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Direct production of an activated matrix metalloproteinase-9 (gelatinase B) from mammalian cells

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Abstract **Matrix metalloproteinase-9 (MMP-9) is produced by the inactive proform and activated by a proteolytic process. However, it has not been reported to produce the active form directly from cells, which has hindered the research to elicit the physiological roles of this enzyme. In this study, we prepared mutant MMP-9 containing the furin-recognizing sequence in the prodomain and showed that the mutant MMP-9 was secreted as the active form directly from CHO-K1 cells and primary hepatocytes after the gene was transfected. The secreted MMP-9 showed proteolytic activity without further activation and degraded collagen IV in vitro. In addition, the transfection of the gene into the liver resulted in the efficient expression of active MMP-9 in the liver and the serum in vivo.**

Key words: Matrix metalloproteinase-9; Furin; Activation; Hepatocyte; Liver

1. Introduction

Matrix metalloproteinases (MMPs) are a large family of zinc-requiring matrix degrading enzymes. They have been implicated in various physiological aspects such as invasive cell behavior, embryonic development and organ morphogenesis. In these processes, the degradation of basement membrane is the critical event. The basement membrane is essentially composed of type IV collagen, the molecule that gelatinases, an MMP subclass, preferentially degrade. Therefore, it is thought that the roles of gelatinases are significant in the physiological processes. The gelatinases, also called type IV collagenases as a result of their activity, include MMP-2 (gelatinase A) and MMP-9 (gelatinase B). These MMPs are thought to be activated by 'cysteine switch' model. In this model, coordination of the zinc molecule in the active site of the catalytic domain with a sulfhydryl-containing cysteine residue in the conserved sequence [1] located at the C-terminus of the propeptide region is required for latency. Disruption of the zinc–sulfhydryl ligation by sulfhydryl reagents or removal of the prodomain by denaturants, oxidants, and proteases allows coordination of the zinc with a water molecule, resulting in the formation of an active enzyme. Compared to MMP-2, which is activated by MT1-MMP, the physiological activation mechanism of MMP-9 has not been clear. Since MMP-9 is produced as an inactive proform and processed to an active form by cleaving the prodomain, the physiological functions of active MMP-9 have not been clear yet. So far, the only way to examine the functional mechanism of MMP-9 is employing MMP-9-deficient mice [2–4]. However, the distribution of these mice is restricted, and without active MMP-9 production from cells it is difficult to perform in vitro cellular experiments. According to the protein structure and the activation mechanism of MMP, some constitutive active mutant forms of MMPs were prepared by modifying the cysteine-switch region [5]. However, modifying the improper amino acids in the region often results in the impairment of normal protein folding, which resulted in the failure of active enzyme production [6]. Thus, the method can not be applicable to any MMP. In fact, preparation by this method of active forms of only a few MMPs such as MMP-3 and MMP-13 have been reported [5–8]. In contrast, some MMPs such as stromelysin-3 (MMP-11) and MT1-MMP (MMP-14) contain the furin-cleavage sequence in the prodomain and are expressed from cells as processed, active forms [9].

Furin, a proprotein-processing enzyme, is a member of the yeast Kex2 family of endoproteases that contain a subtilisin-like serine-protease domain as an active site [10]. Furin cleaves the carboxy-terminus of the unique consensus sequence [RX(K/R)R]. In this paper, we have tried another method to produce active MMPs directly from cells by inserting furin-recognition sequence in the prodomain. Accordingly, we applied this activation mechanism to MMP-9 and demonstrated that transfection of this mutant gene into cultured cells resulted in the direct production of active MMP-9 in vitro and in vivo. Since furin is distributed more or less in virtually all tissues [11], the active MMP-9 can be produced from almost all cells. This mutant form will be a useful tool to examine the roles of MMP-9. In addition, this activation method can be applicable to any MMP since this method modifies the only prodomain after folding but not the critical region, the 'cysteine switch' domain.

2. Materials and methods

2.1. Reagents and cells

Plasmid DNA, pGEL2-SK containing mouse full length MMP-9 gene [12] was kindly provided by Dr. H. Tanaka (Shionogi and Co., Ltd., Osaka, Japan), and mouse full length furin gene in pSVL plasmid [10] and CHO-K1 cells were obtained from American Type Culture Collection (ATCC) (MD, USA). A mammalian expression vector, pTracer-CMV plasmid, was purchased from Invitrogen (San Diego, CA, USA). Primary mouse hepatocytes were isolated from adult ICR mice (8-weeks-old) by the modified methods as described

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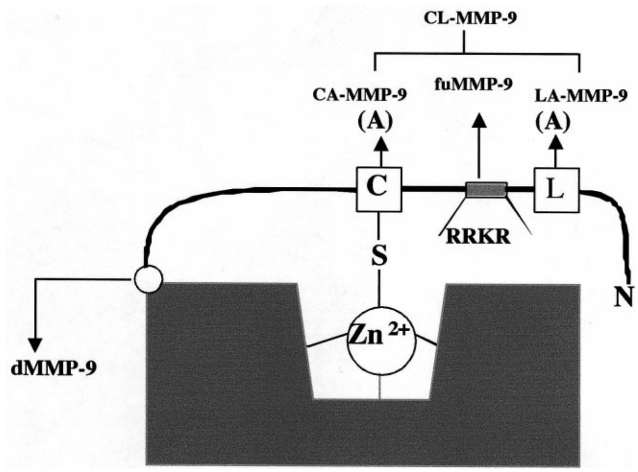

Fig. 1. Design of various mouse mutant MMP-9. Structural image of MMP-9 and the mutation sites are depicted. Five mutants were designed (fuMMP-9, dMMP-9, CA-MMP-9, LA-MMP-9, CL-MMP-9).

[13]. Cells were transfected with the reagents, Lipofectamine (Gibco BRL, Bethesda, MD, USA) for CHO-K1 and FuGene (Roche Diagonostics, Inc., Tokyo, Japan) for primary hepatocytes following the manufacturer's instruction. Other reagents were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, USA) unless specifically described.

2.2. *Preparation of mutant MMP-9 containing a furin-recognition sequence in the prodomain (fuMMP-9) and other mutant genes*

MMP-9-containing plasmid, pGEL2-SK, contains *Sma*I sites in the prodomain (at 167) and hemopexin domain (at 1915). Therefore, *Sma*I digestion generated plasmid lacking 167–1915 of MMP-9. The product was purified and dephosphorylated (*Sma*I-pGEL2). At the same time, using primers containing the RRKR sequence codon in the sense (P1: 5′-GGAGGAAACGTGCCGCCCAGATGATG; P2: 5′-GGGACGACGCGGGAG), the fragment (167–1915) of MMP-9 containing the RRKR sequence in the prodomain was prepared by PCR. The fragment was then ligated to the dephosphorylated *Sma*I-pGEL2. The sequence of the mutant MMP-9 was confirmed by sequence analysis. The MMP-9 fragment was cut out by *Eco*RI-*Not*I digestion and inserted into the expression vector, pTracer-CMV at the multicloning site. The furin insert was also cut out from pSVL by *Eco*RI digestion and inserted into pTracer-CMV. Other point-mutated MMP-9 forms (CA-MMP-9; cysteine^{100} was changed to Arg, LA-MMP-9; leucine^{50} to Arg, LC-MMP-9; both C^{100} and L^{50} to

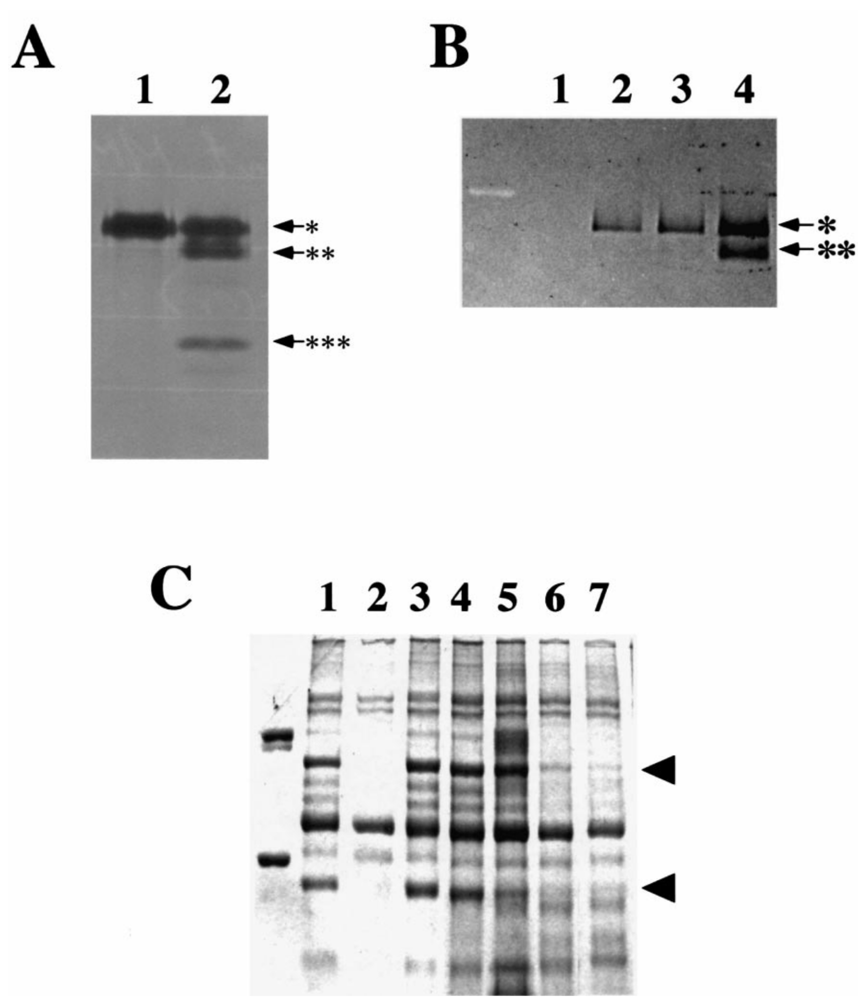

Fig. 2. Production of furin-dependent active MMP-9 with fuMMP-9 gene in different cell types. A: CHO-K1 cells were transfected with wtMMP-9 or fuMMP-9 plasmids. 24 h after transfection, the supernatants were harvested and subjected to zymography. Lane 1: wtMMP-9, lane 2: fuMMP-9. *Latent form, **active form, ***autocleaved fragment. B and C: Isolated hepatocytes were transfected with wtMMP-9, fuMMP-9 alone or fuMMP-9 plus furin plasmids. 24 h after transfection, the supernatants were harvested and subjected to gelatin zymography (B) or proteolytic assay against type IV collagen (C). B: Lane 1, control; lane 2, wtMMP-9; lane 3, fuMMP-9; lane 4, fuMMP-9 plus furin. *Latent form, **active form. C: Lane 1, control; lane 2, gelatinase (100 μg/ml); lane 3, APMA (1.5 mM); lane 4, wtMMP-9; lane 5, fuMMP-9 alone; lane 6, fuMMP-9 plus furin; lane 7, APMA-treated wtMMP-9. The arrows are the major type IV collagen bands.

Arg) (Fig. 1) which are supposedly active according to the modification results of MMP-3 [5] were prepared by QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). The deleted mutant of MMP-9 (dMMP-9) which has the same DNA sequence of the cleaved, active MMP-9 without prodomain was also prepared by PCR and inserted into pTracer-CMV.

2.3. Gelatin zymography

The supernatants from culture cells or lysates from tissues were electrophoresed on 7.5% SDS–polyacrylamide gel containing 0.1% gelatin. The gel was washed with 0.1% Triton X-100 solution for 30 min and then incubated at 37°C overnight in the activation buffer (50 mM Tris–HCl, pH 7.4, 0.2 M NaCl, 5 mM $CaCl_2$, and 0.02% NaN_3). After staining with CBB R-250, the gelatinolytic activities were detected as clear bands against the blue background. In some cases, MMPs in supernatants were concentrated by gelatin–Sepharose (Pharmacia, Uppsala, Sweden). The contrast images of zymography were inverted to facilitate the band detection.

2.4. Type IV collagen proteolytic activity

Active MMP-9 was confirmed by both the mobility shift of the bands in zymography and proteolytic activity of collagen type IV. The supernatants from cultured cells or lysates from tissues (1 μl) were incubated with 3 μg collagen type IV (Sigma) (1 μl) in the activating buffer (8 μl) at 37°C overnight. These samples were then subjected to SDS–PAGE on 7.5% gel (10 μl/lane).

2.5. In vivo gene transfection

In vivo transfection into the liver was performed as described [14]. Briefly, genes in the expression vector, pTracer (100 μg/mouse) were intravenously injected into mice with 6 ml Ringer solution, which results in the specific expression of transfected genes in hepatocytes [14]. 9 h after the administration, the serum and liver tissue lysates were subjected to zymography.

3. Results and discussion

3.1. Furin-dependent, direct production of active MMP-9 from cultured cells

First, we transfected either wild type MMP-9 (wtMMP-9) or fuMMP-9 into CHO-K1 cells and examined the gelatinolytic activities of the supernatants. As shown in Fig. 2A, wtMMP-9 was secreted in the supernatant and appeared as a single band which was corresponding to the latent form. In contrast, fuMMP-9 was spontaneously processed when secreted from the transduced cells and approximately half of the gelatinolytic activity was found in the position of active MMP-9. Part of the active form was autodegraded and appeared as a small fragment at the lower position.

Next, we confirmed furin dependency of fuMMP-9 employing primary mouse hepatocytes since, contrary to the in vivo situation, these cells express little furin in vitro without any stimulation [15]. Primary hepatocytes did not spontaneously express any detectable amount of MMPs in the culture supernatant and the transient transfection of wtMMP-9 gene resulted in production of the intact, inactive form (Fig. 2B, lane 2). Hepatocytes transfected with fuMMP-9 only also produced the inactive form of MMP-9 (Fig. 2B, lane 3). In contrast, cotransfection of fuMMP-9 and furin genes into these cells produced the active form of MMP-9 in the supernatants (Fig. 2B, lane 4). These results demonstrate that the inactive form produced from cells transfected with fuMMP-9 gene was processed to the active form in a furin-dependent manner. The reason why approximately only half of the amount protein was processed to the active form is unclear at present. According to the putative mechanism wherein furin processes the prodomain of the inactive form at the first step, and then the processed protein autoactivates and become the active form at the second step, the autoactivation at the second step may not be sufficient. The details of the mechanism, however, remain to be solved. The processed active form from fuMMP-9 showed strong proteolytic activity against type IV collagen (Fig. 2C). The supernatant from fuMMP-9 and furin cotransfected hepatocytes (lane 6) contained almost the same proteolytic activity as commercial gelatinase (lane 2) or chemically activated (APMA-treated) MMP-9 (lane 7). It is notable that neither wtMMP-9 nor fuMMP-9 without furin cotransfection did not show any proteolytic activity (lanes 4 and 5). These results also demonstrate furin-dependent, direct production of active MMP-9 from cells. Interestingly, despite the production

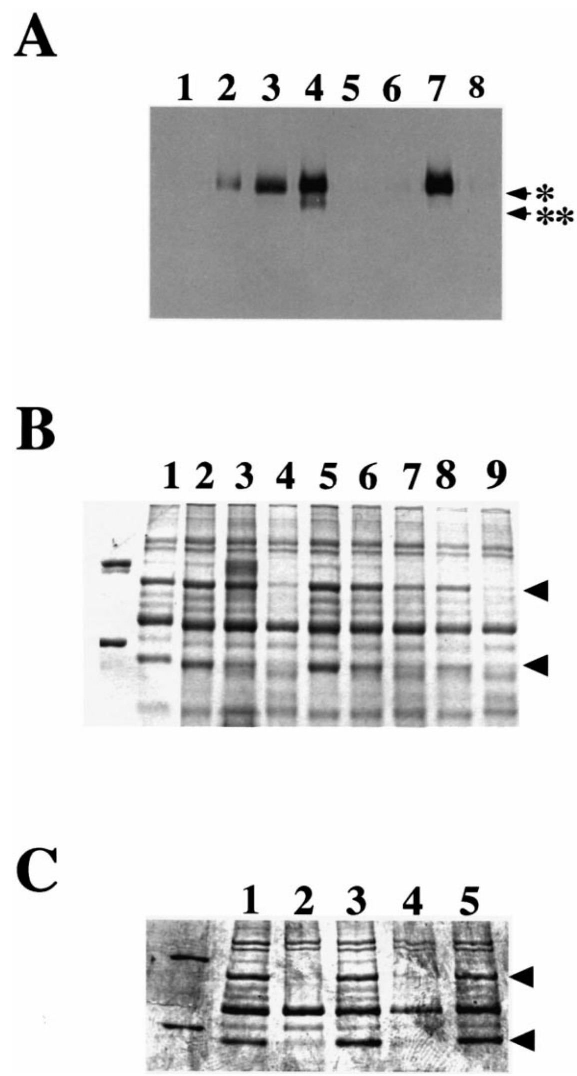

Fig. 3. Comparative production of active MMP-9 by fuMMP-9 gene to other mutants. Mutant MMP-9s were transfected into primary cultured hepatocytes and 24 h later, the supernatants were harvested and subjected to zymography (A) or proteolytic activity assay against type IV collagen (B and C). A: Lane 1, control; lane 2, wtMMP-9; lane 3, fuMMP-9 alone; lane 4, fuMMP-9 plus furin; lane 5, dMMP-9; lane 6, CA-MMP-9; lane 7, LA-MMP-9; lane 8, CL-MMP-9. *Latent form, **active form. B: Lane 1, control; lane 2, wtMMP-9; lane 3, fuMMP-9 alone; lane 4, fuMMP-9 plus furin; lane 5, dMMP-9; lane 6, CA-MMP-9; lane 7, LA-MMP-9; lane 8, CL-MMP-9; lane 9, APMA-treated wtMMP-9. C: Lane 1, control; lane 2, APMA-treated wtMMP-9; lane 3, APMA-treated wtMMP-9 with EDTA (10 mM); lane 4, fuMMP-9 plus furin; lane 5, fuMMP-9 plus furin with EDTA.

of active MMP-9, the morphology of the producing cells did not change when these cells were cultured on either collagen-coated or gelatin-coated plates. This may be because these cells produce a non-substrate extracellular matrix for MMP-9, such as fibronectin or because MT-MMPs rather than secreted MMPs have a more profound effect on cellular functions in the microenvironments as described [16].

3.2. Comparison to other types of mutant MMP-9

Some active MMPs could be prepared by point mutations in cysteine-switch domain or prodomain [5,6]. Therefore, we compared the point-mutated forms of MMP-9 to fuMMP-9 in the production and activation. We prepared three types of point mutants according to the previous report describing point-mutated active MMP-3 [5]. CA-MMP-9 has a mutation in the cysteine of the 'cysteine switch'. LA-MMP-9 has a point mutation in the leucine of the prodomain. CL-MMP-9 has both mutations in the same molecule. Besides, we prepared deleted MMP-9 (dMMP-9) which has the same codon sequence of active MMP-9. When these mutants were transfected into primary hepatocytes (these cells are proper to examine MMP production assay since, as described above, primary hepatocytes do not produce any MMPs in the supernatant), the secretion of CA-MMP-9, CL-MMP-9 or dMMP-9 in small amounts was detected (Fig. 3A). The gelatinolytic activity was detected in the supernatant of only LA-MMP-9-transfected cells. However, the active form was detected in small amounts (lane 6) compared to fuMMP-9 with furin (lane 4). The proteolytic activities in the supernatants of these mutants reflected the production pattern. As shown in Fig. 3B, only fuMMP-9 with furin showed the sufficient proteolytic activity against type IV collagen compared to other mutant forms. These results suggest two important points, i.e. the cysteine in the cysteine switch is important for the protein secretion, probably because the cysteine function as a key amino acid for the regulation of protein folding. The second point is that the point mutation in the prodomain is insufficient for the processing of inactive MMP-9 to active MMP-9, different from the case of MMP-3.

The proteolytic activities of the supernatants were completely blocked by the addition of EDTA in the assay system, which also demonstrated the active function of MMP-9 (Fig. 3C).

3.3. Expression of active MMP-9 by fuMMP-9 gene in vivo

We next tried to induce the expression of active MMP-9 using fuMMP-9 in vivo since in vivo expression is required for the investigation of the physiological functions. Transduction into the liver was performed by intravenous injection of plasmid DNA [14]. As shown in Fig. 4, transfection of MMP-9 genes in the liver resulted in high expression of the proteins in both liver and serum. Mere injection of Ringer solution slightly induced MMP-9 expression in both liver and serum. This is probably because of the liver injury by the massive volume of injection [14], for liver injury is thought to induce MMP-9 expression in the liver [17]. Liver injury is also thought to induce plasmin activation [18]. Interestingly, transduced wtMMP-9 was activated in the liver but not in the serum (Fig. 4A,B, lane 3). It is possible that the localized activation of MMP-9 is due to the plasmin activation since the plasmin system is involved in the processing of proMMP-9 to the active form [19]. In contrast, the activated form of

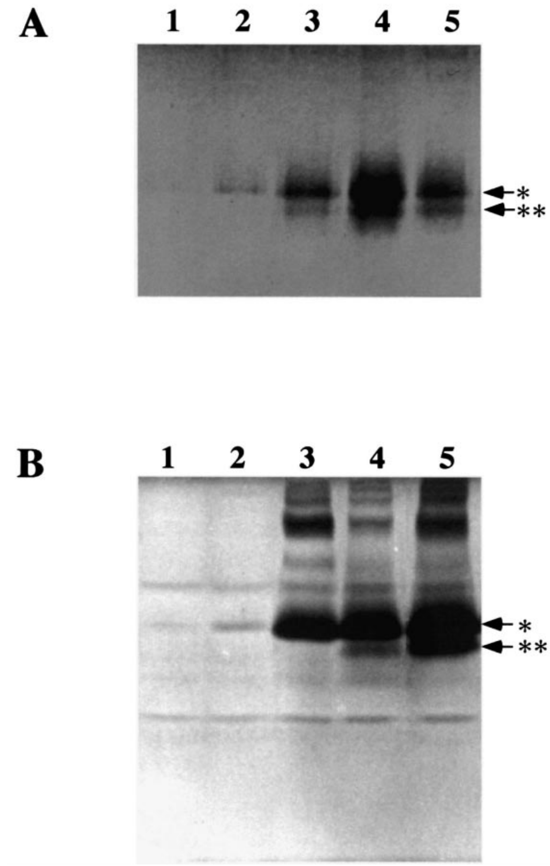

Fig. 4. In vivo expression of active MMP-9 by the transduction of fuMMP-9 in the liver. wtMMP-9, fuMMP-9 or fuMMP-9 plus furin genes in pTracer plasmid were transfected into the liver with 6 ml Ringer solution via the tail vein. 6 h later, the liver and serum were collected. The liver was homogenized and the gelatinase activity was concentrated by a gelatin–Sepharose column. Then, the samples were subjected to gelatin zymography. The serum was diluted and directly subjected to zymography after the protein concentration was adjusted. MMP activities in the liver lysates (A) and in the sera (B). Lane 1, control; lane 2, Ringer only; lane 3, wtMMP-9; lane 4, fuMMP-9; lane 5, fuMMP-9 plus furin. *Latent form, **active form.

fuMMP-9 was detected in both the liver and serum of the mice transfected with fuMMP-9 gene alone although the active form was minor in the serum (lane 4). It is likely that the endogenous furin activity in the liver is not sufficient to process the large amount of transfected fuMMP-9. However, co-transfection of fuMMP-9 plus furin genes resulted in strong expression of active MMP-9 in both the liver and serum (lane 5). These results demonstrate that transduced fuMMP-9 was processed by furin in vivo. To our knowledge, this is the first successful report that active MMP-9 was systematically induced in vivo. This fuMMP-9 can be a promising tool for the physiological investigation of this molecule and therapeutic purposes.

References

[1] Vu, T.H. and Werb, Z. (1998) Gelatinase B: Structure, Regulations, and Function, Academic Press, New York.
[2] Vu, T.H., Shipley, J.M., Bergers, G., Berger, J.E., Helms, J.A., Hanahan, D., Shapiro, S.D., Senior, R.M. and Werb, Z. (1998) Cell 93, 411–422.

[3] Liu, Z., Zhou, X., Shapiro, S.D., Shipley, J.M., Twining, S.S., Diaz, L.A., Senior, R.M. and Werb, Z. (2000) Cell 102, 647–655.
[4] Dubois, B., Masure, S., Hurtenback, U., Paemen, L., Heremans, H., Oord, J.v.d., Sciot, R., Meinhardt, T., Hämmerling, G., Opdenakker, G. and Arnold, B. (1999) J. Clin. Invest. 104, 1507–1515.
[5] Freimark, B.D., Feeser, W.S. and Rosenfeld, S.A. (1994) J. Biol. Chem. 269, 26982–26987.
[6] Park, A.J., Matrisian, L.M., Kells, A.F., Pearson, R., Yuan, Z. and Navre, M. (1991) J. Biol. Chem. 266, 1584–1590.
[7] Neuhold, L.A., Killar, L., Zhao, W., Sung, M.A., Warner, L., Kulik, J., Turner, J., Wu, W., Billinghurst, C., Meijers, T., Poole, A.R., Babij, P. and DeGennaro, L.J. (2001) J. Clin. Invest. 107, 35–44.
[8] Sternlicht, M.D., Lochter, A., Sympson, C.J., Huey, B., Rougier, J., Gray, J.W., Pinkel, D., Bissell, M.J. and Werb, Z. (1999) Cell 98, 137–146.
[9] Vu, T.H. and Werb, Z. (2000) Genes Dev. 14, 2123–2133.
[10] v.d. Ven, W.J.M., Creemers, J.W.M. and Roebroek, A.J.M. (1991) Enzyme 45, 257–270.
[11] Schalken, J.A., Roebroek, A.J.M., Oomen, P.P.C.A., Wagenaar, S.S., Debruyne, F.M.J., Bloemers, H.P.J. and v.d. Ven, W.J.M. (1987) J. Clin. Invest. 80, 1545–1549.
[12] Tanaka, H., Hojo, K., Yoshida, H., Yoshioka, T. and Sugita, K. (1993) Biochem. Biophys. Res. Commun. 190, 732–740.
[13] Morita, M., Watanabe, Y. and Akaike, T. (1994) Hepatology 19, 426–431.
[14] Zhang, G., Budker, V. and Wolff, J.A. (1999) Hum. Gene Ther. 10, 1735–1737.
[15] Hoshino, H., Konda, Y. and Takeuchi, T. (1997) FEBS lett. 419, 9–12.
[16] Hotary, K., Allen, E., Punturieri, A., Yana, I. and Weiss, S.J. (2000) J. Cell Biol. 149, 1309–1323.
[17] Kim, T., Mars, W.M., Stolz, D.B. and Michalopoulos, G.K. (2000) Hepatology 31, 75–82.
[18] Kim, T., Mars, W.M., Stolz, D.B., Petersen, B.E. and Michalopoulos, G.K. (1997) Hepatology 26, 896–904.
[19] Baramova, E.N., Bajou, K., Remacle, A., L'Hoir, C., Krell, H.W., Weidle, U.H., Noel, A. and Foidart, J.M. (1997) FEBS lett. 405, 157–162.