行政院國家科學委員會補助專題研究計畫進度報告

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※動物供應-利用基因轉殖及基因剔除技術研究血液凝固機制及癌轉移(3/3)※
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執行期間：90 年　08 月　01 日至　91 年　07 月　31 日

計畫主持人：林淑華　教授
共同主持人：

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中　華　民　國　91 年 10 月 30 日

行政院國家科學委員會專題研究計畫進度報告

動物供應-利用基因轉殖及基因剔除技術研究血液凝固機制及癌轉移(3/3)

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一、中文摘要

Hepsin 為肝細胞膜上的蛋白(一種 serine protease),至今功能未明，從細胞培養實驗推測與細胞生長有關。最近證明可以活化凝血第七因子，因此 hepsin 是否與凝血起始機制有關急待證明。此外 hepsin 被認為與癌細胞轉移相關，主要因為癌細胞可藉著 fibrin 的屏障躲避免疫系統的清除，而 hepsins 又與 fibrin 生成相關，利用 RT-PCR 分析病人肝癌組織(病理科許輝吉教授提供)，發現 hepsin 大量表現。因此本實驗設計先作出 hepsin 剔除老鼠回答 hepsin 與細胞生長發育及凝血的關係，再利用 hepsin KO 老鼠得到 hepsin null 的肝癌細胞測定癌細胞轉移能力。目前在 Hepsin KO 小鼠的研究發現剔除鼠大致正常(已發表)。因此不易由剔除鼠實驗得知 hepsin 的生理功能。故將研究重點放在當將 hepsin 表達於血管內皮細胞與凝血因子直接接觸時是否影響胚胎發育，為回答此問題，本計劃擬製造 2 種 hepsin 轉殖鼠(Hepsin-Tg)，利用 Tie2 promoter 在血管內皮細胞大量表現人類 hepsin 蛋白，另一種表現 hepsin 突變蛋白(hepsin mutant)，此突變蛋白將具有結合受質但不活化受質的功能。將此 DNA 注射於小鼠受精 0.5 天之原核中後，取出胚胎第 12 天的轉殖鼠，經 β-gal 染色，可清楚見到小鼠胚胎心血管組織部份有陽性染色結果。接著在統計轉殖鼠出生數目後，我們可以發現，無論注射 FVB 或 C57BL/6 strain 原核所出生帶有轉殖基因的轉殖鼠在出生 24 小時內皆有約 50%高死亡率。反之不帶有轉殖基因或是注射 mutate hepsin 基因的轉殖鼠出生都有近八成以上的存活率。加上出生小鼠帶有轉殖基因比例不高（20%），推測胚胎發育期中，亦可能死亡，在分析了各天數的轉殖鼠，發覺於第 13 天的胚胎存活率明顯下降至 18%，相較於注射對照 DNA 第 13 天胚胎依有 47.5%的存活率，且在第 13 天陽性染色胚胎數減少了 60%，推測帶有轉殖基因的轉殖小鼠於第 13 天有胚胎死亡的現象。然而對於小鼠的死亡原因相關的組織及病理分析正在進行。

關鍵詞：基因轉殖、基因剔除、Hepsin、Tie2

Abstract

This project is aimed at dissecting the role of hepsin by transgenic technology. We have created hepsin knockout mice (published). Hepsin null mice were phenotypically normal, fertile and showed no apparent difference from wild-type mice in blood coagulation and blood chemistry. This indicates that hepsin is not essential for embryogenesis. It is also not critical for the maintenance of basal level blood coagulation. To further characterize hepsin's interaction with factor VIIa, a plasma clotting factor essential for the initiation of blood coagulation, we have created hepsin transgenic mice with wild-type and active-site mutated hepsin gene under the control of the Tie2 promoter and enhancer. We generated transgenic mice with overexpressed hepsin in vascular endothelial system, which is in direct contact with plasma coagulation factors, aiming at revealing the relationship between hepsin and these proteins.Hence we generated two

transgenic constructs, each containing Tie2-promoter-driven wild-type and catalytic-site-mutated hepsin cDNA respectively, and had them microinjected into the pronuclei of E0.5 mouse embryos. Subsequently, E12.5 embryos were harvested and subjected to β-gal staining in which the cardiovascular tissue of these embryos showed positive results. We observed a ~50% mortality rate within 24 hours after birth for newborn hepsin transgenic mice compared to ~20% of control and hepsin mutant transgenic mice. There was only ~20% of the newborn mice carrying the transgene, indicating that some might have died in the womb. The survival rate of the E13 embryos injected with wild-type hepsin construct significantly dropped to 18% if compared to 47.5% of control. And compared with earlier stage (E11), positive samples of β-gal staining percentage also demonstrated a reducing of 60%. Presumably, these transgenic mice had died in the womb at 13 days p.c..In conclusion, hepsin overexpression in vascular endothelial cells might cause embryonic lethality, comparable to the short-term survival of only a few of those transgenic mice. And related histological and pathological mechanism are under studing.

Keywords: Gene knockout, Transgene, Hepsin, Tie2

二、緣由與目的

科學研究者目前可利用基因轉殖及基因剔除的技術來研究動物體內生理現象，基因轉殖即是在實驗鼠染色體內嵌入特定基因，而基因剔除則是將染色體內特定基因破壞。將單基因剔除的實驗鼠互相交配，則可製造出雙基因(甚至多基因)剔除的老鼠，這樣的老鼠無疑是研究相關基因群的最佳實驗材料! Hepsin 被認為與細胞生長、細胞移動有關、並可能參與正常血液凝固的起始(藉由活化第七因子)(1,2)、及癌症狀況下高度血栓的機制有關(3,4)。本實驗室已成功地製造出 hepsin 基因剔除鼠。由本實驗室結果得知，缺乏 Hepsin 的老鼠不但可正常生長且可生育，表示 hepsin 在胚胎發育中並不是必要的。然而對於 hepsin 參與血液凝固"起始"的探討，自始是十分重要卻又很難證明的。此外，hepsin 是否因活化第七因子而參與癌細胞轉移也待證實。為了瞭解 hepsin 與凝血的關係，提出本計劃期望達成下列的目標:

(A) 製造在血管表皮細胞過度表現 hepsin 基因的轉殖老鼠。

(B)研究 hepsin 基因剔除鼠及轉殖鼠的表現型並著重探討 hepsin 在凝血機制的角色。

為了直接探究 hepsin 與血液凝固的關係，本計劃將 hepsin 表現於血管內，使 hepsin 與凝血因子(尤其第七因子)直接接觸，以偵測凝血的變化。利用 Tie2(angiopoitin receptor)(5,6)啟動子可達成目標，選擇 Tie2 不僅可將 hepsin 直接暴露於血液中更可以在胚胎發育的更早期時引發 hepsin 的表現(Tie2 在 E7.5 表現，而 hepsin 是 E13.5)，這樣的體內試驗可輕易地藉由觀察老鼠表徵及出生率而得知 hepsin 的影響。

三、結果與討論

本實驗室從 1995 年開始從事 hepsin knockout (KO) mice 的實驗,目前成果有:(Ⅰ)在本院設立了 KO 實驗室,能獨立完成所有的工作,包括 ES 細胞培養,分離並建立 primary fibroblast feeder 及 gene targeting 與顯微注射(Ⅱ)目前已成功剔除 hepsin 基因及 pig-a 基因,已有 2 篇論文發表(文獻 7,8)。

目前在 Hepsin KO 小鼠的研究發現剔除鼠大致正常。因此不易由剔除鼠實驗得知 hepsin 的生理功能。故將研究重點放在當將 hepsin 表達於血管內皮細胞與凝血因子直接接觸時是否影響胚胎發育，表達載體 DNA 經過特別的設計，包含了由 Tie2 啟動子所啟動的 hepsin wild type cDNA 和 IRES-NLS-LacZ reporter gene 以及 Tie2 enhancer；pTHIZ^{Mut}-Su，由 wild type hepsin 經 mutagenesis 後將其活化區突變的 hepsin。在 BEC 細胞中短暫性轉染此基因轉殖質體後，經β-gal 染色，可見細胞核中有藍色濃染，故確認此質體的功能。

將此DNA注射於小鼠受精0.5天之原核中後，取出胚胎第12天的轉殖鼠，經β-gal染色，可清楚見到小鼠胚胎心血管組織部份有陽性染色結果。接著在統計轉殖鼠出生數目後，我們可以發現，無論注射FVB或C57BL/6 strain原核所出生帶有轉殖基因的轉殖鼠在出生24小時內皆有約50%高死亡率。反之不帶有轉殖基因或是注射mutate hepsin基因的轉殖鼠出生都有近八成以上的存活率。加上出生小鼠帶有轉殖基因比例不高（20%），推測胚胎發育期中，亦可能死亡，在分析了各天數的轉殖鼠，發覺於第13天的胚胎存活率明顯下降至18%，相較於注射對照DNA第13天胚胎依有47.5%的存活率，且在第13天陽性染色胚胎數減少了60%，推測帶有轉殖基因的轉殖小鼠於第13天有胚胎死亡的現象。

然而這樣的一個策略是建立在以往對hepsin的研究，對於hepsin的生理功能至今尚未明瞭，在活體中亦沒有直接的實驗結果證明hepsin調控的機制，且hepsin完全缺損的小鼠，依然正常，且可以生長、發育、繁衍，顯示hepsin並非是絕對須要的。在轉殖鼠的基因型分析上可以發現，轉殖鼠在經PCR確認後，不論是FVB或C57BL/6小鼠，其死亡比例皆相當高(60%~90%)，且都是出生後的24小時內死亡；再加上針對胚胎各時期的數目和基因型的分析，也發現在E13天胚胎數目有明顯減少且陽性LacZ染色比例亦下降。相對於無轉殖基因嵌入的小鼠，其死亡比例則較低，另一方面，注射活化區突變的hepsin基因的小鼠，無論基因型是陽性或陰性其死亡率皆相當低，造成此結果可能是hepsin於內皮細胞大量表現而造成生理失衡。然而目前的組織學初部分析尚無預期之血栓產生，所以針對小鼠的死亡原因及機轉仍須有更多的病理研究。此外本實驗室也正製備hepsin單株抗體對於後續的實驗將有一定的幫助。

三、意見及需求

(1)合作支援:主持人已碰到一些問題，當特殊情況需定期由成大醫學院購入小鼠時，發現台灣找不到室溫(22-24 °C)運送老鼠的快遞服務，已往在夏天運送時常有10-20%的死亡率，對使用者不便。對與他校的合作支援也大打折扣，希望這方面能得到國科會的幫助。

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Mice Deficient in Hepsin, a Serine Protease, Exhibit Normal Embryogenesis and Unchanged Hepatocyte Regeneration Ability

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Key words

Hepsin, gene targeting and liver regeneration

Summary

Hepsin, a liver-enriched novel serine protease, has been implicated in participating with normal cell growth, embryogenesis, and blood coagulation pathway. To study its function *in vivo*, we have disrupted the mouse hepsin gene by homologous recombination. Targeted disruption of the hepsin gene and ablation of hepsin message were demonstrated by Southern blotting, Northern blotting and RT-PCR analysis. Homozygous hepsin -/- mice were viable, fertile, and exhibited no gross abnormalities, as judged by the size, weight and blood coagulation (PT) assays. However, the serum concentration of the bone form of alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase of the hepsin -/- mice was mildly elevated, in spite of no obvious pathological change of hepatocytes. To examine whether hepsin is involved in controlling cell growth in adult tissues, 70% hepatectomy was applied to the hepsin -/- mice. Liver regeneration proceeded normally in the hepsin -/- mice as judged by the liver mass restoration rate. These results suggest that loss of hepsin function causes no effect in cell growth and embryogenesis *in vivo*, which is in contradiction to the studies using in vitro cell culturing system. Moreover, gross mass regeneration of liver after damage proceeds normally in the absence of functional hepsin.

Introduction

Hepsin is a type II transmembrane protein with a hydrophobic region of 27 amino acids located at the N-terminus (18 amino acids down from the first methionine) serving as a transmembrane signal, whereas a carboxyl terminus featuring the serine protease function exposed extracellularly (1, 2). The primary amino acid sequence of hepsin is highly homologous to trypsin and trypsin-like blood clotting factors, and it is likely to be synthesized as an inactive zymogen, which is converted to an active enzyme by cleavage at the site of residues Arg162-Ile163 (1, 2). The mechanism regulating hepsin's activity by proteolysis is not quite known, although autocleavage has recently been proposed (3). Enzymatically activated hepsin is a two-chain molecule consisting of the non-catalytic amino-terminus (residues 1-162) held by disulfide bonds to the catalytic carboxyl-terminus (residues 163-417). Expression of hepsin is not tissue-restricted. Its transcript is detected abundantly in liver, while low level of expression is also detectable in kidney, pancreas, testis, lung, thyroid, and pituitary gland in mammals such as human, baboon, and mouse (4, 5). It is barely detectable in human endothelial cells, smooth muscle cells and skin fibroblasts. Several cell lines including hepatoma cell lines and some primary tumors such as ovarian cancer and renal cell carcinoma also have elevated expression of hepsin (5, 6).

Several lines of evidence indicate that hepsin may function in controlling normal cell growth as well as in activating coagulative factor VII to its active form-VIIa (4, 7). Growth arrest was observed in HepG2, a hepatoma cell line expressing hepsin, when the cells were treated with anti-hepsin antibodies or hepsin-specific antisense oligonucleotides (4). The expression of hepsin was first detected in preimplantation mouse embryos as early as the two-cell stage (3), and later in actively growing tissue of mice embryos beyond day E10.5 (8), suggesting its involvement in developmental processes. In addition, the cytoplasmic tail of hepsin might participate in intracellular signal transduction pathway since an alternatively- spliced RNA transcript is found, which codes for an extra 20 amino acids with a twisted structure (3, 9, 10). The role of hepsin in initiating blood coagulation is supported by transfection experiments, which showed that by expression of hepsin in baby hamster kidney (BHK) cells, the cells specifically converted exogenously added factor VII to active factor VIIa, and ultimately leading to the generation of thrombin (11). Thrombin is involved in a lot of physiological and malignant processes, with or without the coupling of its receptor(s) (12-18).

The role of hepsin in development and coagulation was recently examined with hepsin-deficient mice (19). The results indicate that hepsin is not essential for embryonic development and normal hemostasis. Here we report the generation of hepsin-deficient mice by using a targeting vector to cause genomic deletion of hepsin's exon 9 and 10. Exon 9 and 10 of hepsin encode amino acid residues 186 to 270, including the histidine and aspartic acid residues of the catalytic triad (residues 202 and 256, respectively, numbering system following ref. 3). We therefore reasoned that such deletion might cause functionally inactive hepsin, which is confirmed by the absence of hepsin's transcript and protein. The hepsin null mice exhibit no apparent abnormality in embryogenesis and organogenesis, which is consistent with the other

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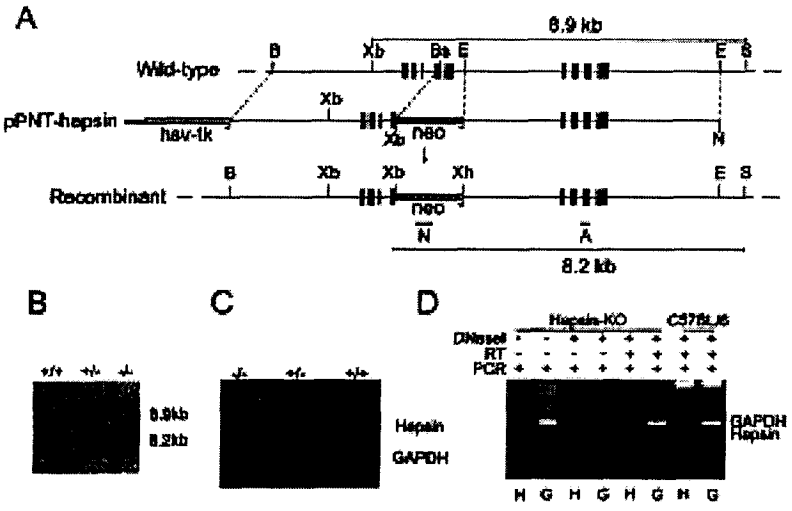

Fig. 1 Targeted disruption of the mouse *hepsin* gene. Panel A. Genomic structure and restriction map of the murine wild-type hepsin, the pPNT-hepsin plasmid, and the recombinant hepsin allele after homologous recombination are shown. Location of the probes (N and A), and the sizes of diagnostic fragments are indicated. Filled blocks, exons; arrowhead, direction of the neo and hsv-tk genes (hatched and gray areas, respectively). Panel B. Genotyping by Southern blot analysis. Genomic tail DNA were isolated from wild-type (+/+), heterozygous (+/-), and homozygous (-/-) hepsin knockout mice and digested with *Xba*I (Xb) and SacI (S). The NC blot was probed with probe A. The bands corresponding to the wild-type (8.9 kb) and mutant (8.2 kb) are indicated. B: *Bam*HI, E: *Eco*RI, N: *Not*I, Xh: *Xho*I, Bs: *Bst*XI. Panel C. Northern blotting using full-length human hepsin and GAPDH probe. RNA was prepared from liver as described in "Materials and Methods". +/+, wild-type mouse; +/-, heterozygous; -/-, homozygous hepsin knockout mice. Panel D. RT-PCR of total liver RNA from hepsin homozygous KO mice and wild-type C57BL/6J mice. Reactions with PCR only, with DNaseI treatment prior to PCR, or with DNaseI treatment prior to RT experiments were indicated above each lane. H and G stand for PCR reaction using hepsin and GAPDH specific primers, respectively

report. We further examined hepsin's role in hepatocyte regeneration in adult mice, since it has been proposed to play a role in controlling normal cell growth in fast-growing tissues (see above). We found that the hepsin null mice have an unchanged liver regeneration rate even after 70% partial hepatectomy (PH) compared with that of normal ones. Our results suggest that hepsin is not required for embryogenesis and also is not essential for regulating cell growth, which is obviously contradictory to the results obtained from the *in vitro* cell culturing system.

Materials and Methods

Targeting vector. Isogenic hepsin genomic DNA was obtained by screening a 129/sv-mouse genomic library (a gift of Dr. Begue, Pasteur Institute, France) with a human hepsin cDNA (obtained by RT-PCR of a human liver RNA with primer 5'-TGAGTGGGCCGGCCACTGTGG AAGAGAGG-3' for reverse transcription and 5'-AGGTCAGCCAGGGAATCATTAACAAGA G-3' and 5'-GTCAGGGCTGAGTTACCAT GCCG-3' for PCR). A clone with an insert size of approximately 18 kb, containing most of the genomic sequence of hepsin except exon 1 to 5'-portion of intron 1 was isolated and used in constructing the targeting vector. Disruption strategy was to interrupt and delete the coding sequence of hepsin from the 186th codon in exon 9 to the 270th in exon 10 by replacing the deleted region with the neomycin resistant gene from pPNT (20). The 5' homologous region was a 3.5-kb *Bam*HI-*Bst*XI fragment, containing sequences from intron 5 to exon 9 truncated at the *Bst*XI site, and the 3' homologous region was a 6-kb *Eco*RI fragment with sequences from intron 10 to intron 14. Both fragments were isolated from the 18 kb insert by digestion with appropriate enzymes, changed to *Bam*HI and *Xho*I sites, respectively, and subcloned to the unique *Bam*HI and *Xho*I sites of pPNT. The resulting targeting construct, pPNT-hepsin, is 16.7 kb in size (Fig. 1A).

Generation of hepsin-deficient mice. Embryonic stem (ES) cells (from Dr. C. Babinet, Pasteur Institute) were grown and maintained as described (21). The pPNT-hepsin was linearized with *Not*I and electroporated into the ES cells, which were then selected in media containing G418 and gancyclovir as described (21). Electroporation condition was 220 V and 975 μF. Genomic DNA isolation and Southern blotting were performed with the survival ES cell clones following standard procedure (22). The ES cell clones with correct homologous recombination were introduced to blastocysts of C57BL/6J mice by microinjection using standard technique (22). Breeding of the chimeric mice was done by crossing the hepsin konckout mice with C57BL/6J to generate F1; F2 was generated by intercrossing of F1 littermates. Tail DNA preparation followed standard procedures (22-24). The identification of the F1 heterzygous and F2 homozygous hepsin knockout mice was by Southern blotting analysis of the tail DNA as described for ES cells. PCR was performed with tail DNA to screen for pups of subsequent breeding of the F1 and F2 mice. The PCR condition was 35 cycles of 94° C, 30 sec, 60° C, 1 min and 72° C, 2 min. The oligonucleotide primers used were 5'-AGGAAGC TGCCGGTGGACCG-CATT-3' (forward) in intron 9 pairing with 5'-AACTGTTCGCCAGGCT-CAAGGC-3' (reverse) in the neo gene for the recombinant allele (the product size was 0.9 kb), and pairing with 5'-CCGAGACAGGACCC GGTTCC-3' (reverse) in the deleted exon 10 for the wild-type allele (product size, 0.27 kb), respectively. The PCR mixtures contained 0.3-1 μg tail DNA, 200 μM dNTP, 200 nM each of the primers, 2.5 U Taq DNA polymerase in 50 μl reaction buffers supplied by the manufacturer (Amersham Pharmacia Biotech Inc., UK).

RNA isolation, Northern blotting analysis, and RT-PCR. Total RNA was extracted from tissues using TriZol reagent (GibcoBRL, USA) following manufacturer's instructions. Northern blotting was performed using the glyoxal method as described (25). In brief, 20 μg of total RNA was fractionated on 1% glyoxal-denaturing gel, transferred onto the nylon membrane (Nytran plus, Schleicher & Schuell, Dassel, Germany), and hybridized at 42° C to ^{32}P-labeled full-length human hepsin cDNA prepared by random priming (rediprime II, Amersham Phamacia Biotech, UK). After washing away the unbound probes, the signal was visualized by autoradiography. The same membrane was subsequently stripped and re-probed with ^{32}P-labeled human glyceraldhyde-3-phosphate dehydrogenase (GAPDH) cDNA as control for RNA integrity. For RT-PCR, 5 μg of total RNA was first reverse transcribed into cDNA using reverse transcriptase (Superscript RT, GibcoBRL, USA) and random hexamers. The cDNA was amplified by PCR for 35 cycles of 94° C, 1 min, 55° C, 2 min and 72° C, 2 min using primers specific for hepsin (forward: 5'-GGCACATCGG-GCT TCTTCTG -3' and reverse: 5'-CCGAGACAGGACCCGGT TCC-3') or

GAPDH (forward: 5'-GACCACAGTC CATGCCATCAC-3', and reverse: 5'-TCCA CCACCCTGTTGCTGTAG-3'). The GAPDH was used as an internal control. The PCR products were analyzed by electrophoresis on 2% agarose gel. Parallel samples were subjected to DNaseI treatment (GibcoBRL) prior to the RT reaction to eliminate the possibility of genomic DNA contamination.

Clinical chemistry and blood clotting assay. Whole blood was collected periodically by puncture of the retro-orbital plexus of mice using capillary tubes and allowed to clot at room temperature. Serum samples were isolated by centrifugation (5000 rpm, 10 min, Eppendorf 5415C, Germany) and were analyzed for blood chemistry items including total protein, albumin, total billirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), amylase, creatine kinase (CK), lactate dehydrogenase (LD), and γ-glutamyltransferase (γ-GT) by the facilities (Hitachi 7170A Automatic Analyzer, HITACHI, Japan) at the Clinical Laboratory of the National Taiwan University Hospital. ALP isoforms were separated by eletrophoresis of 20 μl serum samples on Hydragel ISO-PAL affinity agarose gel at 100 V for 40 min, and visualized by staining with substrates for ALP (Indolyl phosphate and NBT, Nitro-Blue Tetrazolium) according to the manufacturer's instructions (Hydragel ISO-PAL kit, Sebia, France). For blood clotting assay, prothrombin time (PT) was measured with citrated plasma as described (26). Mouse whole blood was collected in 3.8% sodium citrate buffer at a ratio of 9:1 and centrifuged at 10,000 rpm (Eppendorf 5415C) for 5 min to separate plasma from blood cells. PT assays were performed by adding 100 μl plasma to 200 μl of tissue thromboplastin reagent (Thromborel S, Behring, Germany) and the time for fibrin clot formation was monitored and recorded on Fibrin Timer 2 (Behring).

Partial hepatectomy (PH) and evaluation of liver regeneration. Liver regeneration was induced in 8- to 10- week-old mice by 70% partial hepatectomy as described by Higgins and Anderson (27). Under Ketamin anesthesia, the medial and left lateral lobes of the liver were ligated at the vascular stalk and removed. The rate for liver regeneration was estimated by the mass restoration rate and calculated as follows: actual weight of remnant liver divided by the weight of prehepatectomy liver × 100%, where prehepatectomy liver was 3/2 weight of initially resected liver. In vivo BrdU labeling was also used to evaluate hapatocyte DNA synthesis during liver regeneration. BrdU (Sigma, 10 μg/g body weight) was injected intraperitoneally 2 hr before sacrifice. The regenerated liver was harvested at 0, 24, 48, and 96 hr after PH, 4% formaline fixed, sectioned and stained with antibodies to BrdU (BrdU staining kit, Oncogene). Positive, dark-stained hepatocyte nuclei were counted in 10 different 400× fields/tissue section. Tissue sections from 3 hepsin KO mice and wild-type control mice were evaluated at each time point after PH.

Histology. Liver samples were fixed in 10% neutral-buffered formalin and processed according to standard methods. Staining used hematoxylin and eosin (H & E), and microscopic examination was performed on regular microscope (Nikon, Model Microphot-XFA).

Results

Generation of hepsin-deficient mice. As shown in Fig. 1A, the pPNT-hepsin contained the neomysin-resistant expression cassette replacing the part of exon 9 and the entire exon 10 of the hepsin genomic sequence. The resulting targeted recombination event with this vector causes deletion of the mouse hepsin from amino acid residues 186 to 270, which include the Thr-Ala-Ala-His-Cys active-site histidine (residues 199-203) and the Asp-Ile-Ala-Leu-Val active-site aspartic acid (residues 256-260). Southern blotting of *XbaI* and *SacI* double-digested genomic DNA from ES cell clones and their derived mice with 3'probe would detect a 8.2 kb fragment in a recombinant allele, whereas the wild-type allele would yield a 8.9 kb fragment (Fig.1A and 1B). A total of 250 ES cell clones surviving G418 and gancyclovir selection were analyzed by Southern blotting and 5 clones showed heterozygous F1 (hepsin ±) with two fragments of 8.2 kb and 8.9 kb, compared with the wild-type with 8.9 kb signals. Homozygous F2 hepsin null (hepsi -/-) mice were successfully breeded by mating heterozygous F1.

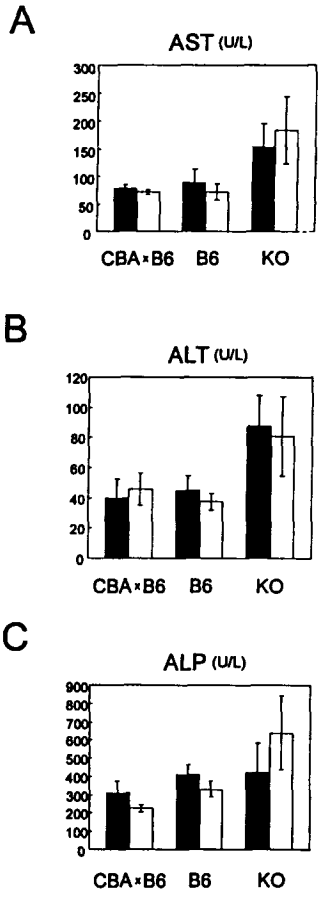

Fig. 2 Comparison of serum AST, ALT and ALP levels in hepsin knockout mice and wild-type mice. Serum samples were prepared from 8 to 10 weeks old male (black bar) and female (white bar) mice as described in materials and methods. Panel A. Serum AST level. Panel B. Serum ALT level. Panel C. Serum ALP level. The numbers of mice used in each group were as follows. CBA × B6 : male, n = 14; female, n = 17. B6 (C57BL/6J): male, n = 11; female, n = 14. Hepsin knockout mice (KO): male, n = 10; female, n = 13. Data are presented as the mean ± SD. CBA × B6: F1 mice of CBA strain crossed with C57BL/6J

To verify that the targeting construct completely eliminated hepsin RNA expression, Northern blotting and RT-PCR were performed with RNA from putatively hepsin-null mouse liver tissues. Northern blotting (Fig. 1C) demonstrated complete absence of the hepsin RNA in the F2 hepsin -/- mice evidenced by the lack of both the 1.8 kb and the 1.9 kb alternatively spliced form (3, 9), which was in turn detected in the liver of wild-type or heterozygous F1 mice. Moreover, we did not detect any truncated or fusion forms of hepsin (size checked from 0.1kb up). A 1.5 kb human GAPDH cDNA fragment was used as an internal control. The results were further confirmed by more sensitive RT-PCR experiments (Fig. 1D). Showing an absence of hepsin message in the liver. We conclude that the hepsin null mice were successfully produced.

Serum levels of ALP, AST, and ALT are mildly increased in hepsin deficient mice. Upon observation over 50 weeks, the hepsin -/- mice were viable and fertile without any gross abnormality, suggesting that lack of hepsin causes no effect in embryogenesis, normal life sustaining process and fertilization. The male to female ratio of the hepsin -/- mice is 1:1. Since hepsin is implicated in the regulation of coagulation, we have examined the bleeding time of the hepsin -/- mice. As judged

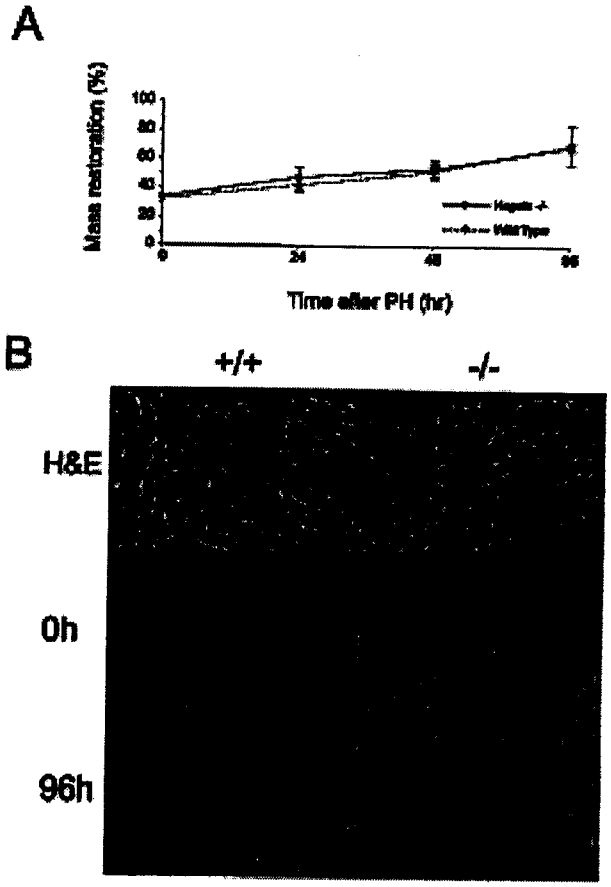

Fig. 3 Evaluation of liver regeneration. Panel A. Rate of liver mass restoration after partial hepatectomy (PH). An average of six animals were examined in each group at each time point. The restoration rate at the indicated time point was calculated as described under "Materials and Methods". Panel B. H & E stain (top panels) and BrdU labeling of liver tissue before (0 h) and after (96 h) PH. Liver samples were taken at 0 h and 96 h after PH. Representative photomicrographs of liver sections are shown from wild type (+/+) and hepsin-KO (-/-) mice. H& E stain were samples prior to PH. The dark-stained nuclei indicated BrdU-labeled nuclei of hepatocytes. (Magnification = × 200)

by a tail bleed, the bleeding time of the hepsin -/- mice was similar to that of wild-type and heterozygous hepsin +/- mice. The PT clotting time was also similar for hepsin -/-, hepsin +/-, and wild type mice. The averaged values were 8.7 ± 0.35 sec (n = 10) and 8.69 ± 0.58 sec (n = 11) for hepsin -/- male (former) and female (latter) mice, compared with 8.71 ± 0.34 sec (n = 10) and 8.5 ± 0.25 sec (n = 10) for wild-type male and female mice, respectively. This indicates that hepsin is not essential for the VIIa-TF dependent extrinsic coagulation pathway. Blood chemistry analysis was performed to search for any pathological condition. The results showed that the homozygous hepsin -/- mice contained elevated levels of alkaline phosphatase (ALP) and mildly elevated AST and ALT, all being the markers of hepatocellular damage, compared with those of the wild-type mice (Fig. 2). The increased levels of ALP, AST, and ALT seem to be gender-independent since both hepsin -/- female and male mice showed the same results. Further analysis for ALP isoenzymes demonstrated that the elevation was due to increase of the bone form ALP (data not shown). The ALP result is in agreement with the data of previously reported hepsin null mice (19). However, the AST and ALT results are not consistent with the previous finding. The reason for increased ALP, AST and ALT of hepsin -/- mice is unclear at this moment. We did not observe any morphological abnormalities of hepatocytes such as inflammatory cell infiltrate, degeneration, necrosis, apoptosis by H&E stains or hepatitis virus infection (by ELISA testing of the presence of circulating antibodies specific for mouse hepatitis virus). Other parameters of liver function such as the level of total protein, albumin, total bilirubin, amylase, creatine kinase, lactate dehydrogenase and γ-GT in hepsin null mice is similar to those of the wild-type mice (data not shown).

Liver regeneration rate is unchanged in hepsin deficient mice. Since hepsin null mice showed no defect in embryogenesis, we further examined whether it is involved in controlling cell growth in the adult stage based on reports from in vitro assay by Tsuji et al. (4). Groups of hepsin -/- and wild-type mice were subjected to a two-thirds partial hepatectomy (PH) to induce liver regeneration. The liver mass restoration was estimated at 24, 48, and 96 hours after PH. As shown in Fig. 3A, the liver regained its mass up to 70% within 96 h after PH in our study. However, no significant difference in liver mass restoration was seen at each time point. The hepatic DNA synthesis rate after PH was also evaluated using BrdU in vivo labeling. Similar numbers of BrdU positive hepatocytes were obtained at 24 h, 48 h and 96 h, after PH from wild-type and hepsin-KO liver section. An example of 96 h after PH is shown in Fig. 3B. The morphology of regenerated livers also exhibit no histologically detectable differences between wild type and hepsin -/- mice. This result indicates that hepsin is not involved in hepatocyte growth and proliferation after PH and is not necessary for liver regeneration in adult mice.

Discussion

To gain a better understanding of the in vivo function of hepsin, a novel serine protease, we have generated mice carrying a disrupted hepsin gene. Mice deficient in hepsin are viable, fertile, and exhibit no gross abnormalities, as judged by the size, weight and blood coagulation assay. These are in agreement with the report of Wu et al. of hepsin knockout mouse that exhibited a normal hemostasis function (19). Blood coagulation is thought to be triggered by the formation of factor VII (VIIa) and TF complex. The presence of basal levels of factor VIIa in circulation could contribute to the physiological hemostasis, but its origination is still under debate. Factor IXa, factor Xa or factor VIIa-TF have been investigated as the key regulators of basal levels of factor VII (VIIa). Factor IXa apparently plays a major role in maintaining this normal plasma concentration of factor VIIa since patients with hemophilia B, but not patients with hemophilia A, have been found to have significantly reduced levels of factor VIIa (28). The implication of hepsin being capable of activating VII to VIIa provide a new and attractive viewpoint to the initiation of blood clotting (7, 11). One explanation for the normal blood clotting ability observed in hepsin -/- mice is that the redundancy of groups of serine proteases regulating the cascades of coagulative processes. Another possibility is that other coagulation factors such as factor IXa compensate for the function of hepsin. Clearly, the phenotype of mice lacking both hepsin and factors in the coagulation pathway will be informative as it directly addresses the question of the origin of basal level factor VIIa in the circulation system.

In addition, the liver of hepsin null mouse developed normally and showed no difference in the hepatocyte regeneration rate, as demonstrated by the partial hepatectomy experiment. Mammalian liver has the unique ability to undergo regeneration after injury (29). The process of

liver regeneration is regulated by lots of growth factors and thus provides a useful model in studying hepatocyte growth and proliferation in vivo (29). The lack of difference in liver mass restoration observed in our hepsin knockout mice indicates that hepsin is not required for liver regeneration in mice. One explanation is that hepsin did not participate in postnatal hepatocyte growth and proliferation as suggested by studies using in-vitro cell culturing system (8). Alternatively, loss of hepsin function may be compensated for other functionally related gene products, presumably other serine proteases.

The expression of hepsin on various cancer cell lines such as the hepatocellular carcinoma cells (HepG2 and the Alexander cells) and the osteosarcoma cells, and its ability to generate thrombin has provided further insight into its role in tumor migration and metastasis, a complex series of multistep processes (30, 31). It is well known that cancer is always complicated by thrombosis due to elevated procoagulative effect and molecules of the blood coagulation cascade have been demonstrated to be involved in thrombin formation (32-36). Consequently, in malignant cells, thrombin mediates the adhesion of cancer cells to endothelial cells through fibrin formation and platelet activation (12-16, 37) and fibrin in turn acts as glue that facilitates tumor cell adhesion and the pathogenesis of tumor growth and metastasis. Support for these ideas comes from observations that anticoagulants (such as warfarin) and fibrinolytic agents (like plasminogen activator and urokinase) could lower the numbers of tumor metastasis (17, 38-40). They either prevent the formation of activated clotting factors like factors VII, IX, X and thrombin (warfarin), or they dissolve tumor-associated fibrin clots and thus destroy the extracellular matrix and basement membrane (t-PA and urokinase). The hypercoagulative phenomenon in malignancy has been divided into tissue factor (TF, the cofactor for factor VIIa)-dependent and -independent activation process of coagulation (34, 35). Hepsin is capable of activating factor VII to VIIa and leading to thrombin generation in the absence of TF, suggesting that it confer a TF-independent procoagulation activity (11). Moreover, there are reports indicated the presence of the different alternatively spliced form of hepsin mRNA in murine hepatocytes that may translate a full-length hepsin molecule with an extra 20 amino acid residues at the cytoplasmic tail shaping it into a signaling peptide (3, 9). We have examined the presence of this hepsin mRNA molecules in human and mouse liver tissues as well as in human primary hepatoma tumors. Our RT-PCR results using species and allele specific primers, suggest that while both the shorter and longer forms of hepsin mRNA can be detected in mouse liver, only the shorter hepsin mRNA can be detected in human liver tissues, either normal or malignant (data not shown). This is in agreement with previously reported results and again suggesting a possible different role of hepsin in human and mouse (3). More experiments have to be performed to address the role of hepsin in human and its relation to the metastasis of tumor cells.

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