever not available produce reagents to demonstrate G-CSFR in rats.

I. Sequence and Probe

Sequence of rat G-CSFR

The sequence of rat G-CSFR has not been published. Conserved regions of G-CSFR were deducted from a comparison of mouse (Fukunaga et al., 1990a) and human (Fukunaga et al., 1990b) G-CSFR. We sequenced part of the extracellular domain of the rat G-CSFR gene, which contained the highly conserved motif “WSXWS” shared by members of the cytokine receptor family. Besides this a G-CSFR specific stretch was found. Specificity was shown by comparing this sequence with other known sequences using BLAST (NCBI). This part of the gene was included in the subsequent design of the G-CSFR probe.

Probe Generation

Detection of G-CSFR mRNA expression is based on the binding of a specific probe. There are several types of probes currently used for the detection of RNA expression by hybridization-based techniques: RNA probes, double-strand DNA (dsDNA) probes and single-strand DNA (ssDNA) probes.

To detect the G-CSFR expression at the mRNA level, we needed a highly specific, and stable probe which could be used in Northern blot hybridization and FISH. Tissue penetration should be good especially since cross-linking occurs during formalin fixation of the tissue, which makes tissue penetration more difficult. Background staining should be as low as possible and single stranded probes should be used to demonstrate that the positive staining result is really due to m-RNA expression and not just due to binding of the probe to DNA.

RNA probes are still the most commonly employed since they were introduced by Casey and Davidson in 1977 (Casey and Davidson, 1977). They are synthesized by *in vitro* transcription (Melton et al., 1984). Even though the sensitivity of RNA probes is very high, their instability and poor tissue penetration limit their use (Bales et al., 1993).

The use of double stranded DNA probes was first reported by Taylor and his colleague (Taylor and Gettman, 1976). They can be produced by random priming (Holtke et al., 1990), PCR (Jansen and Ledley, 1989) or after cloning by plasmid production (Jacquet et al., 1977).

ssDNA probes were first generated in 1990 (Scully et al., 1990). ssDNA are synthesized by asymmetric PCR. This PCR technique is based on unequal concentrations of the forward and reverse primers (Pagratis, 1996). At the end of the PCR reaction, the amount of the sense or antisense strand - depending on the primer used at higher concentrations - of the amplified DNA is three to five times higher. The asymmetric amplification initially proceeds at an exponential rate and then slows to an arithmetic rate when the amount of one oligonucleotide primer becomes the limiting factor. Setting up multiple reactions can help to compensate for the decrease in total yield in a single reaction (Finckh et al., 1991).

Taking all this into consideration the use of ssDNA probes for the detection of G-CSFR mRNA expression was the only logical choice.

A probe is useless unless it is labeled. The sensitivity of a probe depends to a great deal on the attached label itself and the amount of label attached.
Radioactive labeled nucleotides were the first labels used (Feinberg and Vogelstein, 1983) and are still widely used. Their advantage is the high sensitivity. Their disadvantage is due to their radioactivity that can be only handled in special laboratories under special security precautions.
The revolution in nucleic acid labeling was fueled by the wide assortment of new and improved nonisotopic marker molecules. The first molecule used was fluorescein, visualized via fluorescence (Martin et al., 1990). Nowadays multiple fluorochromes are available. These fluorescent labels now offer sensitivity levels similar to those produced by radioisotopes, but without the high backgrounds originally observed (Dirks et al., 1991) . In addition they allow the simultaneous analysis of multiple probes.
Enzymes like alkaline phosphatse and peroxidase can be used as labels. Their downside is that sensitivity is lower compared to fluorochromes.
Lately luminophores have been used. They show high sensitivity but in *in situ* hybridization they cannot be used since their ability to emit light is limited to very quite intervals that are not long enough to screen a slide.
The use of nonradioactive labeles was a prerequisite for us. Fluorochromes seemed to be the appropriate label since we wanted to generate an ssDNA probe showing the highest sensitivity possible in *in situ* hybridization.
Fluorochromes are either directly labeled to nucleotides which are used to label a probe. Another approach makes use of nucleotides which are labeled with a hapten like biotin or carry a chemically active group like aminoallyl-dUTP to which fluorochromes are attached in a subsequent reaction after the nucleotides were inserted into a probe. Actually the first nonradioactive nucleic acid labeling method developed was based on the biotin-avidin/streptavidin complex (biotin-dUTP) (Langer et al., 1981). But this method is based on a high affinity binding reaction of avidin and

biotin that can be the source of unspecific staining since biotin is also present in the tissue itself.

The efficiency of a PCR reaction is defined as the amount of amplicon of expected size produced during a defined number of PCR cycles. We compared the use of Fluorescin-12-dUTP and aminoallyl-dUTP in PCR and found an at least hundredfold higher efficiency using aminoallyl-dUTP compared to Fluorescin-12-dUTP. Fluorescin-labeled dNTPs/NTPs can not be incorporated by reverse transcriptase or polymerases with the same efficiency as unlabeled NTPs, leading to variable incorporation efficiency of different dyes (e.g. FITC-NTP vs. Cy3-NTP) (Gauthier et al., 2002). Aminoallyl-dUTP showed an efficiency that was on the same level as dTTP. So this chemically modified nucleotide seems not to influence the efficiency of the PCR.

In addition amino-allyl dUTP is very stable, there is no loss due to exposure to light or high temperature. So both of the amino-allyl dUTP and the amino-allyl coupled DNA by PCR can be stored for a long time, and be coupled with dye before use.

To the best of our knowledge, no procedure describing the application of two-step labeling method to label ssDNA probes was available at the time the dissertation started.

II. G-CSFR Expression

Saturable high-affinity receptors for G-CSF on human cells, including hematopoietic cells of granulocytic lineage, platelets, monocytes, and lymphocytes were demonstrated by biologically active, radioiodinated G-CSF (Larsen et al., 1990). In 1990 murine and human G-CSFR were cloned (Fukunaga et al., 1990a; Fukunaga et al., 1990b).

Based on the published sequence detection of mRNA expression by RT-PCR, Northern Blotting and in-situ hybridization was possible (Avalos et al., 1990; Bocchietto et al., 1993; Tweardy et al., 1992). In addition antibodies against G-CSFR protein are now available from commercial sources (Santa Cruz, CA).

We employed different methods to detect G-CSFR mRNA in rat tissues. To demonstrate expression of mRNA, RT-PCR was used as the most sensitive tool. Quantitatively mRNA expression was demonstrated by Northern blots.

In all tissues we examined, G-CSFR mRNA were detected by RT-PCR and in Northern Blots. In both methods purified total mRNA is the basis of the tests. This is consistent with the results of Calhoun et al. who observed G-CSFR expression by *in situ* RT-PCR in nearly every organ and tissue examined in human fetus (Calhoun et al., 1999). What limits the interpretation of data acquired through these methods is the fact that the cellular distribution is not known and the mRNA detected could be as well transcribed by bone marrow derived cells located in the tissue.

To demonstrate tissue distribution of mRNA transcription we employed FISH. These data were confirmed demonstrating protein expression by immunohistochemistry. Tissue expression was only regarded positive when mRNA and protein expression could be shown.

It was reported that G-CSF administration modulates the expression of its receptor due to transcriptional activation without synthesis of new protein in mouse (Steinman and Tweardy, 1994). Tsuruta also demonstrated an induction of the expression of G-CSFR mRNA by G-CSF in human neutrophils (Tsuruta et al., 1996). To look at the regulation of G-CSFR expression by way of this autocrine loop we used systemic administration of G-CSF in our rat model.

G-CSF belongs to the group of cytokines. Cytokines very often form interwoven networks affecting each other. Major surgical trauma such as 90% liver resection is a well-defined animal model putting major stress on the organism thereby inducing multiple cytokines and growth factors. We wanted to test whether this “stress” by itself has an effect on the G-CSFR expression and whether there is an additive effect to the systemic G-CSF administration. Changes in the expression of G-CSFR due to administration of G-CSF and partial hepatectomy were demonstrated by immunohistochemistry

Heart

G-CSFR could be detected in myocytes. Protein expression was upregulated by systemic administration of G-CSF and by 90% PH. The additive effect was shown more dramatic at 6 hours.

Cardiac myocytes have been considered as terminally differentiated cells. It has been reported recently that mobilization of bone marrow stem cells by G-CSF leads to regeneration and functional improvement after infarction (Kuethe et al., 2004; Norol et al., 2003). In papers looking at the beneficial effect of bone marrow derived stem cells after cardiac lesions a direct effect of G-CSF on myocytes has never been taken into consideration. Since the receptor was present in cardiomyocytes and the expression was upregulated by G-CSF administration, a direct effect seems possible.

At the same time the pluripotentiality of adult hematopoietic stem cells has been challenged (Holden and Vogel, 2002; Wagers et al., 2002), and the process of transdifferentiation, used to explain how tissue-specific cells can generate cells of other tissues, is controversial (Terada et al., 2002; Ying et al., 2002).

Lung

Early studies revealed the presence of G-CSFR and its ligands in human fetal lung tissues (Calhoun et al., 1999). We found G-CSFR in type 2 pneumocytes in normal animals. A role of G-CSF and its receptor is also indicated by the fact that diffuse alveolar damage, is seen in knock out mice lacking G-CSFR (Seymour et al., 1997). In addition G-CSF was demonstrated to promote lung regeneration and increase bone marrow-derived cell numbers in alveoli (Ishizawa et al., 2004). We found that G-CSF treatment stimulated G-CSFR expression in alveolar cells, and bronchiolar epithelium cells which could indicate an increased effect of G-CSF administration at this point. Whereas PH induced only a slight increase of G-CSFR expression, but there was also a marked additive effect of G-CSF administration and PH

Kidney

In the current study, G-CSFR was detected in tubular epithelial cells. Both after G-CSF treatment and in the 90% PH model we could demonstrate an increased expression in the tubular epithelial cells. An additive effect could be demonstrated at 6 hours and the subsequent decrease was shown.

In a renal ischemia reperfusion model, Zhang showed that there is an increase in the serum concentrations of G-CSF protein, and in G-CSF mRNA and protein in the ischemic kidney (Zhang et al., 2004). So at least the production of the ligand could be demonstrated however no reports on G-CSFR expression in parenchymal cells of the kidney exist. Only in human fetus G-CSFR has been reported (Calhoun et al., 1999).

Intestine

In human fetus G-CSFR was demonstrate in enterocytes (Calhoun et al., 2000). In normal rats, only a small number of enterocytes expressed G-CSFR. After G-CSF treatment, G-CSFR expression was distinctly enhanced in the apical part of the cytoplasm of the enterocytes. Also 90% PH induced marked G-CSFR expression at 6 hours. In PH and GCSF treated animals, there was dramatic increase in the expression of G-CSFR at 6 hours, and slightly decreased expression at 7 days.

Pancreas

Although pancreatic carcinoma has been reported to produce G-CSF (Ohtsubo et al., 1998; Uematsu et al., 1996) little has been determined about the receptor for G-CSF in either normal or tumor cells of pancreas.

We demonstrated G-CSFR expression in acinar cells of the pancreas. Upregulation could be shown after G-CSF treatment and after 90%PH.G-CSF plus 90%PH also led to a induction of G-CSFR expression in islet cells.

A beneficial effect of G-CSF treatment has recently been shown in a rat pancreatitis model (Cui and Bai, 2003) but has been attributed to the increase in bone marrow derived stem cells.

III. G-CSFR and liver regeneration

Liver

G-CSFR expression has been demonstrated in the human fetal liver (Calhoun et al., 1999). We found G-CSFR expression in a few hepatocytes in zone 2 and 3 in normal rats. While G-CSF treatment alone leads only to a minor increase in G-CEFR expression, 90%PH and especially 90%PH plus G-CSF treatment led to a marked increase in hepatocellular G-CSFR expression.

Cytokines have been proposed as the earliest factors triggering activation of several transcription factors during liver regeneration (Diehl, 2000). G-CSFR is closely related to the IL-6R, and overlapping signal transduction pathways exist (Ziegler et al., 1993). It has been shown that G-CSF administration accelerates and enhances proliferation of hepatocytes after partial hepatectomy and chemical injury in rats (Theocharis et al., 1996; Theocharis et al., 1997). Additionally, the administration of G-CSF to rats after chemical injury of the liver increased the biosynthesis and mitotic activity in hepatocytes. In normal rats, G-CSF administration did not increase mitotic activity in hepatocytes (Theocharis et al., 2003). This suggests that G-CSF does not by itself exert mitogenic action, in quiescent hepatocytes. However G-CSF enhances hepatocellular proliferation acting possibly like other cytokines, by modulating the expression of growth factors.

At the same time it could be demonstrated that bone marrow derived stem cells migrate to the liver and transdifferentiate into hepatocytes (Petersen et al., 1999). This transdifferentiation has also been shown in other models (gender different liver or bone marrow transplantation) (Strain, 1999; Theise et al., 2000b). The frequency of transdifferentiated hepatocytes however is discussed quite controversial. Thus the biologic relevance remains doubtful.

Especially after liver resection or chemically induced damage (Theocharis et al., 1996; Theocharis et al., 1997). the positive effect of G-CSF administration could be on one hand due to increase in the number of peripheral stem cells. On the other hand the combination of G-CSF administration plus subtotal liver resection led to a marked increase in the expression of G-CSFR in the early phase after the operation.

Especially this phase is critical for the survival after extended PH and it could be speculated whether G-CSFR is an important signal transducer in hepatocyes.

Further experimental data on the mechanism of action of exogenously administered G-CSF, as well as endogenously produces G-CSF, on the hepatocyte proliferative response to different kind of stimuli are necessary in order to delineate the mechanism of its action and to support the clinical significance of the administration of this growth factor in extensive liver injury.

Conclusion: G-CSFR was expressed in specific cells of the liver and other major nonhematopoetic organs of the rats and was enhanced by either pretreatment with its ligand or a major surgical trauma such as 90% liver resection, which suggests that G-CSF may play a role as early phase protein in the liver regeneration, and the beneficial effect may take advantage from the additive effect.

Tables

Table 1. General reagents and materials

Reagents	Producer/Supplier	Address
10x MOPS	Amresco Co.	Solon, OHIO, USA
20x SSC	VYSIS Inc	Downers Grove, IL, USA
Boric Acid	Carl Roth GmbH	Karlsruhe, Germany
Calcium Chloride	Sigma Chemical Co.	St Louis, MO, USA
Chloroform	Sigma Chemical Co.	St Louis, MO, USA
Diethaonolamine	Sigma Chemical Co.	St Louis, MO, USA
Dimethyl Sulfoxide	Amresco Co.	Solon, OHIO, USA
Ethanol	Mallikrodt Baker B. V	Deventer, Holland
Ethidium Bromide	Sigma	St Louis, MO, USA
Ethylene Diamine Tetraacetic Acid	Amresco Co.	Solon, OHIO, USA
Glycerol	Sigma Chemical Co.	St Louis, MO, USA
Hydrochloric acid	Carl Roth GmbH	Karlsruhe, Germany
Immun-mount	Shandon	Pittsburg, PA, USA
Isopropanol	Sigma Chemical Co.	St Louis, MO, USA
Magnesium Chloride	Amresco Co.	Solon, OHIO, USA
Methylene Blue	Sigma	St Louis, MO, USA
Paraformaldeyde	Merck GmbH	Hohenbrunn, Germany
Peroxide hydrate	Carl Roth GmbH	Karlsruhe, Germany
Phosphate Buffered Saline	Sigma Chemical Co.	St Louis, MO, USA
Sodium Acetate	Amresco Co.	Solon, OHIO, USA
Sodium Chloride	Amresco Co.	Solon, OHIO, USA
Sodium Dodecyl Sulfate	Amresco Co.	Solon, OHIO, USA
Sodium Hydroxide	Amresco Co.	Solon, OHIO, USA
Tris (hydroxymethyl)-aminomethane	Carl Roth GmbH	Karlsruhe, Germany
Trypton/Pepton	Serva Electrophoresis GmbH	Heidelberg, Germany
Xylol	Mallckrodt Baker B.V.	Deventer, Holland
Yeast Extract	Serva Electrophoresis GmbH	Heidelberg, Germany

Table 2. General Equipments and Instruments

Equipments	Producer/Supplier	Address
ABI PRISM 310 Genetic Analyzer	Perkin Elmer Applied Biosystem	Foster City, CA, USA
Biophotometer	Eppendorf GmbH	Hamburg, Germany
CCD camera	VYSIS Inc.	DownersGrove, IL, USA
Centrifuge 5415 D	Eppendorf GmbH	Hamburg, Germany
Consort E132	Peqlab Biotech. GmbH	Erlangen, Germany
Digital camera Nikon CoolPix 995	Nikon Inc.	Japan
Electrophoresis tank	Eppendorf GmbH	Hamburg, Germany
Fastprep FP120	Savant Inc.	Holbrook, NY, USA
Gerhardt Thermoshaker	Peter Oehmen GmbH	Essen, Germany
GFL Water bath	Gesellschaft fur Labortechnik GmbH	Burgwedel, Germany
Heraeus Biofuge	Kendro Laboratory GmbH	Hanau, Germany
Heraeus Oven	Kendro Laboratory GmbH	Hanau, Germany
Hybridization Oven	UniEquip. GmbH	Martinsried, Germany
HYBrite	VYSIS Inc.	DownersGrove, IL USA
Liebherr Refrigerator	Liebherr GmbH	Germany
OLYMPUS BX50 light microscope	Olympus Inc.	Japan
Omni Gene Primus thermal cycler	MWG Biotech GmbH	Ebersberg, Germany
UV light	Roth, GmbH	Karlsruhe, Germany

Table 3. Principles of primer design

Length:	18-30 nucleotides
G/C content:	40-60%
Tm:	Estimate melting temperature (T_m) T_m=2C x (A+T)+4C x (G+C) Whenever possible, design primer pairs with similar T_m values. Optimal annealing temperatures may be above or below estimated T_m. As a starting point an annealing temperature was used 5ºC below T_m.
Sequence:	– Avoid complimentarity of two or three bases at the 3' ends of primer pairs to reduce primer-dimer formation; – Avoid runs of 3 or more Gs or Cs at the 3' end; avoid a 3'end T; – Avoid complementary sequences within a primer sequence and between the primer pair.

Table 4. Conserved regions and primers

	Consensus position	**Leng-th (nt)**	**Consensus sequence**	**T_m (°C)**	**G/C %**	**Modified T_m (°C)**
1	609~627	19	TCTGCCAGTGGGAGCCAGG	63.2	68.4	59.5
2	848~865	18	CCCATGGATGTTGTGAAA	51.7	44.4	
3	1063~1079	17	TGAGCTCTGCGGGCTCC	59.7	70.6	59.7
4	1247~1267	21	GTGCAGCTGTTCTGGAAGCCA	62.4	57.1	59.6
5	1494~1511	18	GACTCCATGCCATGGCCC	60.8	66.7	60.8
6	1789~1803	15	GCTGCATCTAAAGCA	41.1	46.7	
7	1814~1836	23	ACCTGGGCACAGCTGGAGTGGGT	68.9	65.2	61.4
8	1871~1891	21	ACCCACTACACCATCTTCTGG	55.2	52.4	
9	1960~1975	18	CCTGGAGCCCGCCAGT	59.3	75.0	59.3
10	2584~2606	23	TGACTCCACTCAGCCCCTCTTGG	65.6	60.9	59.8

Red color, modified nucleotides; gray color, conserved region not used for primer

Table 5. DNA purification protocol

Reagents	DNA purification kit	QIAGEN, Hilden, Germany
	Proteinase K	Roche, Mannheim, Germany
Equipments	Microfuge	
Procedures		
Homogeniza-tion and lysis	1. Cut tissues into small pieces, place them in a 1.5ml micro-centrifuge tube. Add 180ul of Buffer labeled ATL. 2. Add 20ul Proteinase K, mix with vortex, incubate at 56ºC on a heating platform until complete lysis of tissue 3 hours. 3. Briefly centrifuge the tube (800g 10sec), add 200 µl Buffer labeled AL, pulse-vortexing, mix 15sec, incubate at 70ºCfor 10min, briefly centrifuge again.	
Binding	4. Add 200ul of 100%ethanol, mix by pulse-vortexing 15sec, briefly centrifuge in a 1.5ml tube at 800rpm for 15sec. 5. Apply the mixture to a spin column, centrifuge at 6000g (8000rpm) 1min.	
Washing	6. Add 500ul Buffer AW1, centrifuge at 6000 g (8000rpm) 1min, put the spin column on a clean 2ml collection tube. 7. Add 500ul Buffer AW2, centrifuge at 12000 g (14000rpm) for 3min.	
Elution	8. Place spin column on a 1.5ml microcentrifuge tube, add 200 µl Buffer labeled AE or distilled water, incubate at room temperature for 1-5 min, centrifuge at 6000 g (8000rpmm) min. 9. Repeat incubation and centrifugation. A third elution step using 200 µl Buffer labeled AE increased yield.	

Table 6. RNA purification protocol

Reagents	RNeasy RNA extraction kit	QIAGEN GmbH, Hilden, Gemany
	DNase kit	QIAGEN GmbH, Hilden, Gemany
Equipments	Microfuge	
Procedures		
Homogeniza-tion and lysis	1. Remove stabilized tissue from reagent using forceps. 2. Place sample into 600µl Buffer labeled RLT. 3. Disrupt and homogenize with a pestle-mortar in 1.5ml tube, until uniformly	
Binding	4. Centrifuge the tissue lysate for 3min at 16,1000rpm in a 1.5ml tube in microcentrifuge. 5. Carefully transfer the supernant to a new microcentrifuge tube with a pipett. 6. Add 600ul of 70% ethanol to the lysate, mix immediately by pipetting.	
Washing	7. Apply 700µl sample (including any precipitate) to a RNeasy column, place on a 2 ml collection tube, centrifuge for 15 sec at 8000 g, discard the flow-through. 8. Add 350µl Buffer labeled RW1 to the RNeasy column, centrifuge 15sec at 8000 g to wash the column, discard the flow-through.	
On column DNase Digestion	9. Add 10µl DNase I stock solution and 70ul buffer labeled RDD mixture onto the column and place on benchtop 30ºC for 15 min. 10. Pipet 350µl Buffer RW1 to the RNeasy column, centrifuge 15sec at 8000 g to wash the column, discard the flow-through. 11. Add 500µl buffer RPE onto the column, centrifuge at 8000g (10000rpm) for 15sec to wash the column. (Reuse the collection tube) Repeat again.	
Elution	12. Transfer RNeasy column on a new 1.5ml collection tube, pipette 30-50µl RNase-free water directly onto RNeasy silica-gel membrane, centrifuge for1min at 8000 g to elute..	

Table 7. Electrophoresis Analysis

Reagents	5x TBE	MBI Fermenta
	Marker DNA	MBI Fermenta
	6x loading dye solution	MBI Fermenta
Equipment	electrophoresis tank	
Procedures		
Gel preparation	1. Prepare electrophoresis buffer (0.5 x TBE) to fill in the electrophoresis tank. 2. Add 1g agarose powder per 100ml (1%) electrophoresis buffer in an glass bottle. 3. Heat the bottle in a microwave until the agarose dissolves. 4. Cool the solution to 60ºC (roughly). During this time, place a comb (0.5mm) above the plate so that a complete well is formed when the agarose is added. 5. Pour warm agarose solution into the plastic tray. 6. After the gel was completely solidified (45min at room temperature), carefully remove the comb and mount the gel in the electrophoresis tank.	
Loading samples	7. Mix the samples of DNA 5µl with the 6x gel-loading dye buffer 1.5µl. 8. Add just enough electrophoresis buffer to cover the gel to a depth of about 1mm. 9. Load 6µl of the sample mixture into the slots of the submerged gel using a pipette.	
Running gel and dying	10. Close the lid of the gel tank and attach the electrical leads so that the DNA will migrate toward the anode. Apply a voltage of 100V (5V/cm). Run the gel until the bromophenol blue has migrated at the end of the gel. 11. Turn off the electric current and remove the leads from the gel tank. 12. Stain the gel by soaking it in solution of ethidium bromide (0.5µg/ml) for 30-45 min at room temperature. 13. Examine the gel by ultraviolet light and take a photograph of the gel.	

Table 8. PCR reaction protocol

Reagents	10 x PCR buffer	QIAGEN, Hilden, Germany		
	dNTP (mix)	QIAGEN, Hilden, Germany		
	$MgCl_2$	QIAGEN, Hilden, Germany		
	HotstarTaq	QIAGEN, Hilden, Germany		
	Primer	synthesized by MWG, Ebersberg, Germany		
Equipments	Thermal cycler			
Procedures				
PCR recipe	**PCR reagents**	**Volume**		**Final concentration**
	Template	1 µl		~100ng DNA
	Primer F,R	2 µl		400pmol/ µl
	dNTP mix	2 µl		200uM each
	10x PCR buffer	5 µl		1x
	$MgCl_2$	2 µl		2µM
	HotStarTaq	0.25 µl		1.25U/reaction
	Total volume	50 µl		
PCR program		**Temperature**	**Time**	**Number of cycles**
	Initial denaturation	95ºC	15min	1(first) cycle
	Denaturation	95ºC	30sec	
	Annealing	66ºCx2, 64ºCx2, 62ºCx2, 60ºCx2, 58ºCx2, 56ºCx2, 54ºCx28	30 sec	40 cycles
	Elongation	72ºC	1min	
	Final elongation	72ºC	10min	
Totaltime	35 circles 2h 38min			

Table 9. Two-step RT-PCR Protocol

Reagents	Random primer	Promega, USA	
	dNTP mixure	QIAGEN, Germany	
	RNase inhibitor	MBI Fermentas, Germany	
	5x Reaction Buffer	Roche,Germany	
	M-MuLV Reverse Transciptase	MBI Fermentas, Germany	
Methods	Two-step RT-PCR		
Synthesis of the first strand of cDNA	**Reagents**	**Volume**	**Final concentration**
	Template RNA	1.5µl	*(0.1-5µg)*
	Random primer	1µl	2.5uM
	Deionized water	8.5µl	to 11µl
	1. Incubate the mix at 70ºC for 5 min and chill on ice. Add the reagents in the following order:		
	5x reaction Buffer	4µl	1x
	10mM dNTP mix	2µl	1.0mM each
	RNase inhibitor	1µl	1U/µl
	Deionized water	1µl	to 19µl
	2. Incubate at 25ºC for 5 min.		
	M-MLV reverse transcriptase (10U/µl).	1µl	200 units
	3. Incubate the reaction mixture at 25ºC for 10min and then at 42ºC for 60min		
	4. Stop the reaction by heating at 72ºC for 10min. Chill on ice.		
PCR amplification	**Reagents**	**Volume**	**Final concentration**
	10x PCR buffer (containing 15mM $MgCl_2$)	5µl	1x
	dNTPs (10mM)	1µl	0.2mM each
	Primer F/R (10pmol/µl)	2 µl	0.4µM
	Template cDNA from last step	2.5µl	
	TaqDNA polymerase (5U/µl)	0.25ul	1.25U
	Sterile H_2O	q.s.	
	Total volume	50µl	

Steps	Temperature	Duration
Initial denaturation	95°C	10min
Denaturation during cycles	**95°C**	**30sec**
Primer annealing	58°C	30sec
Extention	72°C	30sec
Final elongation	72°C	10min
Total time (35 cycles)		2h 38min

Table 10. PCR product purification protocol

Reagents	QIAquick Purification Kit	QIAGEN, Hilden, Germany
Equipments	Centrifuge	
Procedures		
Binding DNA	1. Mix PCR product from the same primer in a QIAquick spin column. 2. Add 5 volumes of buffer labeled PB to 1 volume of PCR reaction and mix. 3. Place the spin column on a provided 2-ml collection tube and centrifuge 60 sec at 12,000g. 4. Discard flow-through. Place QIAquick column back onto the same tube.	
Washing	5. Add 0.6ml buffer PE to column and centrifuge 60 sec at 12,000g . 6. Discard flow-through. Place QIAquick column back onto the same tube. Centrifuge another 60 sec at 13200g. 7. Place QIAquick column in a clean 1.5-ml microfuge tube.	
Eluting DNA	8. Add 30µl buffer labeled EB or 50µl H_2O to the center of the QIAquick column and centrifuge for1min at 13200g after incubation at room temperature for 1 min. 9. Store DNA at –20°C.	

Table 11. Cycle Sequencing Reaction Protocol

<table>
<tr><td>Reagents</td><td>BigDye Terminator Ready Reaction</td><td>Perkin Elmer, Foster City, CA</td></tr>
<tr><td></td><td>Template Suppression reagent</td><td>Perkin Elmer, Foster City, CA</td></tr>
<tr><td>Equipments</td><td colspan="2">Microfuge, ABI PRISM 310 Genetic Analyzer, thermal cycler</td></tr>
<tr><td colspan="3">Procedures</td></tr>
<tr><td>Cycle sequencing</td><td colspan="2">1. Prepare the reaction to an end volume of 20µl: Mix well and spin briefly.
Reagents — Final concentration
Terminator Ready Reaction — 25pmol (5µl)
Template PCR product DNA — 8ng
Primer (For/ Rev) — 3.2pmol
Denionized water — q.s.
Total volume — 20µl
2. Cycle sequencing on thermal cycler: Repeat the following for 25 cycles:
– Rapid thermal ramp to 96ºC(1ºC/sec), 96ºC for 10sec.
– Rapid thermal ramp to 50ºC, 50ºC for 5sec.
– Rapid thermal ramp to 60ºC, 60ºC for 4 min.
Rapid thermal ramp to 4ºC and hold until ready to purity.</td></tr>
<tr><td>Purifying extention products</td><td colspan="2">3. Prepare precipitating mixture in a 1.5-ml tube including:
10µl of 0.3M sodium acetate (NaOAc), pH4.6
250µl of 95% ethanol (EtOH)
80µl deionized H_2O
4. Remove the product (20µl) to the precipitated mixture, mix thoroughly.
5. Vortex the tubes and leave at room temperature for 15min at least to precipitate the products.
6. Spin the tubes in microcentrifuge for 20min at maximum speed 13,2000g. Proceed to the next step immediately.
7. Carefully aspirate the supernatant with a pipette tip and discard.
8. Rinse the pellet with 250µl of 70% ethanol (-20ºC). Mix them briefly. Vortex.
9. Place the tubes in microfuge in the same orientation as in step 4. Spin for 15min in microcentrifuge at maximum speed, aspirate supernatant, discard.
10. Dry the pellet in dark with the lids open at room temperature for 1hour.</td></tr>
<tr><td>Sample Electrophoresis</td><td colspan="2">11. Add 20µl Template Suppression reagent to the dried sample pellet, vortex and centrifuge briefly for several times to resuspend all the pellet.
12. Incubate at 90ºC for 2 min in thermal cycler to denature. Then chill on ice and place it on ice until ready to use.
13. Load the samples 20µl (at least 15µl) in the Sequencing Analyzer system.</td></tr>
</table>

Table 12. Plasmid Transformation Protocol

<table>
<tr><td>Reagents</td><td>pCR4-TOPO</td><td>Invitrogen, Carlsbad, CA, USA</td></tr>
<tr><td></td><td>EcoR I</td><td>Invitrogen, Carlsbad, CA, USA</td></tr>
<tr><td>Equipments</td><td colspan="2">Incubator, water bath, Thermal cycler</td></tr>
<tr><td colspan="3">Procedures</td></tr>
<tr><td>Setting up TOPO Cloning Reaction</td><td colspan="2">1. Prepare fresh PCR products as indicated above.
2. Purify PCR product as indicated above.
3. Mix 2µl PCR product with 1µl salt solution, 1µl TOPO vector and 2µl sterile water, incubate for 5min at room temperature (22-23ºC).
4. Place the reaction on ice.</td></tr>
<tr><td>Transforming One Shot Competent Cells</td><td colspan="2">5. Thaw on ice 1 vial of One Shot cells for each transformation.
6. Add 2µl of TOPO cloning reaction into a vial of E.coli and mix gently. Incubate on ice for 5min.
7. Heat-shock the cells for 30 sec at 42ºC without shaking.
8. Immediately transfer the tubes to ice. Add 250µl SOC medium.
9. Shake the tube horizontally (200rpm) at 37ºC for 1h.
10. Spread 50µl from each transformation on a prewarmed agarose plate containing Penicillin (100ug/ml) and incubate over night at 37ºC.
11. An efficient TOPO Cloning Reaction should produce hundreds of colonies. Pick ~10 colonies for analysis.</td></tr>
<tr><td>Restricted digestion</td><td colspan="2">12. Prepare an enzyme digestion reaction, Incubate at 37ºC for 1 hour.
Reagents — Volume — Concentration
EcoR I (50U/µl) — 0.5µl — 25U/reaction
10x Buffer — 2µl — 1x
Plasmid sample — 5µl
Deionized water — 12.5µl
Total volume — 20µl
13. Analyze the digested fragments on 0.7% agarose gel.</td></tr>
<tr><td>Analyzing transformants by PCR</td><td colspan="2">14. Prepare a 20µl PCR cocktail consisting of PCR buffer, dNTPs, primers, and Taq polymerase according to recipe indicated above.
16. Pick 10 colonies and resuspend individually in 20µl of the PCR cocktail.
17. Incubate for 10min at 94ºC to lyse the cells and inactivate nucleases.
18. Amplify for 20~30 cycles (94ºC for 1min, 55ºC for 1min, and 72ºC for 1min). For final extention, incubate at 72ºC for 10min. Hold at 4ºC.
19. Visualize by agarose gel electrophoresis.</td></tr>
</table>

Table 13. Purifying of plasmid protocol

Reagents	QIAGEN Plasmid Purification Kit	QIAGEN, Hilden, Germany
Equipment	Microfuge, shaker	
Methods	Modified Alkaline Lysis Method	
Procedures		
Harvest cells	1. Pick a single colony from a freshly streaked selective plate and inoculate a starter culture of 5ml LB medium containing ampicilin 100µg/ml. Incubate for 8h at 37ºC with vigorous shaking (~300rpm). 2. Dilute the starter culture1/500 into 25ml selective LB medium. Grow at 37°C for 16hours. 3. Harvest bacterial cells by centrifugation at 6000g for 15min at 4ºC.	
Alkaline lysis	4. Resuspend the bacterial pellet in 4ml buffer labeled P1 by vortexing. 5. Add 4ml buffer labeled P2, mix gently but thoroughly by inverting 4-6 times, and incubate at room temperature for 5min, till the lysate appear viscous. 6. Add 4ml chilled buffer labeled P3, mix immediately but gently by inverting 4-6 times, and incubate on ice for 15 min.	
Binding	7. Centrifuge at 15000g for 30min at 4ºC. Remove supernatant. 8. Re-centrifuge the supernant at 15,000g for 15min at 4ºC. Remove supernatant.	
Washing	9. Equilibrate a QIAGEN-tip 100 by applying 4ml Buffer labeled QBT, and allow the column to empty by gravity flow. 10. Apply the supernatant and allow it to enter the resin by gravity flow. 11. Wash the tip with 2x10ml Buffer labeled QC.	
Eluting	12. Elute DNA with 5ml buffer labeled QF, collect the eluate in a 10ml tube. 13. Precipitate DNA by adding 3.5ml room-temperature isopropanol to DNA. Mix and centrifuge immediately at 15000g for 30min at 4ºC. 14. Wash DNA pellet with 2ml 70% ethanol, and centrifuge at 15000g for 10min. 15. Air-dry the pellet for 5-10min, and redissolve the DNA in 100µl of TE buffer.	

Table 14. Aminoallyl-dUTP incorporation protocol

Reagents	allylamine-dUTP	Sigma, St. Louis, MO, USA
	dNTP (individually)	Molecular Probes, Eugene, OR, USA
	Other PCR reagents	Same as touchdown PCR
Methods	Two-step amine-modified dUTP incorporation method	
Procedures		
Incorporate Direct Fluor-dUTP by PCR	1. Dilute primer 1/100 with distilled water further to final concentration 0.1uM irrespectively. Then mix F+R/100 or F/100+R. 2.Set up Fluor-dUTP-PCR reaction mixture (100µl): **Reagents** — **Volume** — **Final concentration** 10xBuffer (with 15mM $MgCl_2$) — 10µl — 1mM 25mM $MgCl_2$ — 10µl — 4mM **Fluorescein labeling dNTP mix** — 10µl — 0.2mM dNTP Taq polymerase (5U/µl) — 1µl — 5U/100µl reaction DNA template (plasmid DNA) — 1µl Primer F (10µM) +R/100 (0.1µM) — 4µl — 0.2µM/0.002µM Primer F/100 (0.1µM) +R (10µM) — 4µl — 0.002µM/0.2µM dH_2O — 60µl Total — 100µl	
Incorporate aminoallyl-dUTP by asymmetric PCR	**Reagents** — **Volume** — **Final concentration** 10xBuffer (inc 15mM $MgCl_2$) — 10µl — 1x 25mM $MgCl_2$ — 10µl — 2.5mM 10 mM d(ACG)TP — 2µl — 0.2mM **5 mM dTTP** — 1.4µl — 0.07mM **10mM Aminoallyl dUTP** — 1.4µl — 0.13mM Taq polymerase (5U/µl) — 1µl — 5U/reaction DNA template (plasmid DNA) — 3µl Primer F (10µM) +R/100 (0.1µM) — 4µl — 0.2µM/0.002µM Primer F/100 (0.1µM) +R (10µM) — 4µl — 0.002µM/0.2µM BSA (10mg/ml) — 4µl — 0.4mM dH_2O — 60µl TOTAL — 100µl 2. Set up PCR reaction mixture. 3. Carry out PCR reaction in thermal cycler: 35cycles, primer annealing at 56C 30 sec,	

Table 15. Purification of amine-modified PCR product protocol

Reagents	QIAquick PCR purification Kit	QIAGEN, Meinheim, Germany
	Activated 5/6 Carboxyfluorescein	emp Biotech, Berlin, Germany
	Cy3 Monofunctional dye	Amersham PharmaciaBiotech, Piscataway, UK
Equipments	Centrifuge	
Procedures: On-column purification method		
Purification of AMINE-modified PCR product Protocol	1. Add 100% ethanol 35ml in to purification kit buffer labeled PE. 2. Add 5 volumes of buffer PB to 1 volume of PCR reaction and mix. 3. Place the spin column in a provided 2-ml collection tube and centrifuge 60 sec at10000g. 4. Discard flow-through. Place QIAquick column back into the same tube. 5. Add 0.6ml buffer labeled PE to the column and centrifuge 60 sec at full speed. Discard flow-through. 6. Place QIAquick column back into the same tube. Repeat PE wash and centrifuge another 2 times 60 sec at 14000g. 7. Place QIAquick column in a clean 1.5-ml microfuge tube. Add 50µl dH_2O to the center of the QIAquick column and centrifuge for 1min at 13200g (16100rpm) after incubation in room temperature for 5min.	
Precipitation	8. Add 1/10 volume 3M NaOAc with 2 volume 100% Ethanol and mix them well. 9. Freeze at –20ºC for 1hour and then centrifuge for 20~30min at full speed. **10.** Wash the pellet with 70% ethanol and allow it to air dry with lid open.	
Resuspension	11. Resuspend DNA-pellets by adding 1µg amine-modified DNA in 12µl distilled water. **12.** Mix it well. The solution could be warmed in 45ºC water bath for 2-3min if necessary	
Labeling with an Amino-reactive Reaction	13. Prepare one labeling reaction buffer: transfer 12µl prepared DNA solution to reaction vial. Transfer 4µl dissolved $NaHCO_3$ into reaction vial. 14. Add 10µl DMSO to Carboxyfluorescein derivates. Mix thoroughly by pipeting. 15. Add 4µl mixture of dye (FITC or Cy3) and DMSO to reaction vial, mix them carefully and gently. 16. Leave the reaction in the dark at room temperature for 1 hour. 17. Add glycine (pH8.0, 20mM final concentration) to stop the reaction.	

Table 16. Probe purification protocol

Reagents	Cot-1 DNA	Invitrogen, Carlsbad, CA, USA
	Salmon Sperm DNA	Invitrogen, Carlsbad, CA, USA
Methods	On-column purification method	
Procedures		
Column hybridization	Each of reaction should be purified on one column. 1. Gently tap the column to insure that the dry del has settled at the bottom of the spin column. 2. Remove the top column cap and reconstitute the column by adding 0.65ml of water 3. Allow at least 30min of RT hydration time before using the columns.	
Removal of Interstitial Fluid	4. Place spin column into water collector. If the liquid on the column could not flow through spontaneously, cover the lid to add some pressure. **5.** Spin the column and C-6 in a centrifuge at 750 g for 2min.	
Sample Processing	6. Transfer 20 to 50µl of sample to the top of the gel. 7. Spin at 750g for 2min and collect the flow through in a 1.5ml tube **9.** Measure the quality and concentration of probe**.**	
Precipitation	10. Mix the following solution, vortex, precipitate at –20ºC overnight or at –70ºC for 1~2hours. labeled probe DNA 100ng 2µl Cot-1 DNA 2.5µg 2.5µl salmon sperm DNA 1µl 3M NaAC 0.5µl 100% ethanol 15µl 12. Centrifuge the precipitated DNA at 12000g at 4ºC for 30min. **13.** Carefully pour off supernatant, and place DNA pellet in a 56~60ºC heat block for 5~10min.	

Table 17. Separation of RNA according to size for Northern blot analysis

Reagents	Formadehyde	Sigma, St. Louis MO, USA
	Formamide	Sigma, St. Louis MO, USA
Equipments	Consort gel box, shaker	
Procedures		
Separation RNA accrding to size	1. Set up the denaturation reaction. Mix: RNA (up to 20µg) 2.0µl <1µg/µl 10x MOPS buffer 2.0µl 1x formaldehyde 4.0µl 2.5M formamide 10.0µl 50% ethidium bromide (200µg/ml) 1.0µl 10µg/ml 2. Close the tubes, and incubate the RNA solution at 55ºC for 60min (or 85ºC 10min). Chill the samples for 10min on ice, then spin 5sec. 3. Add 2µl of 10x loading buffer to the samples placed on ice. 4. Install the gel in a horizontal electrophoresis box (thickness should not exceed 6mm). Add sufficient 1x MOPS running buffer. 5. Run the gel submerged in 1x MOPS buffer at 4~5V/cm until the bromophenol blue has migrated 2/3 (~8cm) for 3hours. 6. Visualize the RNAs by placing the gel on a piece of Saran wrap on a UV transilluminator. 7. Proceed with immobilization of RNA onto a solid support by capillary transfer.	

Table 18. RNA transferring and fixing protocol

Reagents	Hybond-N+ membrane	Amersham Phamacia Biotech Inc, Uppsala, Sweden
Equipments	Glass dish, shaker	
Procedures		
Preparation of the Gel for Transfer	1. Partially hydrolyze the RNA sample, by soaking the gel in soaking solution 0.01N NaOH/ 3M NaCl 10minx2 at room temperature. 2. Move the gel containing fractionated RNA to a glass baking dish, use a sharp scalpel to trim away unused areas of the gel. 3. Place a piece of thick blotting paper on a glass plate to form a support that is longer and wider than the trimmed gel. 4. Fill the dish with transfer buffer until the level of liquid reaches almost to the top of the support. When the blotting paper is thoroughly wet, smooth out all air bubbles with a glass rod or pipette.	
Preparation of the membrane for transfer	5. Use a fresh scalpel or a paper cutter to cut a piece of the appropriate nylon membrane~1mm larger than the gel in both dimensions. 6. Float the nylon membrane on the surface of a dish of deionized H_2O until it wets completely from beneath, and then immerse the membrane in 10xSSC for at least 5min.	
Assembling of the transfer system and transfer of RNA	7. Carefully place the gel on the support in an inverted position. 8. Surround, but do not cover, the gel with Saran Wrap. 9. Wet the top of the gel with transfer buffer. Place wet nylon membrane on top of the gel so that the cut corner is aligned. 10. Wet two pieces of thick blotting paper and place them on top of the wet nylon membrane. Smooth out any air bubbles with a glass rod. 11. Cut a stack of paper towels (5~8cm high) just smaller than the blotting papers. Place the towels on the blotting papers. Put a glass plate on top of the stack and weigh it down with a 400g weight. 12. Allow transfer of RNA for 3 hour in alkaline transfer buffer.	
Fix RNA on Membrane	13. Dismantle the capillary transfer system. Transfer the membrane to a glass tray containing 50~300ml of 6xSSC at 23ºC to rehydrate the blot. Place the tray on a shaker and agitate the membrane very slowly for 5min. 14. Remove the membrane from the 6xSSC and allow excess fluid to drain away. Put the membrane, RNA side upward, on a dry sheet of blotting paper for 2~4 min. Fix the RNA to the membrane and proceed directly to prehybridization.	

Table 19. Northern hybridization protocol

Reagents	Sheared DNA	Sigma, St. Louis MO, USA
	Formamide	Sigma, St. Louis MO, USA
	DENHARDT's solution	Sigma, St. Louis MO, USA
	Stringent wash solution	DAKO Cytomation, CA, USA
Equipments	Hybridize oven, shaker	
Procedures		
Pre-hybridization	**1.** Prewarm pre-hybridization solution in 68ºC water bath for 30min. **2.** Prehybridization-Incubate the membrane for 2 hours at 68ºC in 20ml of prehybridization buffer (0.2ml/cm^2). **3.** Boil 200µg/ml of sheared salmon sperm DNA for 5min and chill on ice. Add formamide 5ml to the hybridization buffer.	
Hybridization	**4.** Add the denatured single-stranded probe 5µl (~100ng/ml) to 20ml fresh hybridization solution. **5.** Continue incubation for 12-16 hours at 45ºC.	
Stringent Washes	**6.** Remove blots from hybridization solution and immerse in stringent wash solution TBST at 55ºC in a roller tube. **7.** Wash blots 10min while maintaining 55ºC for 3 times, using 1ml per cm^2 of membrane for each wash. Gently shake the blot during the wash.	

Table 20. Chromogenic development Protocol

Reagents	Chemiluminescent Detection system for Nuclei Acid Blotting	DAKO Cytomation, CA, USA
Method	Alkaline phosphatase-conjugated anti-fluorescein antibody system	
Procedures		
Blocking	1. During the stringent wash step, prepare the blocking buffer at 1:10 dilute with water in an incubation tray. 2. Remove blots from the stringent wash and immerse in the diluted blocking solution at room temperature.	
Anti-FITC AP Conjugate Incubation	3. Leave the blots in the tray, decant the blocking solution into a clean incubation tray. 13. Add anti-FITC AP conjugate solution to the blocking solution to a final dilution of 1: 3200. Mix the solution in the tray, add the blocked blots. 14. Incubate the blots in the conjugate solution for 30min at room temperature while gently shaking the tray.	
Washing	15. Decant the conjugate solution from the tray and rinse the blots twice using 25ml of TBST solution per blot for each time. 16. Transfer the blots to a larger tray and wash an additional 3x 10min at room temperature, using 2ml fresh TBST per cm^2 of membrane for each wash. Gently shake during the washes.	
Chemilumine-scent Substrate Incubation	17. Transfer washed blots to a clean hybridization tube containing 0.05ml of chemiluminescent substrate per cm^2 of the membrane. 18. Incubate for 5min at room temperature with gently shaking.	
Signal Development	19. Drain the majority of the substrate from the blots by briefly holding blot up by a corner. Place the drained blots in individual plastic folders. 20. Gently squeeze excess substrate and bubbles from the folder and seal the edges. The blots should still appear damp. 21. Let the sealed blots incubate for 1hour at room temperature.	
Film Exposure	22. Place the sealed blots in a film exposure holder. 23. Working in the dark, overlay the blot with X-ray film and close the holder. Expose 45min. Process the film.	

Table 21. FISH Protocol

Reagents	Proteinase: 0.1% pepsin in 0.2N HCl	DAKO, Carpinteria, CA, USA
	DAPI II counterstain	VYSIS, DownerGrove, IL, USA
	NP-40	VYSIS, DownerGrove, IL, USA
	Dextran Sulfate	Merck, Damstadt, Germany
	Formalmide	Sigma, St. Louis MO, USA
Equipment	HYBrite	
Method	Separate Denaturation Method	
Procedures		
Tissue preparation	1. Cut tissue sections of 5um, place on Superfrost + slides and air dry at room temperature. 2. Deparaffinize in xylenes three times for 5 minutes each. Hydrate sections gradually through graded alcohols: wash in 100% ethanol twice for 10 minutes each, then 95% ethanol twice for 10 minutes each. Wash in deionized H_2O for 1 minute with stirring. Aspirate excess liquid from slides. 3. Fix tissue sections in 4% paraformaldehyde in PBS (pH 9.5) for 60min at room temperature. Wash twice with PBS for 3min each. 4. Permeablize with 0.1% pepsin in HCl at 37ºC for 15min, wash with PBS 5 min. 5. Soak sections with 50% formamide in 5x SSC.	
Denaturation and Hybridization	6. Heat probe to 80ºC for 1min and iced, add to hybridization solution (50% formamide, 50% dextran sulfate), mix thoroughly. 7. Apply probe mixture to slides. 8. Place slides into a humifide chamber to hybridize at 55ºC over night.	
Post-hyb washing and chromogenic development	9. Wash slides in 2xSSC, 50% formamide 15min at 55ºC to remove coverslip. 10. Wash in 2xSSC, 50% formamide 30min at 55ºC. 11. Wash in 0.2xSSC, 50% formamide 30min at 55ºC twice 12. Equilibrate slides in 0.2xSSC 5min RT. 13. Mount slides with DAPI. 14. Coverslip and observe.	

Table 22. Immunohistochemical Staining Protocol

Reagents	Anti-G-CSFR m-20 (sc-0694)	Santa Cruz Biochemicals, Santa cruz, CA
	Rabbit ABC staining system (sc-2018)	
	Permount	Shandon, UK
Equipments	Water bath	
Methods	avidin-biotin horseradish based method (ABC)	
Slides preparation	1. Cut 4-6 micron thick tissue sections. 2. Deparaffinize in xylenes three times for 5 minutes each. Hydrate sections gradually through graded alcohols: wash in 100% ethanol twice for 10 minutes each, then 95% ethanol twice for 10 minutes each. Wash in deionized H_2O for 1 minute. Aspirate excess liquid from slides. 3. Heat treatment: Place slides in a container and cover with 10 mM sodium citrate buffer, pH 6.0 Allow slides to cool in the buffer for approximately 20 minutes. Wash in deionized H_2O three times for 2 minutes each. Aspirate excess liquid from slides. 4. Incubate for 5 minutes in 0.3% hydrogen peroxide in deionized H_2O to quench endogenous peroxidase activity. Wash in PBS twice for 5 minutes. 5. Incubate specimens for 1 hour in 1.5% goat blocking serum in PBS.	
Antigen-Antibody Reaction	6. Incubate with 1/200 diluted primary antibody (1 µg/ml) for 30 minutes at room temperature or overnight at 4° C. 7. Incubate for 30 minutes with biotin-conjugated secondary antibody (1 µg/ml). 8. Incubate for 30 minutes with avidin biotin enzyme reagent. Wash sections in PBS for 5 minutes three time in between.	
Colorimetric staining and counterstain	9. Incubate in peroxidase substrate as provided for 2-5 minutes, wash sections 5 minutes. 10. Counter-stain in hematoxylin for 10 seconds. Immediately wash with several changes of deionized H_2O. 11. Dehydrate through alcohols and xylenes. Wipe off excess xylene. 12. Immediately add 1-2 drops of permanent mounting medium, cover with a glass coverslip and observe by light microscopy.	

Table 23. Group distribution of tested animals

Observation time	Untreated	Animal	G-CSF treated	Animal
No operation	N=5	REG 138~142	N=5	G-CSF 134~138
90% PH S 6h	N=5	REG-102~106	N=5	G-CSF 60~64
90% PH S 24h	N=5	REG-158~162	N=6	G-CSF 116~121
90% PH S 7d	N=5	REG-170, 181,182, 184,185	N=5	G-CSF 84, 87, 89, 90, 98
Total	N=20		N=21	

Table 24 Comparison of RT-PCR and PCR product

Primer	Len -gth (nt)	GC %	T_m (°C)	Pred-icted length	RT-PCR result	PCR result
1F 5'- TCT GCC AGT GGG AGC CAG	18	66%	60°C	480bp	500bp	500bp
3R 3'-GGA GCC CGC AGA GCT CA	17	58%	58°C			
3F 5'- TGA GCT CCC ACT GGC AGA	18	71%	58°C	200bp	700 bp	200bp,
4R 3'- GGC TTC CAG AAC AGC TGC AC	20	60%	64°C			700 bp
4F 5'- GTG CAG CTG TTC TGG AAG CC	20	60%	64°C	270bp	/	/
5R 3'- GGG CCA TGG CAT GGA GTC	18	67%	60°C			
5F 5'- GAC TCC ATG CCA TGG CCC	18	67%	60°C	350bp	500bp	800bp
7R 3'- CAC TCC AGC TGT GCC CAG G	19	68%	64°C			
7F 5'- CCT GGG CAC AGC TGG AGT G	19	68%	64°C	150bp	600bp	600bp,
9R 3'- ACT GGC GGG CTC CAG G	16	75%	56°C			800bp
9F 5'- CCT GGA GCC CGC CAG T	16	75%	56°C	650bp	800bp	700bp
10R 3'- CAA GAG GGG CTG AGT GGA GTC	21	62%	68°C			

Table 25. G-CSFR expression in normal rat tissues

Organs	Tissues	Cell components		
Mesenchy-mal cells	Submucosa	Endothelial cells	Smooth muscles	Fibroblast
	FISH	+	+	+
	IHC	+	+	+
Liver	Parenchyma	Hepatocyte	Bile duct cells	Kupffer cells
	FISH	Zone 2,3 +	--	--
	IHC	Zone 2,3 +	extremely rare	Trace level
Heart	Cardio	Myocytes		
	FISH	+		
	IHC	+		
Lung	Parenchyma	Respiratory tract cells	Type I pneumocyte	Type II pneumocyte
	FISH	--	--	+
	IHC	+	--	+
Kidney	Parenchyma	Proximal Tubular	Outer medullary	Inner medullary
	FISH	+	+	+/-
	IHC	+	+	--
Intestine	Mucous	Crypt	Villi	
	FISH	Single cell +	-	
	IHC	+	+	
Pancreas	Parenchyma	Acinar	Islet	Duct
	FISH	Consistently +	--	--
	IHC	+	+	+
Lymph node		Lymphocyte	FDC	
	FISH	-	+	
	IHC	-	+/-	
Spleen		Lymphocyte	IDC	DRC
	FISH	-	-	+
	IHC	-	+/-	+
Bone marrow		myeloid precursor cells	granulocytes and monocytes	erythroid cells and megekaryocytes --
	FISH	moderate density	+	--
	IHC	+	+	--

FISH, fluorescent *in situ* hybridization; IHC, immunohistochemistry; IDC, interdigitating cell; DRC, dendritic reticulum cell

Table 26 Comparison of G-CSFR protein expressions in different conditions.

Organs	Tissue/ Cell type	Normal	90%PHS 6h	90%PHS 24h	90%PHS 7d	G-CSF treated	G-CSF 90%PHS 6h	G-CSF 90%PHS 24h	G-CSF 90%PHS 7d
Blood Vessels	Endothelium	+	+~++	+~++	+~++	+~++	+~++	+~++	+~++
Liver	Hepatocyte	+	+	++	+	+~++	++	+~++	+
	Biliary cell	-	+/-	+/-	+/-	+	+	+	+
Heart	Myocyte	+/-	++	+	+/-	+	++	+	+
Lung	Airways	-	+/-	+/-	+	+	++	+~++	+
	Pneumocyte	-	+	+	+	+	++	+~++	+
Kidney	Cortex	+	+~++	+~++	++	+~++	++~+++	++~+++	+
	Outer Medu	-	++~+++	+~++	+~++	+	++	++	+
	Inner Medu	-	++	+	+	+	+~++	++	-
Small Bowel	Mucosa-Villi	+/-	+~++	+	+	+	+~++	+~++	+/-
	Crypt	+/-	+~++	+	+	+	+~++	+~++	+
Pancreas	Acinar	+	++	++	++	++	+++	++	++
	Islet	-	+/-	+	+/-	+	+	+	+
	Ducts	+	++	++	+	+	++	++	+

S, sacrifice; d, day; h, hour; Medu, medullary

Table 27. Cell Types Expressing G-CSFR *in vitro* or *in vivo*.

	Cell type	Species	Reference
Tumorigenic cell lines	Squamous carcinoma	Human	(Noda et al., 1999)
	Colon adenocarcinoma	Human	(Berdel et al., 1989)
	Glioma	Human	(Mueller et al., 1999)
	Lung adenocarcinoma	Human	(Pei et al., 1998)
	Ovary carcinoma	Human	(Brandstetter et al., 1998)
	Oral carcinoma	Human	(Tsuzuki et al., 1998)
	Small cell carcinoma of lung	Human	(Tweardy et al., 1992)
	Bladder transitional cell carcinoma	Human	(Tachibana et al., 1995)
Immune cells	Hematopoietic progenitor cells	Human	(Shimoda et al., 1992)
	Neutrophils	Human	(Fukunaga et al., 1990a)
	Monocytes	Human	(Boneberg et al., 2000)
	Macrophages	Human	(Kuwaki et al., 1990)
	Megakaryocytes and platelet	Human Mouse	(Shimoda et al., 1993) (Saito et al., 1996)
	B-lymphocytes	Human	(Morikawa et al., 2002)
	T-lymphocytes	Human	(Matsushita and Arima, 1998)
Structural cells	Endothelium	Human	(Bussolino et al., 1989)
	Fibroblast	Human	(Bussolino et al., 1989)
	Neuron And glial cells	Rat	(Schabitz et al., 2003)
Fetus	Intestine	Human	(Calhoun et al. 2000)
	Liver	Human	(Calhoun et al. 2000)
	Kidney	Human	(Calhoun et al. 2000)
	Eye	Human	(Calhoun et al. 2000)
	Molar	Rat	(Otsuji et al., 1999)
Others	Placenta	Human	(Larsen et al., 1990)

Figures

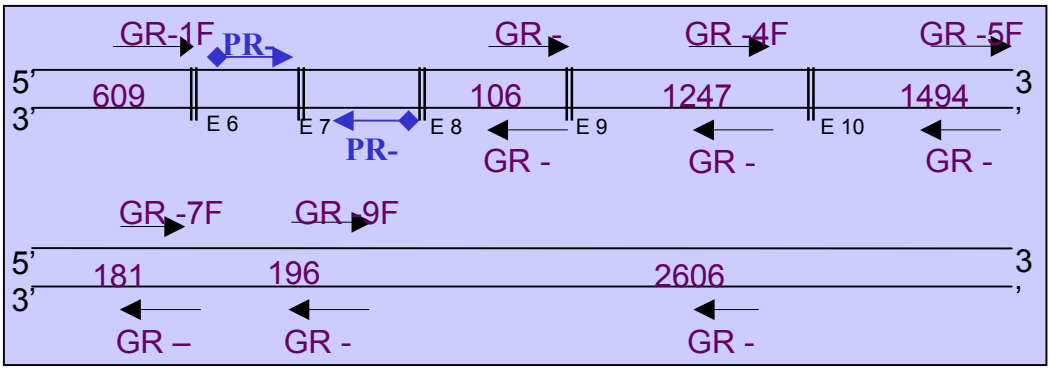
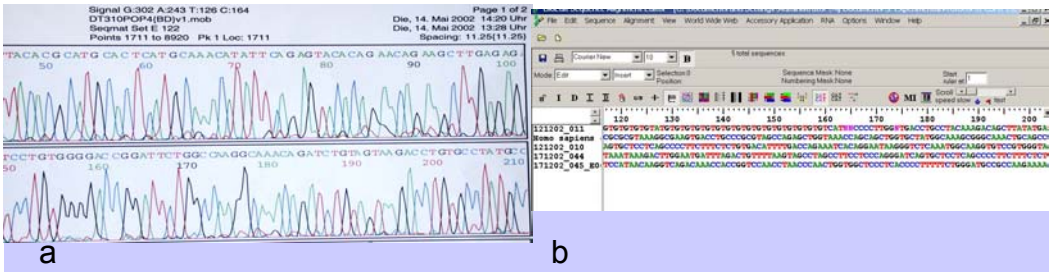


Figure 1A. Schematic diagram of human G-CSFR gene structure and primers designed for the rat gene. Six pairs of primers were designed based on the 10 conserved regions, named GR. Another pair of primer was designed for the G-CSFR probe, named PR.
E, exon; F, forward primer; R, reverse primer.



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TCCAAAGGGAAGACTCTTGTGGAGCCCATGGAGCTATACATCCC
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TGGCATGGGCTGCAAAGGCCTCAGGTCTGCCTGCTGCTAGCTTG
CCTCACCCTCTGCCTCTCCATCCACACCCTAGATTAAGGAGATTG
TAGTGACTCTCAGATGTCCCTAA**ACGGGATCCGTGAGACGC**GCT
GAACTCCTCTGCCTCTCTGGATTCTGCCTGTTGTTGCCCACAGGC
TCTCTCTCTCCCTTAGGCTACGCTAGTGTGGTCTTGAGACTGAAC
TTTCTTATTGGTGACACATCTGCGTGGGAAGGATGAAGGGATGG
AGCTTAGAGTGGGTCAGTGCCCTGGAGGCCCAGGCCAGAGGGG
CATGCCCTTCCCTGTATGCTGGGA**CTTCTCAATTACCAGCGATG**

c

Figure 1B. Partial sequence of the rat G-CSFR gene. A 500 nt-length nucleotide was sequenced (a). Results were compared with the database EMBL/Genbank/DDJB and aligned with the cDNA sequence of human and mouse G-CSFR gene (b). In the nucleotide sequence (c) red color indicates the consensus “WSXWS” (Trp-Ser-any residue-Trp-Ser) motif, violet color indicates a stretch that is highly specific for the rat G-CSFR. The lined sequence indicates the primers used in the amplification of G-CSFR probe for FISH.

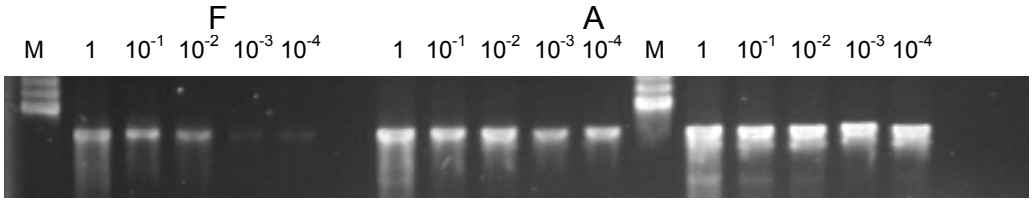

Figure 2A. The PCR reaction using Fluorescein-12-dUTP (F), aminoallyl-dUTP (A), and unlabeled nucleotides (U). Hundred-fold higher efficiency using unlabeled nucleotides or aminoallyl-dUTP compared to

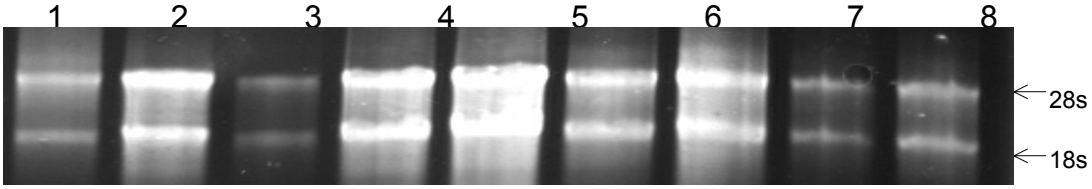

Fig 2B. Electrophoresis of total RNA extracted from rat liver (lane 1), heart (lane 2), lung (lane 3), kidney (lane 4), intestine (lane 5), pancreas (lane 6), lymph node (lane 7), spleen (lane 8), and bone marrow (lane 9). 28s rRNA and 18s rRNA are indicated by arrows. No major degradation of the RNA was detected.

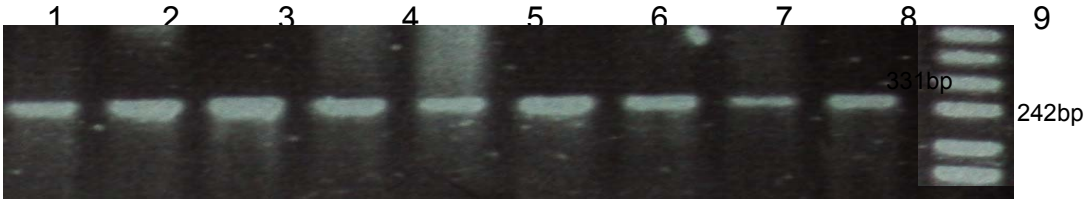

Fig 2C. Detection of G-CSFR mRNA by RT-PCR. Total RNA extracted from normal rat tissues of liver (lane 1), heart (lane 2), lung (lane 3), kidney (lane 4), intestine (lane 5), pancreas (lane 6), lymph node (lane 7), spleen (lane 8), bone marrow (Lane 9) was tested. Amplicons were analyzed on 2% agarose gel and visualized by ethidium bromide fluorescein. M indicated the marker.

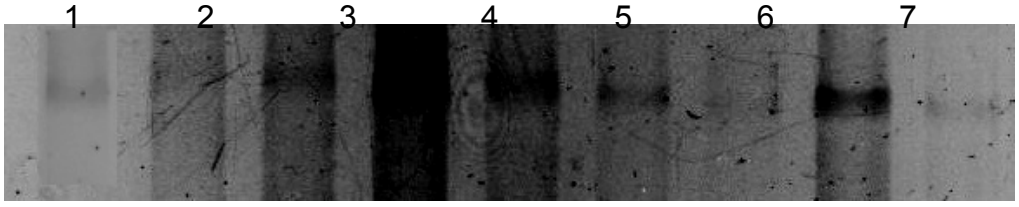

Fig 2D. Northern blot analysis of G-CSFR mRNA. Rat total cellular RNAs electrophoresis on formaldehyde agarose gels, hybridized with G-CSFR probe. All lanes contained 10ug of total RNA from : liver (lane 1), heart (lane 2), lung (lane 3), kidney (lane 4), intestine (lane 5), pancreas (lane 6), lymph node (lane 7), bone marrow (lane 8), spleen (lane 9).

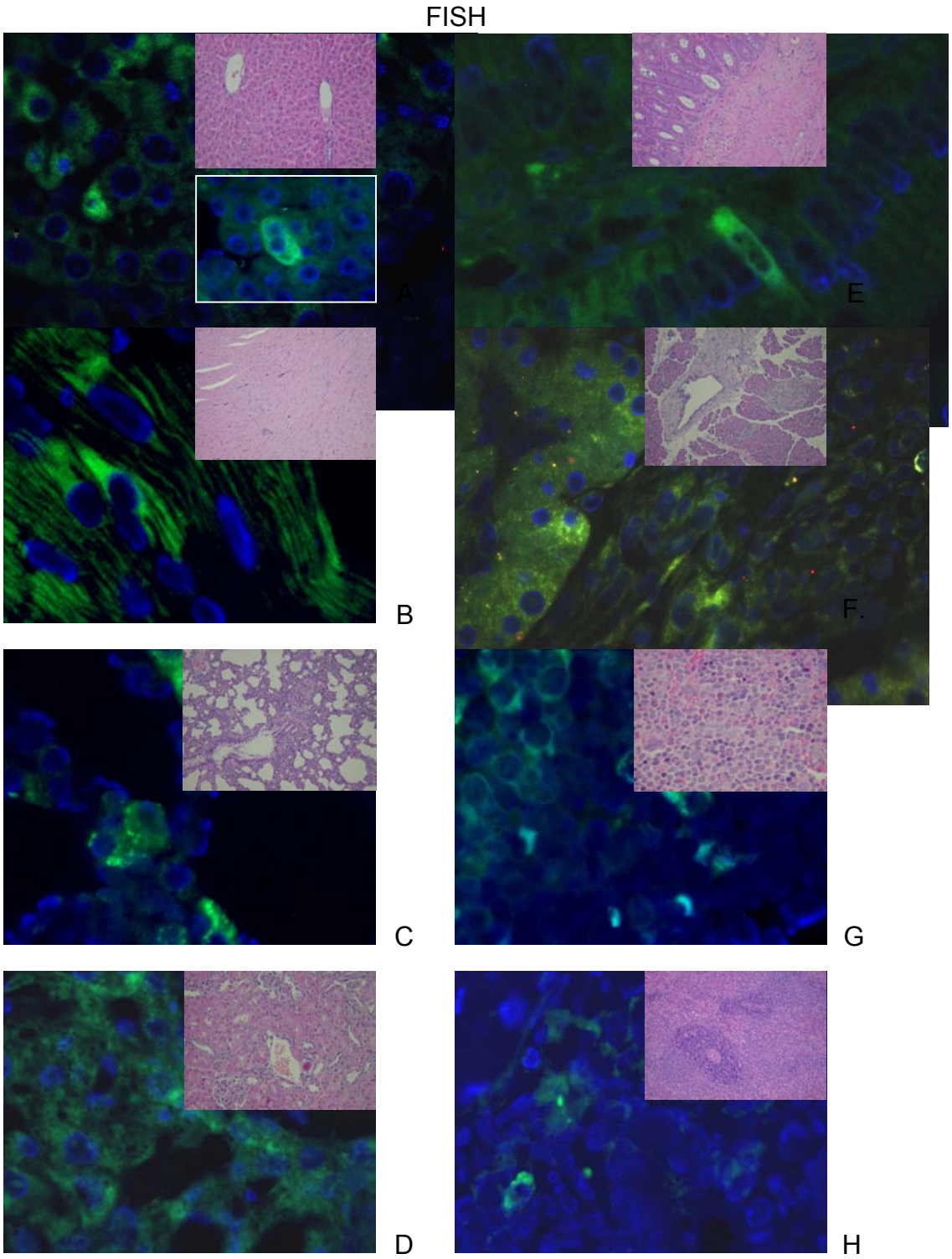

Figure 3. FISH analysis for G-CSFR mRNA in a normal rat. The blue areas represent the DAPI-I staining in the nuclei, green color represents FITC and indicates G-CSFR positivity. G-CSFR mRNA was detected in liver (A); heart (B); lung (C); kidney (D); intestine (E); pancreas (F); spleen (G); and bone marrow (H).
Original magnification: A-H, 600x; insert, haematoxylin-eosin 100x

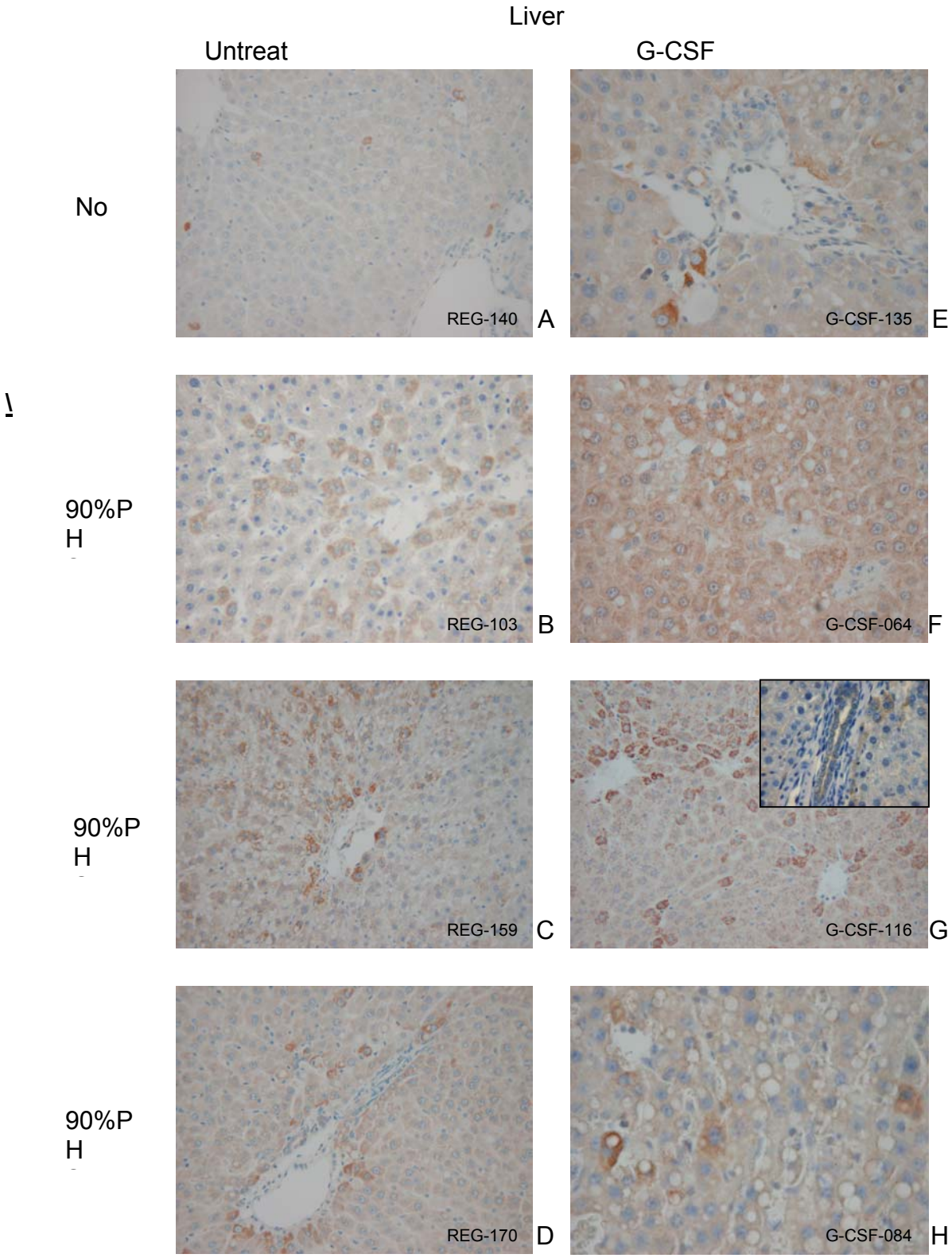

Figure 4. Immunohistochemical analysis of the G-CSF receptor in the rat livers. Immunoperoxidase stain, haematoxylin counterstain. Original magnifications: 100x (A); 200x (C, D, G); 400x (B, E, F,

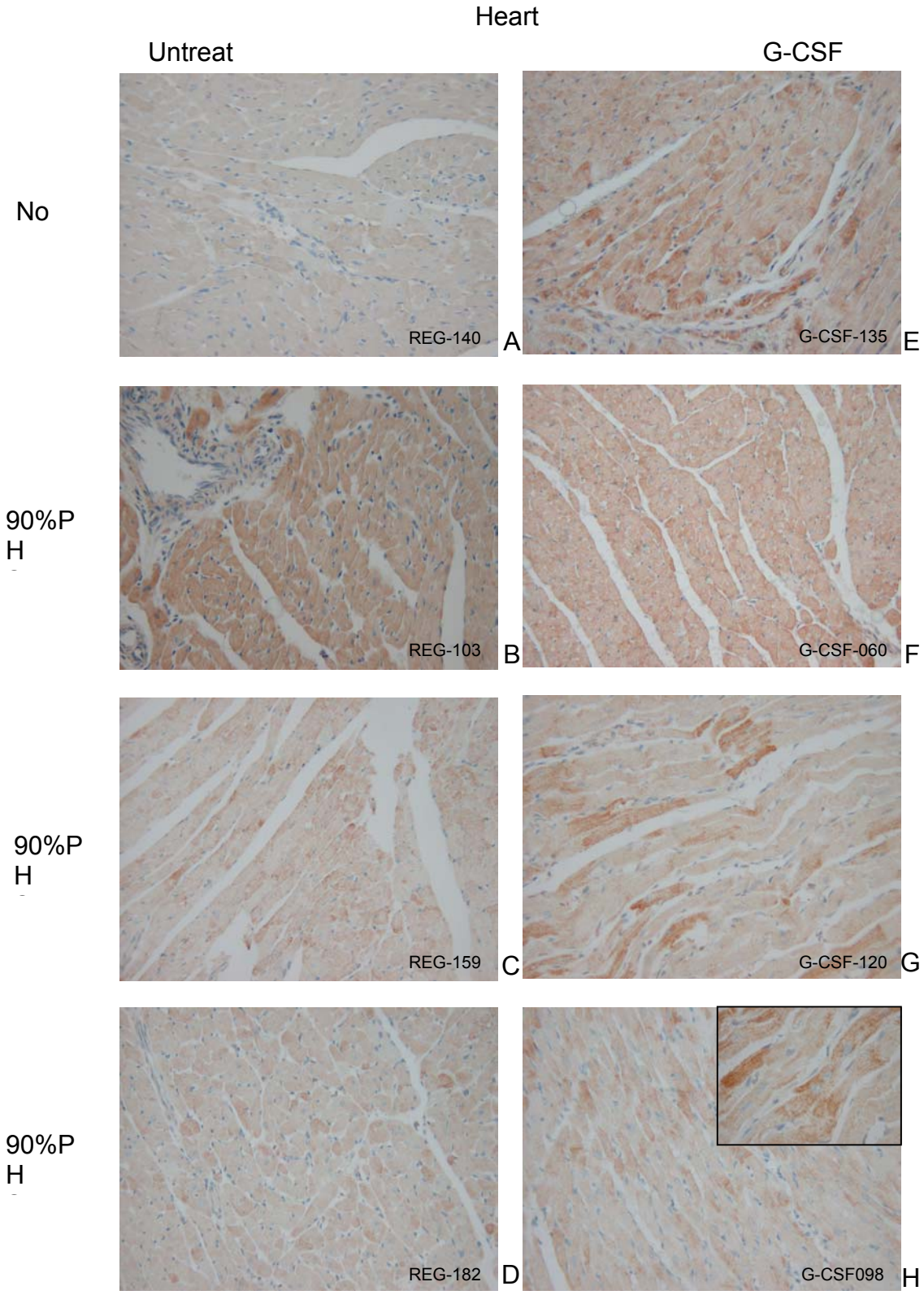

Figure 5. Immunohistochemical analysis of the G-CSF receptor in the rat hearts. Immunoperoxidase stain, haematoxylin counterstain. Original magnifications: 200x (A, C, D, E, F,H); 400x (B, G, H-insert).

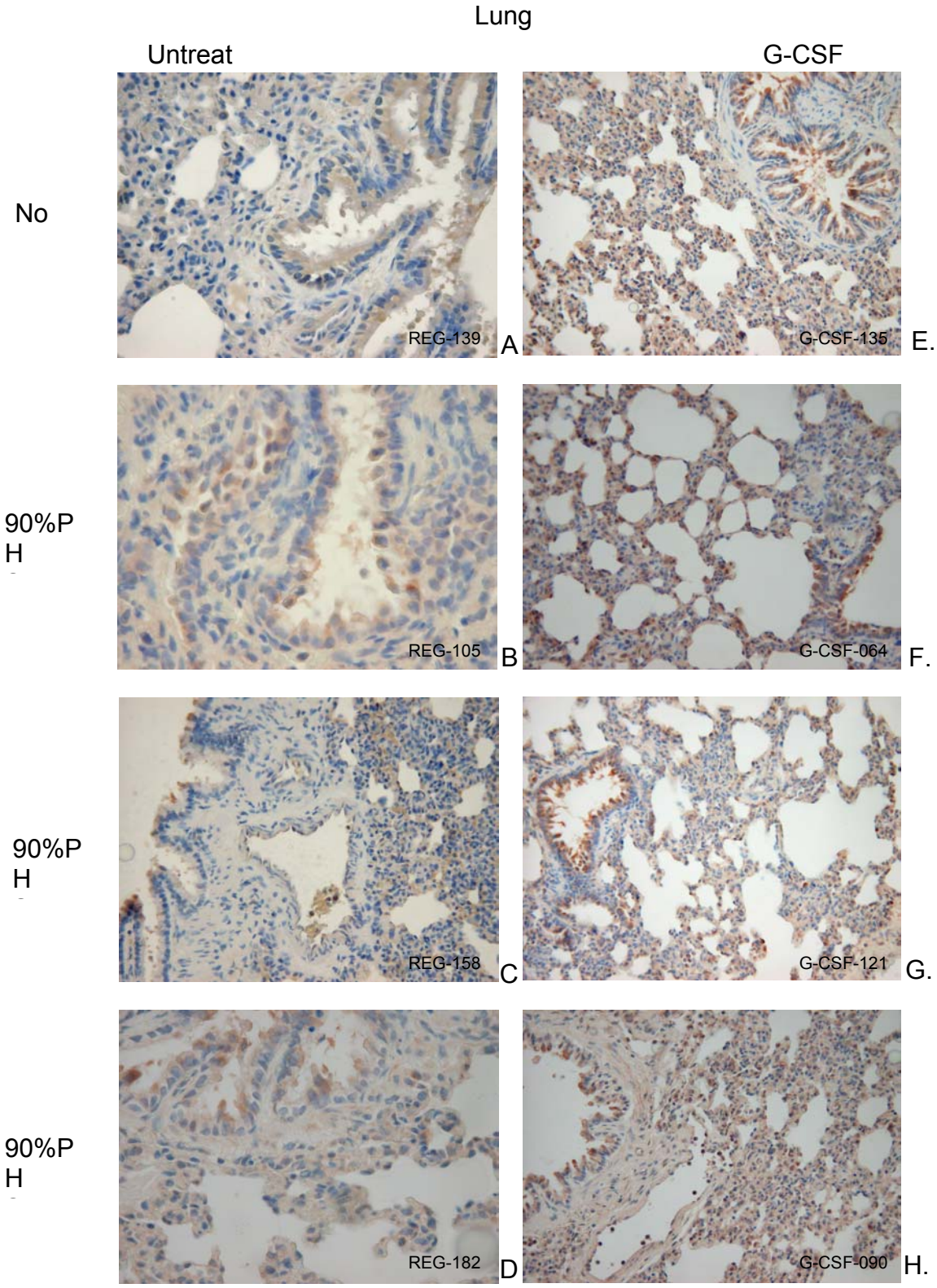

Figure 6. Immunohistochemical analysis of the G-CSF receptor in the rat lungs. Immunoperoxidase stain, haematoxylin counterstain. Original magnifications: 200x (C, E, F, G, H); 400x (A, B, D).

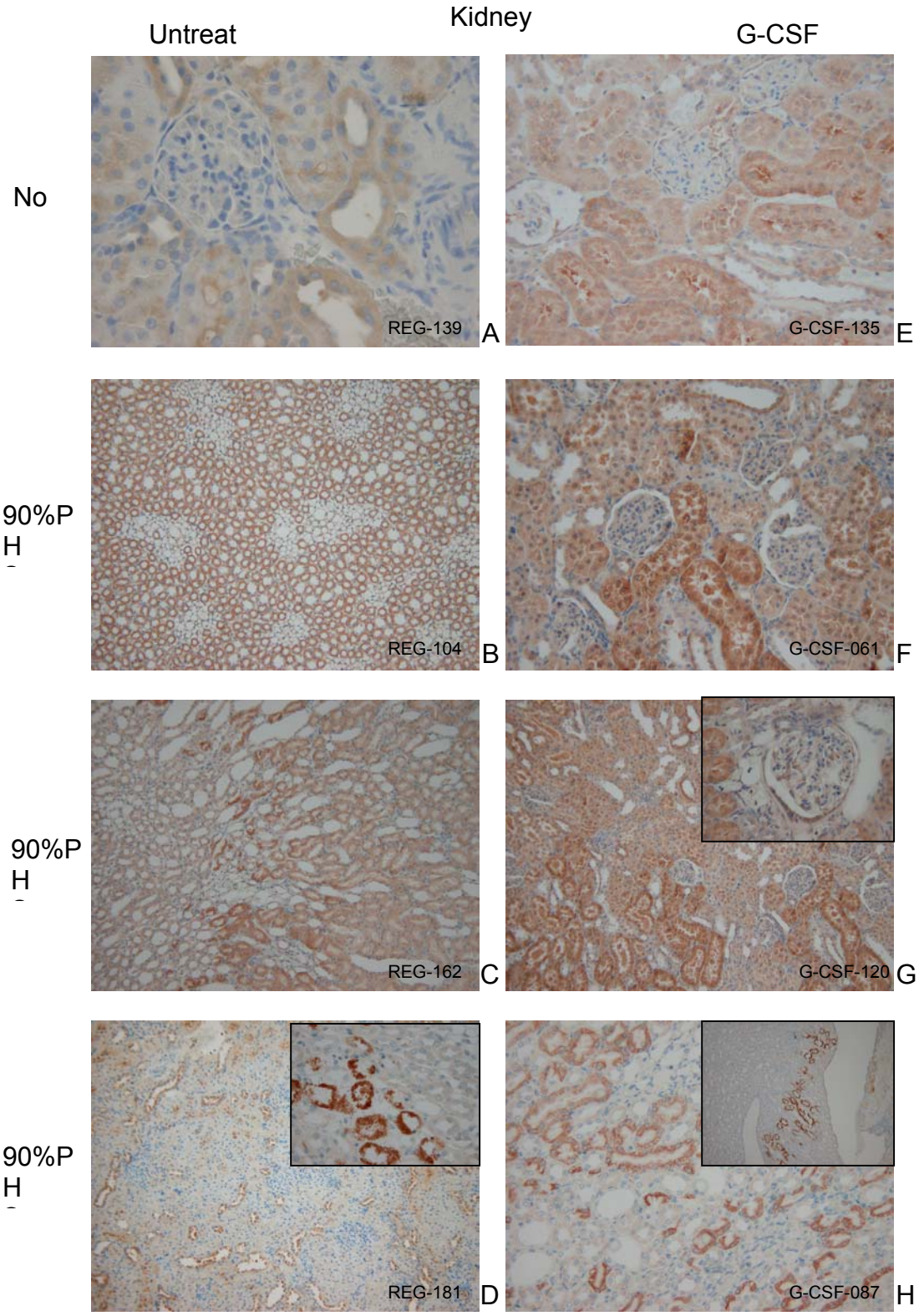

Figure 7. Immunohistochemical analysis of the G-CSF receptor in the rat kidneys. Immunoperoxidase stain, haematoxylin counterstain. Original magnifications: 100x (B, C, D); 200x (G, H); 400x (A, D-

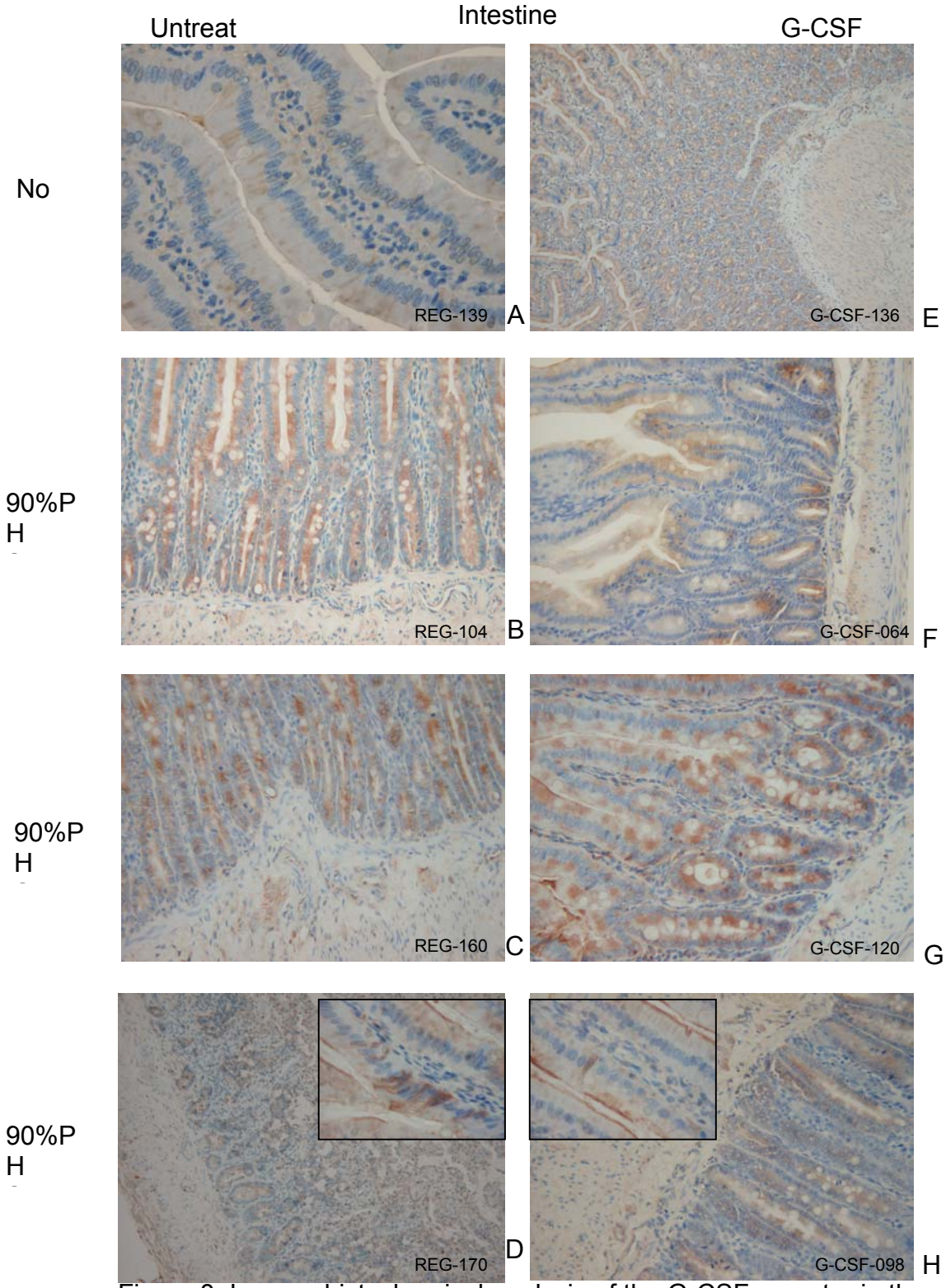

Figure 8. Immunohistochemical analysis of the G-CSF receptor in the rat intestines. Immunoperoxidase stain, haematoxylin counterstain. Original magnifications: 200x (B, C, D, E, F, G, H); 400x (A, D-insert, H-insert).

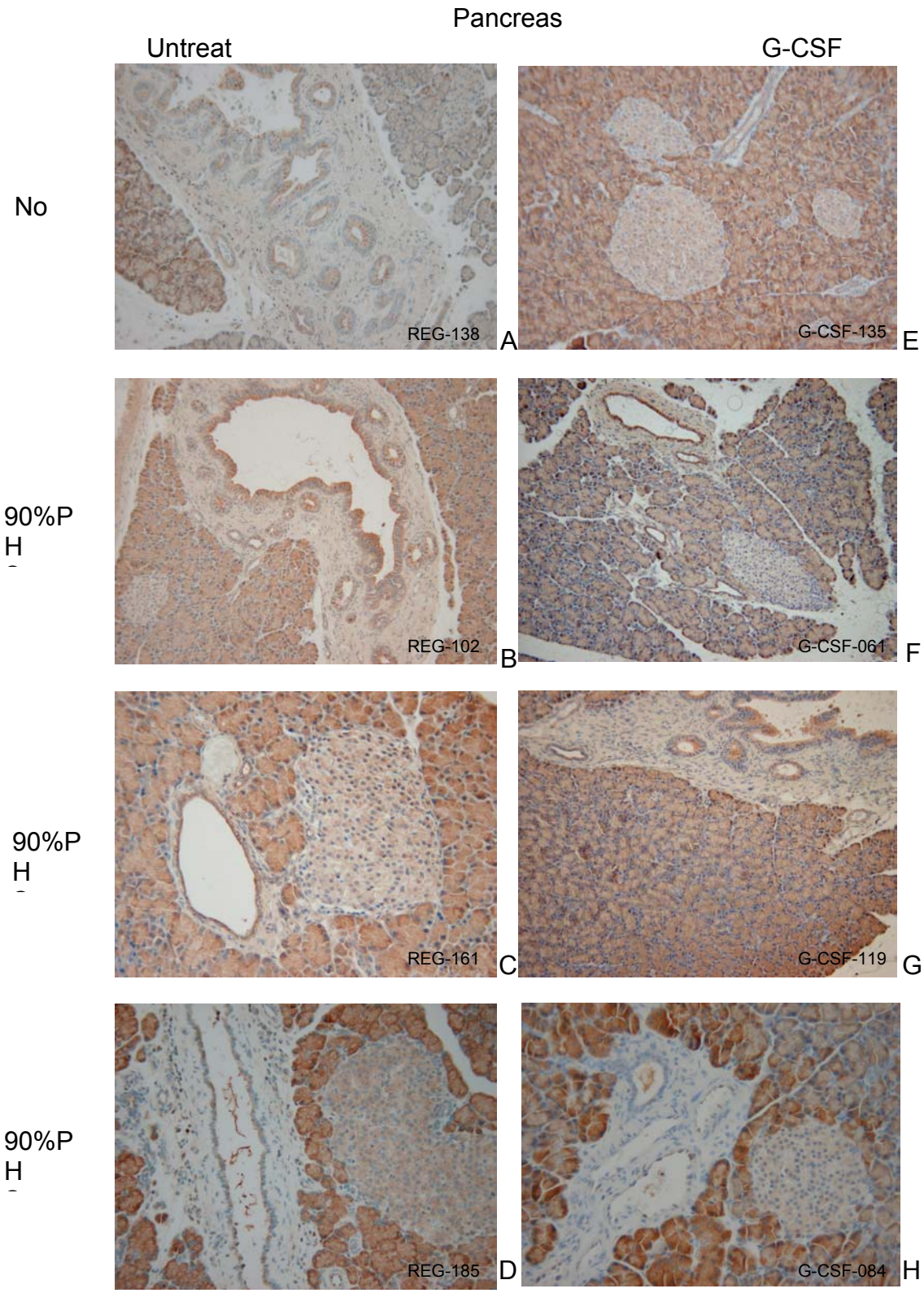

Figure 9. Immunohistochemical analysis of the G-CSF receptor in the rat pancreas. Immunoperoxidase stain, haematoxylin counterstain. Original magnifications: 200x (A, B, C, D, E, F, G); 400x (H).

Abbreviations

aa-dUTP	5-(3-Aminoallyl)-2'deoxyuridine 5'-triphosphate
ABC	avidin-biotin complex method
ALL	acute lymphocytic leukaemia
ATL	adult T-cell leukaemia
BM	bone marrow
BMSCs	bone marrow stem cells
CD	collecting duct
CCD	Couple charged decive
cDNA	Complementary DNA
CCEBP	CCAAT/enhancer-binding protein
CFU-Meg	colony-forming units of megakaryocyte
CRH	cytokine receptor homologous
DAPI	4,6 diamidino-2-phenylindole in phenylenediamine dihydrochloride
DEPC	diethylprocarbonate
DNA	Deoxyribonucleic acid
dNTP	deoxy-N-triphosphate
DRCs	Dendritic reticulum cells
dTTP	2'deoxyuridine 5'-triphosphate
E. coli	Escherichia coli
EB	ethidium bromide
EDTA	ethylene diamine tetraacetic acid
FDC	follicle dendritic cells
FISH	Fluorescence *in situ* hybridization
FITC	Fluorescein isothiocyanate
fMLP	f-met-leu-phe
G-CSF	Granulocyte colony-stimulating factor
G-CSFR	**Granulocyte colony stimulating factor receptor**

GFs	growth factors
GM-CSF	Granulocyte macrophage colony-stimulating factor
H&E	Hematoxylin-Eosin
HPF	high power field
HSC	hematopoietic stem cell
I/RI	ischemia/reperfusion injury
IDCs	Interdigitating cells
IFN- γ	interferon gamma
IHC	immunohistochemistry
IL-1 β	interleukin-1 beta
IL-12	interleukin-12
IL-6	interleukin-6
IL-6R	Interleukin-6 receptor
IMCD	inner medulla collecting duct
JTK-1	Janus tyrosine kinase-1
JTK-2	Janus tyrosine kinase-2
KAc	potassium acetate
LN	lymph node
LPS	lipopolyssacharide-
MAPK	mitogen-activated protein kinase
MF	mitotic figures
MNC	mononucleocyte
mRNA	message
MZ	marginal zone
NaCl	sodium chloride
NaOH	sodium hydroxide
NF-kB	nuclear factor kB
nt	nucleotide
OMCD	outer medulla collecting duct

PCR	polymerase cycle reaction
PH	partial hepatectomy
PMN	polymorphonucleocyte
rhG-CSF	recombinant human-G-CSF
RNA	Ribonucleic acid
rRNA	ribosome
RT-PCR	Reverse transcript-PCR
SDS	sodium dodecyl sulfate
ssDNA	single stranded DNA
ssDNA	single strand DNA
STAT 3	signal transduction and activators of transcription 3
TCR	T-cell receptor
TGFb-1	tissue growth factor beta-1
Th	T-helper/inducer lymphocytes
Th2	T-cell helper 2 type
TK-2	tyrosine kinase-2
TNF-α	tumor necrosis factor-alpha
TPA	phorbol ester
uPA	urokinase-type plasminogen activator

Summary

Background: Binding of the ligand**,** Granulocyte colony-stimulating factor (G-CSF) to its receptor, the G-CSF receptor (G-CSFR), is instrumental for the action of G-CSF in the different issues.

Aim: This study was designed to demonstrate the cellular distribution of G-CSFR in nonhematopoietic tissues of adult rats and the regulation of G-CSFR expression by administration of G-CSF and 90% partial hepatectomy (PH).

Methods: Part of the extracellular domain of G-CSFR was sequenced. A G-CSFR specific ssDNA probe was designed. Asymmetric PCR using amino-allyl dUTP and secondary labeling with FITC was established. G-CSFR mRNA expression in bone marrow and nonhematopoietic organs (liver, heart, lung, kidney, intestine, lymph node, spleen and bone marrow) of normal rats was assessed by RT-PCR and Northern hybridization. The cellular distribution of G-CSFR mRNA was detected by fluorescent in situ hybridization (FISH). The G-CSFR protein expression was visualized using immunohistochemistry (IHC). Regulation of the receptor expression subsequent to 90% liver resection (6h, 24h, 7d) with and without G-CSF-treatment were investigated by IHC.

Results: G-CSFR mRNA was found in all tested organs of normal rats by RT-PCR, and by Northern blot analysis. FISH and IHC on nonhemaopoietic tissue revealed G-CSFR receptor expression in the cytoplasma of hepatocytes, cardiomyocytes, type II pneumocytes, proximal convoluted tubular cells, single enterocytes in the intestine, acinar cells of the pancreas. G-CSFR expression was induced by G-CSF administration. Upregulation of G-CSFR expression was prominent 6 hours after 90%PH and an additive effect of G-CSF administration and 90%PH could be demonstrated.

Conclusions: G-CSFR is expressed in parenchymal cells in adult rats. G-CSFR expression can be upregulated by the administration of G-CSF and by a major surgical trauma as shown in the model of 90% liver resection. G-CSFR expression was most prominent 6 hours after PH and an additive effect of combined G-CSF

treatment was revealed. This could indicate a possible biologic significance of G-CSFR in the early phase of repair and regeneration processes.

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