

Masahiko Kobayashi · Yoshio Naomoto · Tetsuji  
Nobuhisa · Takaomi Okawa · Munenori Takaoka ·  
Yasuhiro Shirakawa · Tomoki Yamatsuji · Junji  
Matsuoka · Takaaki Mizushima · Hironori Matsuura ·  
Motowo Nakajima · Hiroshi Nakagawa · Anil Rustgi ·  
Noriaki Tanaka

## Heparanase regulates esophageal keratinocyte differentiation through nuclear translocation and heparan sulfate cleavage

Received October 10, 2005; accepted in revised form March 6, 2006

**Abstract** Heparanase is an endo- $\beta$ -glucuronidase that specifically cleaves heparan sulfate (HS) chains. Heparanase is involved in the process of metastasis and angiogenesis through the degradation of HS chains of the extracellular matrix and cell surface. Recently, we demonstrated that heparanase was localized in the cell

nucleus of normal esophageal epithelium and esophageal cancer, and that its expression was correlated with cell differentiation. However, the nuclear function of heparanase remains unknown. To elucidate the role of heparanase in esophageal epithelial differentiation, primary human esophageal cells were grown in monolayer as well as organotypic cultures, and cell differentiation was induced. Expression of heparanase, HS, involucrin, and p27 was determined by immunostaining and Western blotting. SF4, a novel pharmacological inhibitor, was used to specifically inhibit heparanase activity. Upon esophageal cell differentiation, heparanase was translocated from the cytoplasm to the nucleus. Such translocation of heparanase appeared to be associated with the degradation of HS chains in the nucleus and changes in the expression of keratinocyte differentiation markers such as p27 and involucrin, whose induction was inhibited by SF4. Furthermore, these *in vitro* observations agreed with the expression pattern of heparanase, HS, involucrin, cytokeratin 13, and p27 in normal esophageal epithelium. Nuclear translocation of heparanase and its catalytic cleavage of HS may play a critical role in the differentiation of esophageal epithelial cells. Our study provides a novel insight into the role of heparanase in an essential differentiation process.

Masahiko Kobayashi · Yoshio Naomoto (✉) · Tetsuji  
Nobuhisa · Takaomi Okawa · Munenori Takaoka · Yasuhiro  
Shirakawa · Tomoki Yamatsuji · Junji Matsuoka · Noriaki  
Tanaka  
Department of Gastroenterological Surgery  
Transplant, and Surgical Oncology  
Graduate School of Medicine and Dentistry, Okayama  
University, 2-5-1 Shikatacho, Okayama 700-8558, Japan  
Tel: +81 86 235 7257  
Fax: +81 86 221 8775  
E-mail: ynaomoto@md.okayama-u.ac.jp

Takaaki Mizushima  
Department of Laboratory Medicine  
Okayama University Graduate School of Medicine and  
Dentistry  
Okayama, Japan

Hironori Matsuura  
Department of Dermatology  
Okayama University Graduate School of Medicine and  
Dentistry  
Okayama, Japan

Motowo Nakajima  
Novartis Institute for Biomedical Research  
Tsukuba, Japan

Hiroshi Nakagawa · Anil Rustgi  
Gastroenterology Division  
University of Pennsylvania, Philadelphia, PA, USA

**Key words** heparanase · nuclear translocation ·  
differentiation · esophageal keratinocyte · heparan sulfate

### Introduction

Heparan sulfate (HS) proteoglycans (HSPGs) are a family of macromolecules in which each member consists of a core protein and at least one HS

glycosaminoglycan chain. HSPGs are key components of the cell surface and extracellular matrix. They are classified into several families according to the amino acid sequence of the core protein, and include perlecan, which are basement-membrane-bound HSPGs, and syndecans, which are cell-surface-bound HSPGs. HS chains bind to and regulate the activities of numerous signaling molecules, such as fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF). HSPGs have been implicated in diverse processes such as cell proliferation, cell–matrix and cell–cell adhesion, migration, angiogenesis, and wound healing (Schlesinger et al., 1995; Filla et al., 1998; Iozzo, 1998; Bernfield et al., 1999; Allen et al., 2001). A recent report showed that loss of HS in human exostosis was associated with cell differentiation (Hecht et al., 2005).

Heparanase is an endo- $\beta$ -glucuronidase that specifically cleaves carbohydrate chains of HSPG (Nakajima et al., 1983, 1984). Heparanase is involved in the degradation of both cell-surface and extracellular matrix HS chains in normal and neoplastic tissues (Toyoshima and Nakajima, 1999; Vlodaysky et al., 1999). Many studies have demonstrated that heparanase is expressed in a variety of normal (Bernard et al., 2001; Haimov-Kochman et al., 2002; Chen et al., 2004; Goldshmidt et al., 2004) and malignant cells (Ginath et al., 2001; Gohji et al., 2001; Ikuta et al., 2001; Koliopoulos et al., 2001; Ikeguchi et al., 2002; Shinyo et al., 2003; Simizu et al., 2003; Stadlmann et al., 2003; Zetser et al., 2003), and is involved in tumor cell metastasis, angiogenesis, inflammation, tissue repair, wound healing, and embryonic morphogenesis by degrading cell-surface and extracellular HS chains and by releasing HS-binding factors such as VEGF (Saijo et al., 2003; Zcharia et al., 2004). We previously demonstrated a correlation between heparanase expression and poor prognosis, cell invasion and angiogenesis in gastric cancer, esophageal cancer, and colon cancer (Takaoka et al., 2003; Nobuhisa et al., 2004; Ohkawa et al., 2004; Okawa et al., 2005).

It has long been reported that HS is present in the nuclei of several cells (Fedarko and Conrad, 1986; Ishihara et al., 1987; Fedarko et al., 1989; Ishihara and Conrad, 1989; Ishihara and Mamiya, 1989; Liang et al., 1997; Richardson et al., 2001; Hsia et al., 2003). However, neither the mechanism underlying the regulation of HS nor its role in the nuclei are well understood, despite the suggestion that HS might regulate the nuclear processes (Kovalszky et al., 1998; Hsia et al., 2003). Recently, the expression of heparanase in the nucleus and its involvement in degrading HS in the nucleus were reported, although heparanase is already well known as a secreted protein involved in the degradation of cell-surface and extracellular HS chains (Schubert et al., 2004). We have recently demonstrated that the expression of heparanase in the nucleus correlated with the expression of differentiation markers in normal

esophageal keratinocytes and in esophageal cancer tissues (Ohkawa et al., 2004). Overexpression of heparanase in the nucleus induces differentiation of human breast cancer cells (Nobuhisa et al., 2005). However, the biological mechanisms and significance of the nuclear expression of both heparanase and HS is still unclear.

To clarify these mechanisms, we used primary cultures of esophageal keratinocytes established from normal human esophageal epithelium. In order to induce differentiation of esophageal keratinocytes, we used calcium and organotypic cell culture. It is well known that the program of keratinocyte differentiation induced by calcium is a multistep process defined by a cascade of interrelated changes in the expression of growth-regulatory and differentiation-specific genes (Hennings et al., 1980; Pillai et al., 1990; Jetten and Harvat, 1997; Bikle et al., 2001; Komuves et al., 2002). In the present study, the expression of heparanase in the nucleus was detected in normal esophageal epithelium and in an esophageal keratinocyte differentiation model. By using this esophageal keratinocyte differentiation model and a heparanase-specific inhibitor, we examined the changes in expression and localization of heparanase and HS chains and elucidated a correlation between the nuclear translocation of heparanase and the expression of growth-regulatory and differentiation-specific genes.

## Materials and methods

### Tissue samples

Tissue samples were surgically resected at the Department of Gastroenterological Surgery, Okayama University Hospital, Okayama, Japan. Normal esophageal tissues were stained by Lugol's iodine, and strongly stained tissues were resected at a distant point from the tumor. Haematoxylin & Eosin (H&E) staining was performed to document the normal histology. From these tissues we made paraffin-embedded sections and frozen sections, and also established normal esophageal keratinocytes. The study protocol was approved by the Human Ethics Review Committees of the participating universities.

### Cell lines

Primary esophageal keratinocytes from normal human esophageal tissues were established as described previously (Andl et al., 2003; Ohkawa et al., 2004). Surgical specimens from normal esophagi were removed promptly and transported aseptically in Hanks' solution (Invitrogen) with 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 5  $\mu$ g/ml gentamicin (Invitrogen, Carlsbad, CA). Each tissue specimen was incubated with 1.5 U/ml dispase at 4°C overnight and then incubated for 10 min with trypsin. The reaction was stopped with a soybean trypsin inhibitor (Sigma, St. Louis, MO) and centrifuged. The pellet was resuspended in keratinocyte-SFM medium (KFSM) (Invitrogen) supplemented with 40  $\mu$ g/ml bovine pituitary extract, 1.0 ng/ml EGF, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 5  $\mu$ g/ml gentamicin (Invitrogen). All three cell lines were subjected to experiments at passage 3.

To induce terminal differentiation in a monolayer culture, cells were treated with 1 mM of calcium chloride. After incubation for various periods at 37°C, cells were solubilized in SDS-containing

buffer for Western blotting or fixed in 20% formalin for immunohistochemistry.

Organotypic cell culture was performed as described previously (Andl et al., 2003). In brief,  $5 \times 10^5$  esophageal keratinocytes were seeded onto a collagen matrix containing  $7.5 \times 10^4$  human skin fibroblast cells. After cell culture under submerged conditions for 4 days, cells were exposed to the air-liquid interface to induce terminal differentiation and cultured for an additional 6 days. The reconstituted epithelial sheets and the collagen matrix were fixed with 10% formaldehyde, embedded in paraffin, and subjected to histology and immunohistochemistry (Andl et al., 2003).

#### Induction of esophageal keratinocyte differentiation by calcium

Keratinocytes were cultured in chamber slides or 10 cm dishes. When 50%–60% confluence was reached, the medium was exchanged for KSFM containing 1 mM calcium chloride. After incubation for various periods at 37°C, cells were dissolved for Western blotting or fixed in 20% formalin for immunohistochemistry.

#### Heparanase-specific inhibitor (SF4) assay

A specific enzyme inhibitor of heparanase, uronic acid-type gem-diamine 1-*N*-iminosugar compound, called SF4, was obtained from Dr. Nishimura, Institute of Microbiological Chemistry (Tokyo, Japan). SF4 was used to inhibit heparanase as an already established pharmacological inhibitor at 100  $\mu$ M, which is the established optimal concentration (Nishimura et al., 2000; Ohkawa et al., 2004). Keratinocytes were cultured in chamber slides or in six-well dishes. When the cell culture reached 50%–60% confluence, the medium was exchanged for KSFM containing 0.09 or 1 mM calcium chloride with or without 100  $\mu$ M SF4. After incubation for 2 or 24 hr at 37°C, cells were dissolved for Western blotting or fixed in 20% formalin for immunohistochemistry.

#### Antibodies

Anti-human mouse heparanase monoclonal antibody was obtained from Novartis Pharma (Tsukuba, Japan); the monoclonal antibody reacted with both the 65 kDa proform and the 50 kDa mature form of human heparanase, as described previously (Takaoka et al., 2003).

Anti-mouse monoclonal antibody for keratin 13 was purchased from Monosan (Am Uden, the Netherlands). Anti-mouse monoclonal antibody for involucrin (SY5) was obtained from Sigma. Anti-rabbit polyclonal antibodies for p27 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mouse monoclonal antibody for HSPG (10E4) was obtained from Seikagaku Co. (Tokyo, Japan).

#### Immunohistochemistry

Fresh-frozen sections of normal esophageal tissue were cut at a thickness of 5  $\mu$ m and mounted on silanized slides (DAKO Japan Co., Tokyo, Japan). Frozen sections were cut and fixed in 20% formalin for 10 min at 4°C. Primary keratinocyte cells were seeded into chamber slides and fixed in 20% formalin for 10 min at 4°C. The fixed sections or cells were washed in PBS and incubated with a blocking reagent (DAKO Japan Co.) for 10 min. Primary antibodies were incubated for 1.5 hr at room temperature (dilution 1:50 for keratin 13) or overnight at 4°C (dilution 1:500 for heparanase and involucrin, 1:200 for p27). After washing with PBS, the primary antibody-reacted sections or cells were incubated with fluorescein- or rhodamine-conjugated secondary antibody (Chemicon, Temecula, CA) for 30 min at 37°C. Stained sections or cells were exam-

ined under an OLYMPUS microscope and imaged with a digital camera.

Paraffin sections (5  $\mu$ m thick) were mounted on silanized slides (DAKO Japan Co.), de-paraffinized in xylene for 20 min, and rehydrated in graded ethanol solutions. Endogenous peroxidase was blocked by incubating the sections in 3.0% H<sub>2</sub>O<sub>2</sub> in methanol for 10 min. Antigen was retrieved from paraffin sections twice by heating in 10 mM citrate buffer solution (pH 6.0) in a microwave for 5 min. After the blocking of nonspecific reactivity with rabbit serum for 15 min at 37°C (Histofine SAB-PO kit; Nichirei, Tokyo, Japan), primary antibodies were incubated for 1.5 hr at room temperature (dilution 1:100 for HSPG) or overnight at 4°C (dilution 1:500 for heparanase). The distribution of the primary antibody was identified by subsequent application of a biotinylated anti-primary antibody (Histofine SAB-PO kit) and streptavidin-peroxidase (Histofine SAB-PO kit). Immunostaining was developed using DAB/H<sub>2</sub>O<sub>2</sub> solution (Histofine DAB substrate kit; Nichirei, Tokyo, Japan).

To confirm the specific immunoreactivity of the anti-HS antibody, we performed immunohistology of the tissue specimens before and after treatment with flavobacterial heparitinase. Paraffin sections were treated with 50 mU/ $\mu$ l of heparitinase I (Seikagaku Co.) for 1 hr at 37°C before the incubation with anti-HS antibody.

#### Confocal microscopy

We used a confocal laser-scanning microscope (LSM-510, Carl Zeiss, Oberkochen, Germany) to observe the localization of heparanase in cultured keratinocytes and imaged with a digital camera at specific magnifications.

#### Western blotting

Western blotting was carried out as described previously (Ohkawa et al., 2004). Cells were lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, and protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml pepstatin, and 1  $\mu$ g/ml aprotinin). Lysis was carried out at 4°C for 30 min and centrifuged at 15,000 rpm.

Nuclear extracts of esophageal cancer cells were collected using NER-PER™ extract reagent (Pierce, Rockford, IL). The protein concentration of the supernatant was determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Equal amounts of proteins were electrophoresed under reducing conditions on 12% or 8% polyacrylamide gels. Proteins were electrophoretically transferred on Hybond-PVDF transfer membranes (Amersham, Arlington Heights, IL). Each membrane was blocked with 5% skim milk in 0.2% Tween-20 in TBS before incubation with primary antibodies (diluted 1:500 for heparanase and involucrin, 1:200 for p27, 1:1,000 for  $\beta$ -Actin, and 1:100 for TFIIF as a control for nuclear protein) overnight and then treated with a peroxidase-linked secondary antibody. The Amersham Enhanced Chemiluminescence Western Blotting System (Amersham, Tokyo, Japan) was used to detect secondary probes.

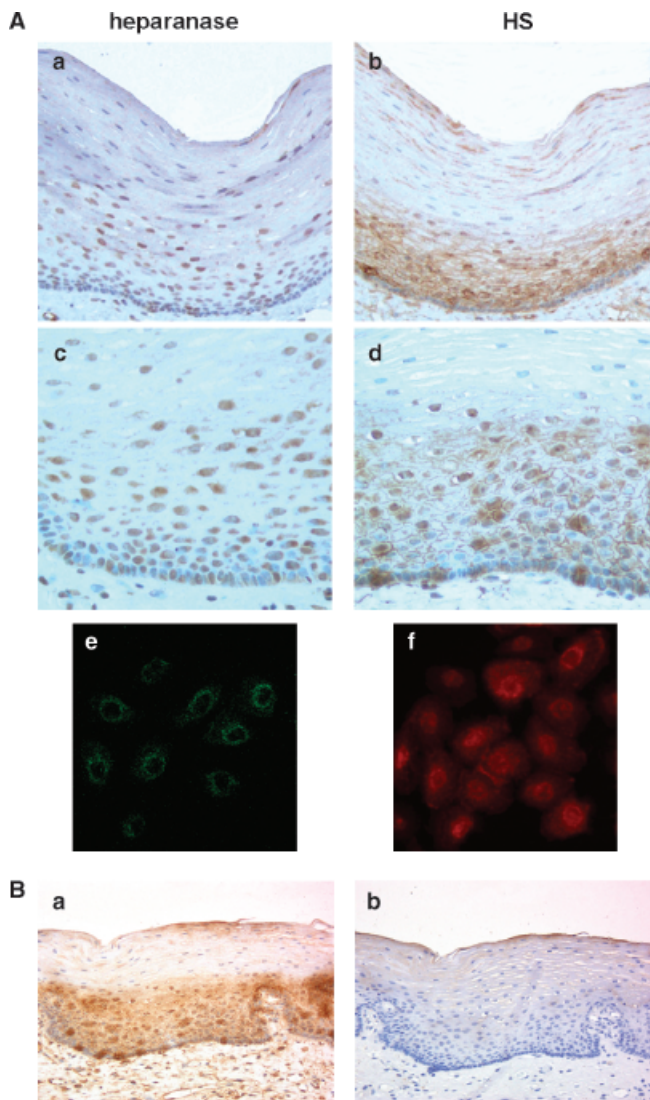
## Results

### Expression of heparanase and HS in normal esophageal epithelium

Heparanase was detected from the basal to parabasal layers, and was expressed especially strongly in the nucleus (Figs. 1A a,c). HS was expressed both on the cell surface and in the nucleus. The expression of

HS in both the cell surface and nucleus gradually weakened and finally disappeared in the upper half of the epithelium (Figs. 1A b,d). The nuclear HS level was reduced in the cells expressing a higher level of heparanase.

Immunoreactivity of the anti-HS antibody was totally abrogated by pre-treatment with bacterial heparitinase I (Fig. 1B).



**Fig. 1** (A) Expression and distribution of heparanase protein and heparan sulfate (HS) in normal esophageal epithelium (a–d). Immunofluorescence shows the expression and localization of heparanase and HS in undifferentiated normal esophageal keratinocytes (e and f). Magnification:  $\times 100$  (a, b),  $\times 200$  (c, d),  $\times 400$  (e, f). (B) To confirm specific immunostaining with an anti-HS antibody, the paraffin-embedded tissue sections of normal esophageal epithelium were subjected to immunohistology using anti-HS antibody before (a) and after (b) treatment with bacterial heparitinase I.

### Expression and localization of heparanase and HS in normal esophageal keratinocytes

The expression and localization of heparanase and HS were examined by immunohistochemistry on a confocal microscope. Heparanase showed perinuclear expression in undifferentiated normal esophageal keratinocytes (Fig. 1Ae). HS was detected in the nucleus and cytoplasm (Fig. 1Af).

### Change in heparanase localization during keratinocyte differentiation induced by calcium

Previously, it was reported that calcium induces terminal differentiation of cultured keratinocytes. We increased the concentration of calcium in the medium to 1 mM, and made a differentiation model of esophageal keratinocytes. The changes in the expression and localization of heparanase protein were examined by confocal microscopy and Western blotting. In the calcium-induced differentiation model, heparanase translocation into the nucleus was observed 30 min after differentiation induction, and its expression level remained high between 1 and 3 hr. After 6 hr, heparanase was detected in both the cytoplasm and the nucleus (Fig. 2A).

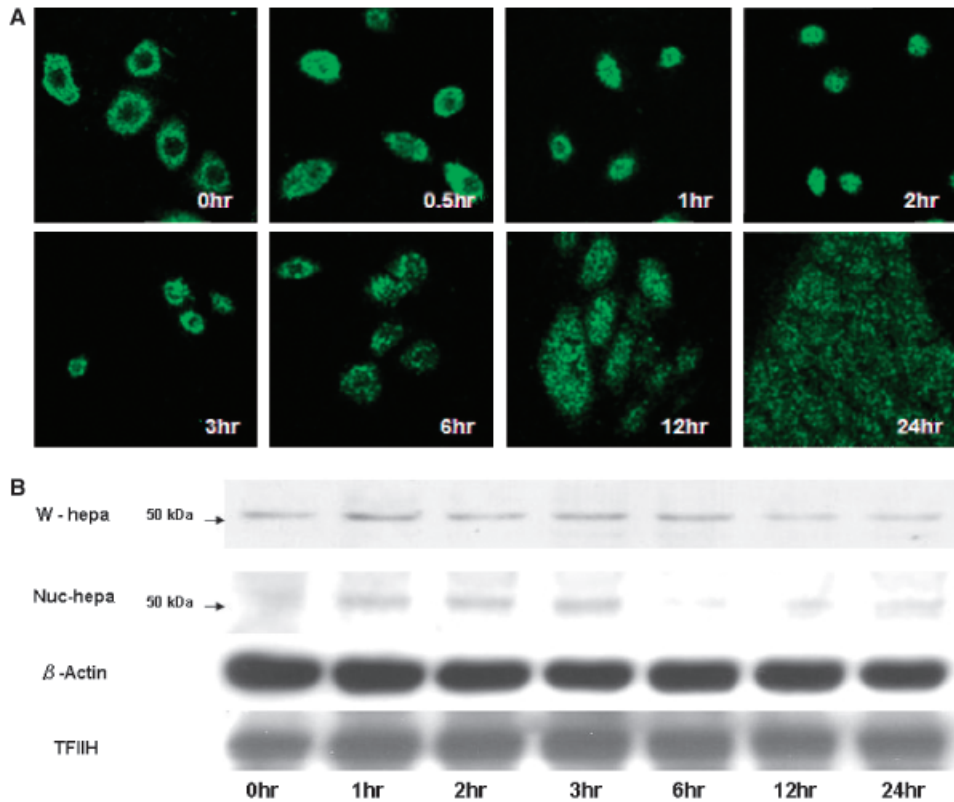
The results of Western blotting (Fig. 2B) confirmed the translocation of heparanase observed in the immunohistochemistry. In whole-cell lysates, there were no significant differences in the heparanase protein levels during the keratinocyte differentiation. However, similar to the results from immunohistochemistry of the esophageal epithelium, the nuclear protein level of heparanase increased 1–3 hr after differentiation induction. Heparanase was detected in the nucleus in the early stage of keratinocyte differentiation. In our study, a significant amount of the 50 kDa form of heparanase was consistently detected in the nucleus and cytoplasm; however, very low levels of the 65 kDa form also appeared in some cases.

### Changes in HS expression during calcium-induced keratinocyte differentiation

In the esophageal keratinocytes, HS was detected in the nucleus and cytoplasm. In the calcium-induced esophageal keratinocyte differentiation model, HS in the nucleus was thoroughly reduced after 24 hr compared with HS in the cytoplasm (Fig. 3A).

### Disappearance of HS in nucleus was inhibited by SF4

By using the heparanase-specific inhibitor SF4, we investigated the relationship between the appearance of HS and the expression of heparanase. The inhibition of heparanase activity blocked the disappearance of HS



**Fig. 2** Heparanase protein was determined by immunohistochemistry using confocal microscopy (A) and Western blotting (B). Cells were treated with calcium chloride for the time period shown. After 30 min, heparanase started to move from the perinucleus into the nucleus. Its nuclear expression was especially strong between 1 and

3 hr.  $\beta$ -Actin was used as the control protein of whole cells. TFIIH was used as the nuclear protein (Harada et al., 1999). Magnification:  $\times 400$ . W-hepa, heparanase in the whole-cell lysate, Nuc-hepa, heparanase in the nuclear extract.

in the nucleus after 24 hr. However, SF4 did not inhibit translocation of heparanase into the nucleus (Fig. 3B).

This result clearly suggested that heparanase migrated into the nucleus and degraded the HS in the nucleus.

#### P27 and involucrin expression in a calcium-induced esophageal keratinocyte differentiation model

We used p27 and involucrin as differentiation-related factors of keratinocyte (Jetten and Harvat, 1997). The increase in p27 expression started 2 hr after differentiation induction by adding 1 mM calcium chloride, and the involucrin protein level increased after 24 hr (Fig. 4A). In the calcium-induced differentiation of keratinocytes, the increase in involucrin protein was observed after up-regulation of p27.

#### Inhibition of p27 and involucrin appearance stimulated by SF4

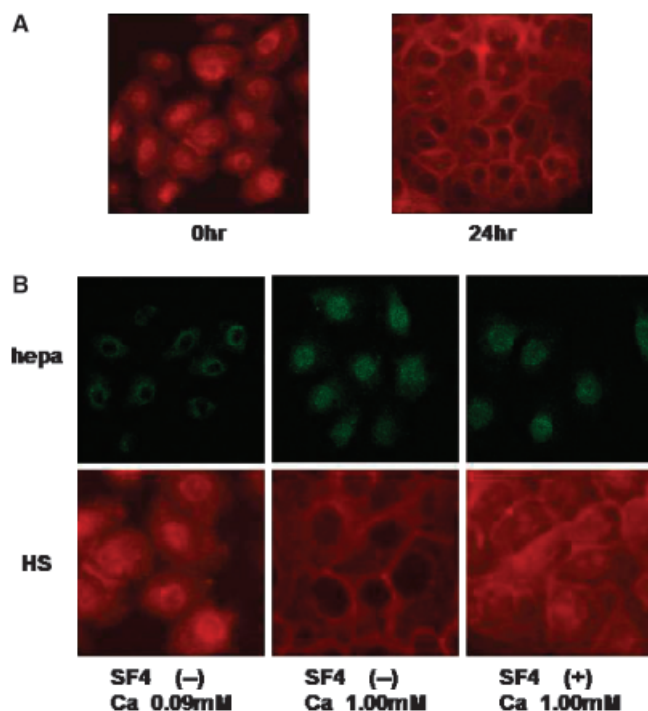
Next, we used SF4 to investigate the relationship between the degradation of nuclear HS and the expression of genes related to the differentiation, such as p27 and

involucrin. When SF4 inhibited heparanase activity, p27 did not appear even after 2 hr. At 24 hr after differentiation induction, SF4 likewise inhibited the involucrin expression (Fig. 4B).

The results suggested that the degradation of nuclear HS correlated with the expression of differentiation-related factors, such as p27 and involucrin, in the calcium-induced esophageal keratinocyte differentiation model.

#### Heparanase is also translocated into the nucleus by keratinocyte differentiation in an organotypic cell culture condition

In order to elucidate the role of heparanase in esophageal cell differentiation, we determined the expression of heparanase in reconstituted esophageal epithelial cells grown as an organotypic culture. As shown in Fig. 5, heparanase was detected at perinuclear site in several cells of the basal layer. From the basal layer to the parabasal layer, heparanase was strongly expressed in the nucleus. These results indicated that heparanase also translocated into the nucleus by keratinocyte differentiation in the organotypic culture condition.



**Fig. 3** In calcium-induced esophageal keratinocyte differentiation, heparan sulfate (HS) in the nucleus almost completely disappeared after 24 hr compared with that in the cytoplasm (A). SF4 is a specific enzyme inhibitor of heparanase. The inhibition of heparanase activity blocked the disappearance of HS in the nucleus after 24 hr. However, SF4 did not inhibit the translocation of heparanase into the nucleus (B). Magnification:  $\times 400$ .

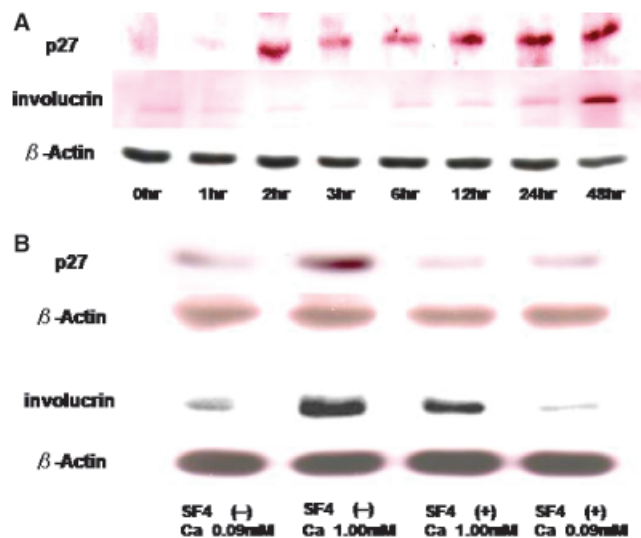
Relationship between heparanase expression and expression of differentiation-related factors such as keratin 13, involucrin, and p27 in normal esophageal epithelium

Heparanase was expressed from the basal layer, and p27 appearing in the early stage of differentiation was also expressed from the basal layer. The distribution of keratin 13 showed that the keratin expression pattern changed in the second layer. Involucrin, as a specific marker of early stage of differentiation, was detected from the third or fourth layer (Fig. 6).

The distribution patterns of keratin, involucrin, and p27 demonstrated that the differentiation of esophageal epithelium arose from the basal layer. The *in vitro* data suggest that heparanase expression correlated with these differentiation markers.

## Discussion

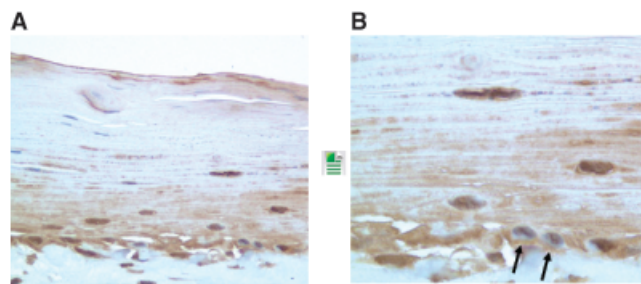
Our study demonstrates three major findings on heparanase. Firstly, heparanase was found to translocate into the nucleus during the normal esophageal keratinocyte differentiation induced by calcium and in the organotypic cell culture condition. This study confirmed the localization of heparanase protein in



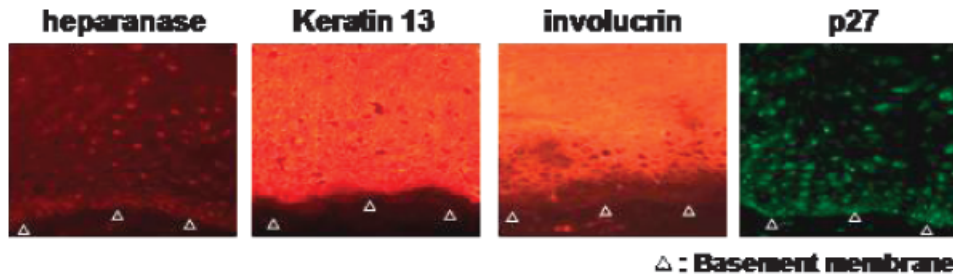
**Fig. 4** In the calcium-induced differentiation of keratinocyte, the increase in p27 expression started at 2 hr. The involucrin protein level increased after 24 hr (A). The concentration of calcium ion used in the differentiation induction experiment was 1 mM. (B) Shows that p27 did not appear after 2 hr, when heparanase activity was inhibited by SF4. After 24 hr, the expression level of involucrin was likewise inhibited.

normal esophageal epithelium and normal esophageal keratinocyte. *In vivo* data showed that heparanase protein was strongly detected in the nucleus, as we demonstrated previously (Okawa et al., 2005). *In vitro* data showed that heparanase protein translocated from the area around the nucleus into the nucleus and from the nucleus to the entirety of the cell in the calcium-induced differentiation model and in the organotypic cell culture.

Several studies reported that heparanase was localized within lysosomes perinuclearly and that processing and activation of latent heparanase occur in lysosomes (Klein et al., 1995; Goldshmidt et al., 2002; Zetser et al., 2004). In our study, heparanase localized at perinuclear sites in keratinocytes when they were not stimulated by calcium, suggesting that heparanase in undifferentiated



**Fig. 5** Immunohistochemistry for heparanase of the reconstituted human esophageal epithelium in the organotypic culture. Heparanase was shown at the perinuclear site in several cells of the basal layer ( $\uparrow$ ). From the basal layer to the parabasal layer, heparanase was strongly expressed in the nuclei. Magnification: A:  $\times 200$ ; B:  $\times 400$ .



**Fig. 6** Relationships between the expression of heparanase and differentiation-related factors such as keratin 13, involucrin, and p27 in normal esophageal epithelium. The distribution patterns of ker-

atin, involucrin, and p27 demonstrated that the differentiation of esophageal epithelium arose from the basal layer.

esophageal keratinocytes is stored in lysosomes. Heparanase was secreted from lysosomes and moved to nuclei by stimulation of keratinocyte differentiation. Although the exact mode of heparanase translocation into the nucleus requires further investigation, our recent study demonstrated that nuclear translocation of heparanase occurred by passive transport (unpublished result).

Secondly, heparanase in the nucleus was found to mediate the degradation of HS in the nucleus. The active form of heparanase in the nucleus is known to degrade HS (Schubert et al., 2004). In this study, the active 50 kDa form of heparanase was detected in both the nucleus and cytoplasm, suggesting that the nuclear translocation of heparanase leads to the degradation of HS in the nucleus. Hiscock et al. (1994) reported that HS did not exist in nuclei in rat ovarian granulose cells. They analyzed the nuclear fraction using Superose-6 chromatography and concluded that HS was not detected in the nuclei pool. Such a controversial conclusion might be due to the difference in analytical methods. We also have to consider the unique variation of biological roles of HS depending on the organs and cells. We will discuss more about this issue in the near future, by applying new techniques. To clarify the specificity of the anti-HS antibody used in our experiment, we treated the paraffin-embedded sections of normal esophageal epithelium with heparitinase I before the reaction with the anti-HS antibody. As the result, HS was not detected by the anti-HS antibody after treatment with heparitinase I (Fig. 1B). Therefore, we confirmed the specificity of the anti-HS antibody and the localization of HS in the nuclei.

Next we used SF4, an uronic acid-type gem-diamine 1-*N*-iminosugar, as a heparanase-specific inhibitor. Although SF4 did not inhibit the nuclear translocation of heparanase, it inhibited the degradation of HS in the nucleus. This result clearly demonstrated that the disappearance of nuclear HS in the calcium-induced keratinocyte differentiation model was caused by heparanase translocated into the nucleus. In the immunohistochemistry of normal esophageal epithelium, HS in both the cell surface and nucleus gradually disappeared under the upper half of the epithelium. The

*in vitro* data indicate that the disappearance of HS in the normal esophageal epithelium correlated with the expression of heparanase, which was expressed mainly in the nucleus.

Thirdly, the role of heparanase in the normal esophageal epithelium strongly correlated with cell differentiation. Previously, we showed that the appearance of heparanase in the nucleus correlated with differentiation of esophageal cancer and breast cancer cells (Ohkawa et al., 2004). The results of the present study are compatible with these previous findings.

In the present study, the block of heparanase-mediated degradation of HS in the nucleus caused significant changes in the expression of genes related to keratinocyte differentiation, such as p27 and involucrin, in the calcium-induced esophageal keratinocyte differentiation.

It has been reported that the growth arrest induced by the increase of a cdk inhibitor such as p27 occurred at an early stage of calcium-induced keratinocyte differentiation, and that at a later stage a differentiation marker such as involucrin is expressed (Jetten and Harvat, 1997). The results obtained from the esophageal keratinocyte differentiation model in our study are compatible with these previous findings. Our study also showed that the degradation of HS in the nucleus by heparanase correlated with the calcium-induced differentiation of esophageal keratinocyte from the early to late stages of cellular differentiation. The *in vitro* data indicate that the expression of heparanase in the nucleus and the disappearance of HS in normal esophageal epithelium correlated with the expression of p27, which was detected from the basal layer, and with the expression of involucrin, which was detected from the third or fourth layer.

In this study, it is still unclear why the nuclear translocation of heparanase is correlated with gene expression involved in keratinocyte differentiation. However, it was shown previously that nuclear fibroblast growth factor receptor-1 worked as a transcriptional regulator and correlated with cAMP-induced differentiation of human neuronal progenitor cells (Stachowiak et al., 2003a, 2003b). HS in the nucleus was known to accumulate and regulate HS-binding proteins, such as FGF

and topoisomerase 1, in the nucleus (Kovalszky et al., 1998; Richardson et al., 2001; Hsia et al., 2003). The release and action of these HS-binding proteins in the nucleus, induced by the nuclear translocation of heparanase, may be the cause of the gene expression related to keratinocyte differentiation.

HS exists very widely in the cell surface, nucleus, and extracellular matrix. Heparanase is a very important enzyme for the regulation of HS and HS-binding proteins. In conclusion, this study presented new findings indicating that heparanase regulates the differentiation of normal esophageal epithelium through nuclear translocation and nuclear HS cleavage, and that heparanase is very important for normal esophageal epithelium development.

**Acknowledgments** We thank Touru Nakai, Tae Yamanishi, and Yoshiko Shirakiya for their excellent technical assistance and our colleagues for valuable scientific advice. This work was supported by a grant from the Ministry of Education, Science, and Culture, Japan and the Ministry of Health and Welfare, Japan, No. 16591319.

## References

- Allen, B.L., Filla, M.S. and Rapraeger, A.C. (2001) Role of heparan sulfate as a tissue-specific regulator of FGF-4 and FGF receptor recognition. *J Cell Biol* 155:845–858, Epub 2001 Nov 2026.
- Andl, C.D., Mizushima, T., Nakagawa, H., Oyama, K., Harada, H., Chruma, K., Herlyn, M. and Rustgi, A.K. (2003) Epidermal growth factor receptor mediates increased cell proliferation, migration, and aggregation in esophageal keratinocytes in vitro and in vivo. *J Biol Chem* 278:1824–1830, Epub 2002 Nov 1814.
- Bernard, D., Mehul, B., Delattre, C., Simonetti, L., Thomas-Colignon, A. and Schmidt, R. (2001) Purification and characterization of the endoglycosidase heparanase 1 from human plantar stratum corneum: a key enzyme in epidermal physiology? *J Invest Dermatol* 117:1266–1273.
- Bernfield, M., Gotte, M., Park, P.W., Reizes, O., Fitzgerald, M.L., Lincecum, J. and Zako, M. (1999) Functions of cell surface heparan sulfate proteoglycans. *Annu Rev Biochem* 68:729–777.
- Bikle, D.D., Ng, D., Tu, C.L., Oda, Y. and Xie, Z. (2001) Calcium- and vitamin D-regulated keratinocyte differentiation. *Mol Cell Endocrinol* 177:161–171.
- Chen, G., Wang, D., Vikramadithyan, R., Yagyu, H., Saxena, U., Pillarisetti, S. and Goldberg, I.J. (2004) Inflammatory cytokines and fatty acids regulate endothelial cell heparanase expression. *Biochemistry* 43:4971–4977.
- Fedarko, N.S. and Conrad, H.E. (1986) A unique heparan sulfate in the nuclei of hepatocytes: structural changes with the growth state of the cells. *J Cell Biol* 102:587–599.
- Fedarko, N.S., Ishihara, M. and Conrad, H.E. (1989) Control of cell division in hepatoma cells by exogenous heparan sulfate proteoglycan. *J Cell Physiol* 139:287–294.
- Filla, M.S., Dam, P. and Rapraeger, A.C. (1998) The cell surface proteoglycan syndecan-1 mediates fibroblast growth factor-2 binding and activity. *J Cell Physiol* 174:310–321.
- Ginath, S., Menczer, J., Friedmann, Y., Aingorn, H., Aviv, A., Tajima, K., Dantes, A., Glezerman, M., Vlodavsky, I. and Amsterdam, A. (2001) Expression of heparanase, Mdm2, and erbB2 in ovarian cancer. *Int J Oncol* 18:1133–1144.
- Gohji, K., Hirano, H., Okamoto, M., Kitazawa, S., Toyoshima, M., Dong, J., Katsuoka, Y. and Nakajima, M. (2001) Expression of three extracellular matrix degradative enzymes in bladder cancer. *Int J Cancer* 95:295–301.
- Goldshmidt, O., Nadav, L., Aingorn, H., Irit, C., Feinstein, N., Ilan, N., Zamir, E., Geiger, B., Vlodavsky, I. and Katz, B.Z. (2002) Human heparanase is localized within lysosomes in a stable form. *Exp Cell Res* 281:50–62.
- Goldshmidt, O., Yeikilis, R., Mawasi, N., Paizi, M., Gan, N., Ilan, N., Pappo, O., Vlodavsky, I. and Spira, G. (2004) Heparanase expression during normal liver development and following partial hepatectomy. *J Pathol* 203:594–602.
- Haimov-Kochman, R., Friedmann, Y., Prus, D., Goldman-Wohl, D.S., Greenfield, C., Anteby, E.Y., Aviv, A., Vlodavsky, I. and Yagel, S. (2002) Localization of heparanase in normal and pathological human placenta. *Mol Hum Reprod* 8:566–573.
- Harada, H., Tanaka, H., Shimada, Y., Shinoda, M., Imamura, M. and Ishizaki, K. (1999) Lymph node metastasis is associated with allelic loss on chromosome 13q12–13 in esophageal squamous cell carcinoma. *Cancer Res* 59:3724–3729.
- Hecht, J.T., Hayes, E., Haynes, R., Cole, W.G., Long, R.J., Farach-Carson, M.C. and Carson, D.D. (2005) Differentiation-induced loss of heparan sulfate in human exostosis derived chondrocytes. *Differentiation* 73:212–221.
- Hennings, H., Michael, D., Cheng, C., Steinert, P., Holbrook, K. and Yuspa, S.H. (1980) Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell* 19:245–254.
- Hiscock, D.R., Yanagishita, M. and Hascall, V.C. (1994) Nuclear localization of glycosaminoglycans in rat ovarian granulosa cells. *J Biol Chem* 269:4539–4546.
- Hsia, E., Richardson, T.P. and Nugent, M.A. (2003) Nuclear localization of basic fibroblast growth factor is mediated by heparan sulfate proteoglycans through protein kinase C signaling. *J Cell Biochem* 88:1214–1225.
- Ikeguchi, M., Ueta, T., Yamane, Y., Hirooka, Y. and Kaibara, N. (2002) Quantitative analysis of heparanase messenger RNA expression in hepatocellular carcinoma. *J Surg Oncol* 81:148–154; discussion 154.
- Ikuta, M., Podyma, K.A., Maruyama, K., Enomoto, S. and Yanagishita, M. (2001) Expression of heparanase in oral cancer cell lines and oral cancer tissues. *Oral Oncol* 37:177–184.
- Iozzo, R.V. (1998) Matrix proteoglycans: from molecular design to cellular function. *Annu Rev Biochem* 67:609–652.
- Ishihara, M. and Conrad, H.E. (1989) Correlations between heparan sulfate metabolism and hepatoma growth. *J Cell Physiol* 138:467–476.
- Ishihara, M., Fedarko, N.S. and Conrad, H.E. (1987) Involvement of phosphatidylinositol and insulin in the coordinate regulation of proteoheparan sulfate metabolism and hepatocyte growth. *J Biol Chem* 262:4708–4716.
- Ishihara, M. and Mamiya, G. (1989) Role of heparan sulfate proteoglycan for control of cell growth. *Tanpakushitsu Kakusan Koso* 34:853–862.
- Jetten, A.M. and Harvat, B.L. (1997) Epidermal differentiation and squamous metaplasia: from stem cell to cell death. *J Dermatol* 24:711–725.
- Klein, O., Staroselsky, A.N., Savion, N., Donin, N., Michowitz, M. and Leibovici, J. (1995) Metastasis-associated cell functions in AKR lymphoma malignancy variants. *Invasion Metastasis* 15:211–221.
- Koliopanos, A., Friess, H., Kleeff, J., Shi, X., Liao, Q., Pecker, I., Vlodavsky, I., Zimmermann, A. and Buchler, M.W. (2001) Heparanase expression in primary and metastatic pancreatic cancer. *Cancer Res* 61:4655–4659.
- Komuves, L., Oda, Y., Tu, C.L., Chang, W.H., Ho-Pao, C.L., Mauro, T. and Bikle, D.D. (2002) Epidermal expression of the full-length extracellular calcium-sensing receptor is required for normal keratinocyte differentiation. *J Cell Physiol* 192:45–54.
- Kovalszky, I., Dudas, J., Olah-Nagy, J., Pogany, G., Tovary, J., Timar, J., Kopper, L., Jeney, A. and Iozzo, R.V. (1998) Inhibition of DNA topoisomerase I activity by heparan sulfate and modulation by basic fibroblast growth factor. *Mol Cell Biochem* 183:11–23.

- Liang, Y., Haring, M., Roughley, P.J., Margolis, R.K. and Margolis, R.U. (1997) Glypican and biglycan in the nuclei of neurons and glioma cells: presence of functional nuclear localization signals and dynamic changes in glypican during the cell cycle. *J Cell Biol* 139:851–864.
- Nakajima, M., Irimura, T., DiFerrante, D., DiFerrante, N. and Nicolson, G.L. (1983) Heparan sulfate degradation: relation to tumor invasiveness and metastatic potential of mouse B16 melanoma sublines. *Science* 220:611–613.
- Nakajima, M., Irimura, T., DiFerrante, N. and Nicolson, G.L. (1984) Metastatic melanoma heparanase. Characterization of heparan sulfate degradation fragments produced by B16 melanoma endoglucuronidase. *J Biol Chem* 259:2283–2290.
- Nishimura, Y., Shitara, E., Adachi, H., Toyoshima, M., Nakajima, M., Okami, Y. and Takeuchi, T. (2000) Flexible synthesis and biological activity of uronic acid-type gem-diamine 1-N-iminosugars: a new family of glycosidase inhibitors. *J Org Chem* 65:2–11.
- Nobuhisa, T., Naomoto, Y., Ohkawa, T., Takaoka, M., Ono, R., Murata, T., Gunduz, M., Shirakawa, Y., Yamatsuji, T., Haisa, M., Matsuoka, J., Tsujigiwa, H., Nagatsuka, H., Nakajima, M. and Tanaka, N. (2004) Heparanase expression correlates with malignant potential in human colon cancer. *J Cancer Res Clin Oncol* 30:30.
- Nobuhisa, T., Naomoto, Y., Takaoka, M., Tabuchi, Y., Ookawa, K., Kitamoto, D., Gunduz, E., Gunduz, M., Nagatsuka, H., Haisa, M., Matsuoka, J., Nakajima, M. and Tanaka, N. (2005) Emergence of nuclear heparanase induces differentiation of human mammary cancer cells. *Biochem Biophys Res Commun* 331:175–180.
- Okawa, T., Naomoto, Y., Nobuhisa, T., Takaoka, M., Motoki, T., Shirakawa, Y., Yamatsuji, T., Inoue, H., Ouchida, M. and Gunduz, M., et al. (2005) Heparanase is involved in angiogenesis in esophageal cancer through induction of cyclooxygenase-2. *Clin Cancer Res* 11:7995–8005.
- Ohkawa, T., Naomoto, Y., Takaoka, M., Nobuhisa, T., Noma, K., Motoki, T., Murata, T., Uetsuka, H., Kobayashi, M. and Shirakawa, Y. (2004) Localization of heparanase in esophageal cancer cells: respective roles in prognosis and differentiation. *Lab Invest* 84:1289–1304.
- Pillai, S., Bickle, D.D., Mancianti, M.L., Cline, P. and Hincenbergs, M. (1990) Calcium regulation of growth and differentiation of normal human keratinocytes: modulation of differentiation competence by stages of growth and extracellular calcium. *J Cell Physiol* 143:294–302.
- Richardson, T.P., Trinkaus-Randall, V. and Nugent, M.A. (2001) Regulation of heparan sulfate proteoglycan nuclear localization by fibronectin. *J Cell Sci* 114:1613–1623.
- Saijo, M., Kitazawa, R., Nakajima, M., Kurosaka, M., Maeda, S. and Kitazawa, S. (2003) Heparanase mRNA expression during fracture repair in mice. *Histochem Cell Biol* 120:493–503, Epub 2003 Nov 2015.
- Schlessinger, J., Lax, I. and Lemmon, M. (1995) Regulation of growth factor activation by proteoglycans: what is the role of the low affinity receptors? *Cell* 83:357–360.
- Schubert, S.Y., Ilan, N., Shushy, M., Ben-Izhak, O., Vlodaysky, I. and Goldshmidt, O. (2004) Human heparanase nuclear localization and enzymatic activity. *Lab Invest* 84:535–544.
- Shinyo, Y., Kodama, J., Hongo, A., Yoshinouchi, M. and Hiramatsu, Y. (2003) Heparanase expression is an independent prognostic factor in patients with invasive cervical cancer. *Ann Oncol* 14:1505–1510.
- Simizu, S., Ishida, K., Wierzba, M.K., Sato, T.A. and Osada, H. (2003) Expression of heparanase in human tumor cell lines and human head and neck tumors. *Cancer Lett* 193:83–89.
- Stachowiak, M.K., Fang, X., Myers, J.M., Dunham, S.M., Berezney, R., Maher, P.A. and Stachowiak, E.K. (2003a) Integrative nuclear FGFR1 signaling (INFS) as a part of a universal “feed-forward-and-gate” signaling module that controls cell growth and differentiation. *J Cell Biochem* 90:662–691.
- Stachowiak, E.K., Fang, X., Myers, J., Dunham, S. and Stachowiak, M.K. (2003b) cAMP-induced differentiation of human neuronal progenitor cells is mediated by nuclear fibroblast growth factor receptor-1 (FGFR1). *J Neurochem* 84:1296–1312.
- Stadlmann, S., Moser, P.L., Pollheimer, J., Steiner, P., Krugmann, J., Dirnhofer, S., Mikuz, G., Margreiter, R. and Amberger, A. (2003) Heparanase-1 gene expression in normal, hyperplastic and neoplastic prostatic tissue. *Eur J Cancer* 39:2229–2233.
- Takaoka, M., Naomoto, Y., Ohkawa, T., Uetsuka, H., Shirakawa, Y., Uno, F., Fujiwara, T., Gunduz, M., Nagatsuka, H., Nakajima, M., Tanaka, N. and Haisa, M. (2003) Heparanase expression correlates with invasion and poor prognosis in gastric cancers. *Lab Invest* 83:613–622.
- Toyoshima, M. and Nakajima, M. (1999) Human heparanase. Purification, characterization, cloning, and expression. *J Biol Chem* 274:24153–24160.
- Vlodaysky, I., Friedmann, Y., Elkin, M., Aingorn, H., Atzmon, R., Ishai-Michaeli, R., Bitan, M., Pappo, O., Peretz, T. Michal, I., Spector, L., and Pecker, I. (1999) Mammalian heparanase: gene cloning, expression and function in tumor progression and metastasis. *Nat Med* 5:793–802.
- Zcharia, E., Metzger, S., Chajek-Shaul, T., Aingorn, H., Elkin, M., Friedmann, Y., Weinstein, T., Li, J.P., Lindahl, U. and Vlodaysky, I. (2004) Transgenic expression of mammalian heparanase uncovers physiological functions of heparan sulfate in tissue morphogenesis, vascularization, and feeding behavior. *Faseb J* 18:252–263.
- Zetser, A., Bashenko, Y., Miao, H.Q., Vlodaysky, I. and Ilan, N. (2003) Heparanase affects adhesive and tumorigenic potential of human glioma cells. *Cancer Res* 63:7733–7741.
- Zetser, A., Levy-Adam, F., Kaplan, V., Gingis-Velitski, S., Bashenko, Y., Schubert, S., Flugelman, M.Y., Vlodaysky, I. and Ilan, N. (2004) Processing and activation of latent heparanase occurs in lysosomes. *J Cell Sci* 117:2249–2258.