

# Increased Furin Activity Enhances the Malignant Phenotype of Human Head and Neck Cancer Cells

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**Many proteins are synthesized as inactive proforms requiring a proteolytic processing to render them active. A variety of proteases catalyze these cleavage reactions. Proprotein convertases are a family of serine proteases capable of activating substrates that will subsequently intervene in extracellular matrix (ECM) degradation, cell growth, differentiation and viral pathogenesis. Furin, the prototype of this family, has been implicated in many physiological and pathological processes. Some of its substrates such as TGF- $\beta$ , MT-MMP's, and IGFR-1 have been identified. Overexpression of furin has been observed in several human tumors. In this report we demonstrate that overexpression of furin causes a significant increase in the invasive potential of human tumor cells of low and moderate aggressive potential *in vitro* and *in vivo*. SCC12 and SCC15 were transfected with furin cDNA, resulting in efficient processing of furin substrates. An *in vivo* invasion assay showed enhancement of invasive ability. Inhibition of furin activity with the synthetic inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethyl-ketone, CMK, showed a significant decrease in both processing and *in vitro* invasiveness. A moderate enhancement in proliferation rate was observed when cells were transfected with furin. CMK treatment resulted in a marked reduction of this effect. Tumors obtained after subcutaneous (s.c.) inoculation of furin-overexpressing cells were larger and developed earlier than the controls. Furin overexpression caused an imbalance in the activation of invasion and proliferation-related substrates leading to the acquisition of an advanced malignant phenotype. In addition, inhibition of furin activity decreases substrate activation, proliferation rate, and invasive potential of cancer cells, suggesting that furin is a potentially useful target for therapeutics. (Am J Pathol 2003, 162:439–447)**

Proteolytic processing is one of the key mechanisms for the production of mature proteins. A wide variety of precursors including hormones, receptors, and growth factors require cleavage to display full activity. Many en-

zymes capable of activating substrates by proteolytic cleavage at specific sequences have been described. Proprotein convertases (PC) are a family of serine proteases that cleave proteins at the carboxy terminus of a sequence of basic paired amino acids, RXR/KR.<sup>1</sup> A wide variety of substrates have been described that contain this particular motif at the carboxy terminal end of their pro-domains including prohormones, viral envelope proteins, anthrax endotoxin, metalloproteinases, etc.<sup>2</sup>

Furin, a prototypic PC, is ubiquitously expressed and has been implicated in many physiological and pathological processes.<sup>3</sup> Overexpression of this PC in human cancer has been well documented. It has been shown that furin levels are increased in lung,<sup>4</sup> breast,<sup>5</sup> and head and neck tumors.<sup>6</sup> In addition, furin has been proposed as a marker of differentiation between non-small cell lung carcinomas (NSCLC) and small cell lung carcinomas (SCLC) since an elevated expression of this PC has been observed in the former.<sup>4</sup>

Metalloproteinases are a family of endopeptidases implicated in the degradation of most components of basement membranes and, hence, directly related to tumor cell invasion.<sup>7</sup> Among the six groups in which these enzymes have been subdivided, membrane-bound metalloproteinases, MT-MMP's, are characterized by their localization to the plasma membrane and intracellular activation.<sup>7</sup> Six members have been described so far and some of them have been associated with cancer. Interestingly, all of them bear the recognition sequence for furin in the boundaries of their pro-domains.<sup>8</sup> MT1-MMP, one of the best-studied invasion-related substrates associated with furin, plays a key role in the activation of progelatinase A.<sup>9,10</sup> This MT-MMP, may represent the main bridge between furin and, ultimately, the biological effect, ie, degradation of the extracellular matrix.<sup>11</sup> Furin inhibition with  $\alpha$ -1 PDX, a specific inhibitor of this PC,<sup>12</sup> demonstrated that the levels of processed MT1-MMP as well as the invasion ability of cells was greatly diminished in head and neck squamous cell carcinoma (HNSCC) cell lines.<sup>13</sup> On the other hand, some reports argue that MT1-MMP can be activated in a furin-independent manner suggesting that, at least in some systems, furin may

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not be crucial to tumor progression.<sup>14</sup> Furin can process another metalloproteinase, stromelysin-3,<sup>15</sup> which is able to degrade  $\alpha$ -1 proteinase inhibitor, a serin protease inhibitor,<sup>16</sup> contributing to enhance extracellular matrix turnover.

In addition, furin can affect cell growth through processing of growth factors and growth factor receptors. Reduction in the levels of functional insulin growth factor receptor-1 (IGF-1R) by PDX-mediated inhibition of furin led to a drastic reduction in tumorigenesis and cell growth rate in HT-29, an aggressive colon cancer cell line.<sup>17</sup>

Other experiments, however, pointed to a potentially opposite effect. A furin-transfected MCF-7 breast carcinoma cell acquired greater dependence on estradiol and exhibited growth impairment<sup>18</sup> at low estradiol concentration. Since the effects of furin overexpression on invasion and tumorigenesis were not previously examined, we transfected the furin cDNA into two moderately aggressive head and neck cancer cell lines. Invasion ability and proliferation of transfectants were analyzed *in vitro* and *in vivo*. Our results demonstrate that overexpression of furin significantly enhanced the advanced malignant phenotype of HNSCC cells.

## Materials and Methods

### Cell Lines

The human cell lines from HNSCC, SCC12, and SCC15 used in these experiments were obtained from the American Type Culture Collection (Rockville, MD). They were cultured in suspension minimal essential medium (S-MEM) (Sigma, St. Louis, MO) with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, and 100 units/ml penicillin and 100  $\mu$ g/ml streptomycin. For selection and culture of transfected cell lines, 400  $\mu$ g/ml G418 were added.

### Transfection of Cell lines

Cells were transfected with the mammalian expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA) or pcDNA3.1-furin. The latter plasmid was constructed as follows. Full-length coding sequence from human furin was amplified by PCR from the plasmid pSVL-fur (American Type Culture Collection).

The primers used were: 1) 5' primer: 5' GCCAAGCT-TACCCCATGGAGCTGAGGCCCTGGTTG 3' and 2) 3' primer: 5' GCGAATTCAGTGGGCTCATCAGAGGGCGCTCTGG 3'.

The primers were designed to include the <sup>217</sup>ATG (5' primer) and the stop codon <sup>259</sup>TGA, which amplified a 2400-bp fragment. In addition, a Hind III site in the 5' and an EcoRI site in the 3' primer restriction sites were added to facilitate directional cloning. PCR conditions used consisted of an initial step at 94°C for 2 minutes, 30 cycles of annealing/synthesis at 72°C for 3 minutes 30 seconds, a denaturation step at 94°C for 1 minute, followed by a final extension of 68°C for 7 minutes. Sequencing checked the identity of the PCR product after elution of a 2.4-kb band

in agarose gels. This HindIII/EcoRV fragment was cloned directionally in the corresponding sites in the multi-cloning site of the vector. Transfections were performed according to the manufacturer's instructions (Lipofectamine, GIBCO, Carlsbad, CA). Colonies were selected from the transfection mixture with G418 800  $\mu$ g/ml. For maintenance and culturing of transfectant clones, 400  $\mu$ g/ml G418 were added to the medium. Separate clones of stable transfectants were used for further studies.

### Cell Growth Assay

[<sup>3</sup>H]-thymidine incorporation was evaluated after plating in  $2.0 \times 10^4$  cells/well in 96-well culture plates. Twenty-four hours after plating, cells were incubated in a growth arrest medium (0.5% FBS-MEM, L-Glu, Pen-Strep) for 24 hours. Then, cells were incubated an additional 24 hours either in the presence of serum or IGF-1 (Sigma, St. Louis, MO). When indicated, the furin inhibitor, decanoyl-Arg-Val-Lys-Arg-chloromethyl-ketone (CMK), 100  $\mu$ mol/L was included in the incubation mixture. For the last 4 hours of incubation, 0.5  $\mu$ Ci/well [<sup>3</sup>H]-methylthymidine (Moravak, Biochemicals, Brea, CA) was added. After this period of time, cells were washed three times with phosphate-buffered saline (PBS) and treated with 10% trichloroacetic acid at 4°C for 30 minutes and washed three times with water. Cells were then rinsed with 70% ethanol and air-dried. Labeled DNA was dissolved with 200  $\mu$ l 10 mmol/L NaOH, 1% SDS and counted with Scintiverse (Fisher, Pittsburgh, PA). Results are expressed as percent with respect to the vector-alone-transfected cells (when incubations were performed in the presence of serum) or to the vector-alone-transfected cells in serum-free medium (in the studies with IGF-1).

### Western Analysis

Cells were grown to 80% confluency and washed three times with cold PBS. 0.1 to 1 ml of RIPA lysis buffer (1X PBS, 0.1% SDS, 0.5% Na deoxycholate, 1% Nonidet P-40) with the protease inhibitors 1 mmol/L aprotinin, 100 mmol/L PMSF, and 100 mmol/L Na<sub>3</sub>VO<sub>3</sub> was added to the culture and incubated at room temperature for 2 minutes. Cells were scraped, passed through a 21-G needle and incubated at 4°C for 30 minutes. After a centrifugation at  $10,000 \times g$  for 5 minutes, the supernatant was separated. Aliquots of the cell lysates were fractionated by electrophoresis in SDS-PAGE, 8% for the analysis of furin and IGF-1R, and 10% for the analysis of MT1-MMP. The proteins were electroblotted to a nitrocellulose membrane and probed with the antifurin monoclonal antibody MON-152 (Alexis, San Diego, CA) and polyclonal anti-MT1-MMP antibody AB815 (Chemicon, Temecula, CA) and polyclonal anti-IGF-1R $\beta$  (C-19) (Santa Cruz, Santa Cruz, CA). Loading controls were performed using an  $\alpha$ -actin polyclonal antibody (I-19) (Santa Cruz). Densitometric analysis was performed with Imagequant 3.1 densitometer (Molecular Dynamics Amersham, Piscataway, NJ) using the software Iq3.15.

## Zymography

A total of  $5 \times 10^6$  cells were grown overnight in serum-free S-MEM with 2 mmol/L L-glutamine. The conditioned media were concentrated to 200  $\mu$ l by ultrafiltration (Centriprep 30, Amicon Millipore, Bedford, MA) and 40  $\mu$ g were loaded in a 10% precast zymogram gel (Invitrogen). Proteins were renatured, developed, and stained according to the manufacturer's suggestions. Zymography standards were purchased from Chemicon (Temecula, CA).

## In Vitro Invasion Assay

The invasive ability of cells was assessed with Biocoat Matrigel invasion chambers (Becton Dickinson, Bedford, MA) and used following the manufacturer's suggestions. Briefly, cells were trypsinized and 25,000 cells were suspended in serum-free S-MEM with 0.1% bovine serum albumin (BSA). In the inhibition studies, 100  $\mu$ mol/L CMK (Alexis Corp, San Diego, CA.) was added to this medium. Medium containing 5% serum was used as chemoattractant. Incubation was performed for 22 hours. Cells that degraded the reconstituted extracellular matrix (ECM) and passed through the invasion chamber were fixed, stained, and counted. Experiments were performed in triplicate. Fifteen to 20 fields were counted per invasion chamber (a total of 3000 to 6000 cells per transfectant). Control inserts (Becton Dickinson, Bedford, MA) were used to assess the ability to migrate through the chambers.

## In Vivo Invasion Assay

Tracheal transplants were prepared as described,<sup>19,20</sup> Cells from each transfected cell line ( $5 \times 10^5$ ) were inoculated into de-epithelialized rat trachea (Zivic-Miller, Zelienople, PA). Eight to 16 tracheas were used. After cell inoculation, the tracheas were sealed and transplanted into the dorsal s.c. tissues of severe combined immunodeficient (Scid) mice. Tracheal transplants were removed surgically at 6 or 8 weeks, sectioned into 3-mm thick rings, and fixed in 10% formalin. After hematoxylin/eosin staining, the degree of invasion of the tracheal wall was determined by measuring the length of maximum penetration of the tumor cells into the tracheal wall. All microscopic images of cross-sections of tracheal transplants were digitized at  $\times 40$ . The lengths were determined by measuring the distance between the luminal center and the most distant point of tumor invasion. If the lumen was obliterated, the distance measured was between the geometric center of the tumor mass inside the tracheal lumen and the most distant point of tumor invasion either inside or outside the tracheal wall. Each tracheal transplant was represented by two to six measurements corresponding to the number of cross-sections containing tumor cells. A mean was calculated for each tracheal transplant and for each group of transfected cells. The results were expressed in  $\mu$ m of penetration depth.

## In Vivo Tumorigenicity

Either furin- or vector-alone-transfected cells ( $5 \times 10^6$ ) were injected into the s.c. tissues of Scid mice (five mice per cell line). Mice were shaved weekly to assess time of tumor development, appearance, and volume. Tumors were measured twice a week after the appearance of the first tumor for the pair of transfected cells by using a Vernier caliper. Volumes ( $V$ ) of the tumors were obtained by using the following equation:  $V = [(L_1 + L_2)/2] \times L_1 \times L_2 \times 0.526$ , where  $L_1$  and  $L_2$  are the length and width of the s.c. tumor.<sup>21</sup>

## Immunohistochemistry

Furin immunohistochemistry of tumors was performed using paraffin-embedded material. All paraffin sections were subjected to antigen retrieval for 10 minutes in distilled water. MON-152 was used as primary antibody at a 1:100 dilution. An avidin-biotin-peroxidase kit (Vectastain Elite, Vector, Burlingame, CA) was used, followed by the chromagen 3,3-diaminobenzidine to develop the immunostain. Negative controls, not incubated with furin antibodies, were incubated with normal mouse IgG. All sections were counterstained with hematoxylin and mounted.

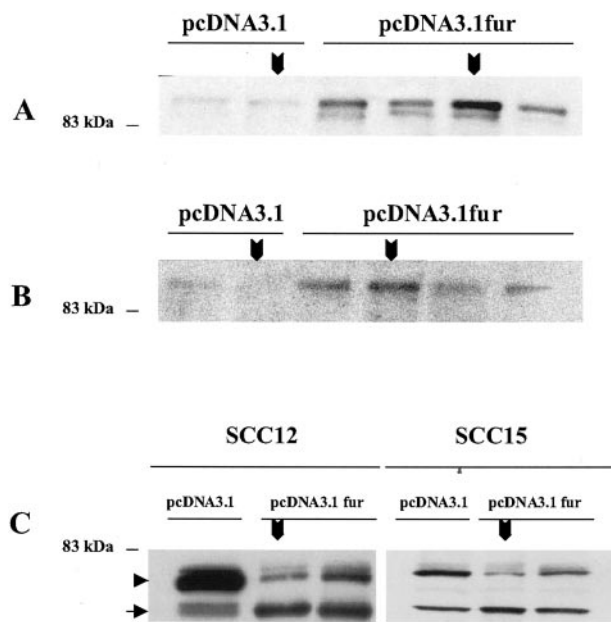
## Results

### *Furin Transfection Enhances the Malignant Phenotype of HNSCC Cells*

In a previous study we demonstrated that expression of endogenous furin correlated with the aggressiveness of head and neck and lung SCC cell lines.<sup>6</sup> Similarly, metastatic tumors exhibited higher levels of furin mRNAs and protein than their non-metastatic counterparts. These results suggested that elevated furin expression could be associated with aggressive behavior.

To demonstrate this association, stable transfection with the complete coding sequence of furin cDNA was performed. Based on the correlation between furin expression<sup>6</sup> and the known *in vivo* growth behavior in immunosuppressed mice,<sup>22</sup> two HNSCC cell lines (SCC12 and SCC15) were selected for transfection with the complete furin cDNA. SCC12 cells display a less malignant behavior than the moderately aggressive cell line, SCC15. Seven to 10 transfectants were analyzed in each case and selection of the clone to be used for future studies was based on the levels of furin expression and ability to process MT1-MMP, a key furin substrate directly related to degradation of ECM and to invasive behavior (Figure 1).

Selected clones corresponding to vector-alone (pcDNA3.1) or furin (pcDNA3.1 fur) are shown in Figure 1, A (SCC12) and B (SCC15). MT1-MMP processing was analyzed in cell lysates from vector-alone- and furin-transfected cell lines. As shown in Figure 1C, the ratio of processed to unprocessed MT1-MMP in furin transfectants is higher than in their vector-alone counterparts. The vector-alone-transfected cells showed a ratio of 0.3



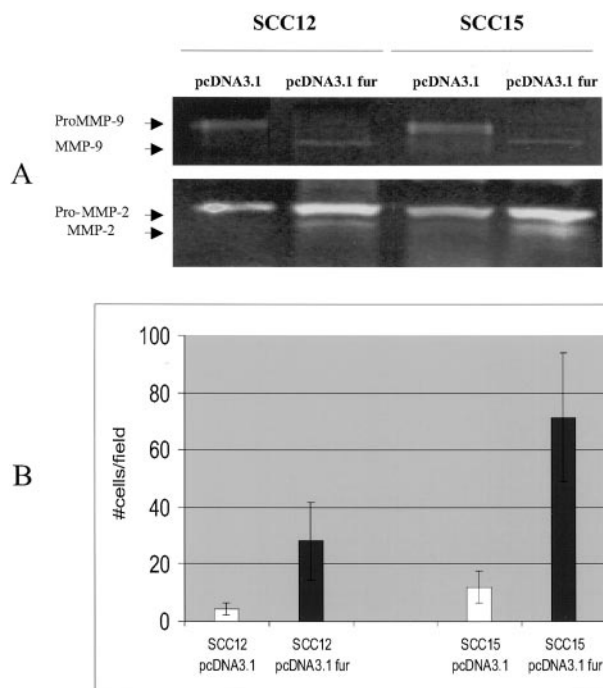
**Figure 1.** Western blot analysis of furin expression in cells transfected with vector-alone (pcDNA3.1) or full-length furin cDNA (pcDNA3.1 fur). Vertical **arrows** indicate clones selected for further studies. **A:** SCC12. **B:** SCC15. **C:** Western analysis of MT1-MMP showing the processed form (60 kd, full **arrow**) and the pro-form (63 kd, **arrowhead**). Cell lysates from SCC12 cells (100  $\mu$ g) and SCC15 (30  $\mu$ g) were fractionated in 10% SDS-PAGE. Note the higher proportion of processed form in the furin-transfected cells.

(SCC12) and 0.7 (SCC15). Transfection of furin and enhanced processing of MT1-MMP is reflected in the higher ratio of 5 and 1.5 for the SCC12 and 2.9 and 1 for the SCC15 furin transfectants. This processing of a key furin substrate demonstrates that, in these cell lines, furin transfection led to an enhanced activation of the metalloproteinase.

Since enhanced MT1-MMP activation results in activation of progelatinase A (MMP-2),<sup>7</sup> the enzyme that directly degrades collagen IV, the gelatinolytic potential of transfectants was evaluated. Gelatinase activity was consistently and significantly enhanced in the furin transfectants. Overexpression of furin resulted in a moderate increase in the levels of the mature MMP-2 form. The levels of MMP-2 are very low in these cell lines, in agreement with their low level of aggressiveness. However, a slight increase in the expression of MMP-2 was observed in the furin-transfected cells.

The collagenolytic activity corresponding to MMP-9 was also analyzed. Its activity was very low but detectable. Interestingly, only the unprocessed form of MMP-9 was observed in vector-alone transfectants (Figure 2A) suggesting that furin may participate in MMP-9 activation.

The invasive ability of cells was evaluated *in vitro* using invasion chambers. Furin-overexpressing SCC12 and SCC15 cells exhibited a remarkable increase in invasiveness when compared to the respective vector-alone-transfected cells (Figure 2B). A fourfold increase in the number of SCC15 furin-transfected cells that were able to invade was observed. Similar results were obtained with SCC12, a less aggressive cell line. Although the absolute number of cells that invaded through the filter were lower than those seen in SCC15, the invasive ability enhance-



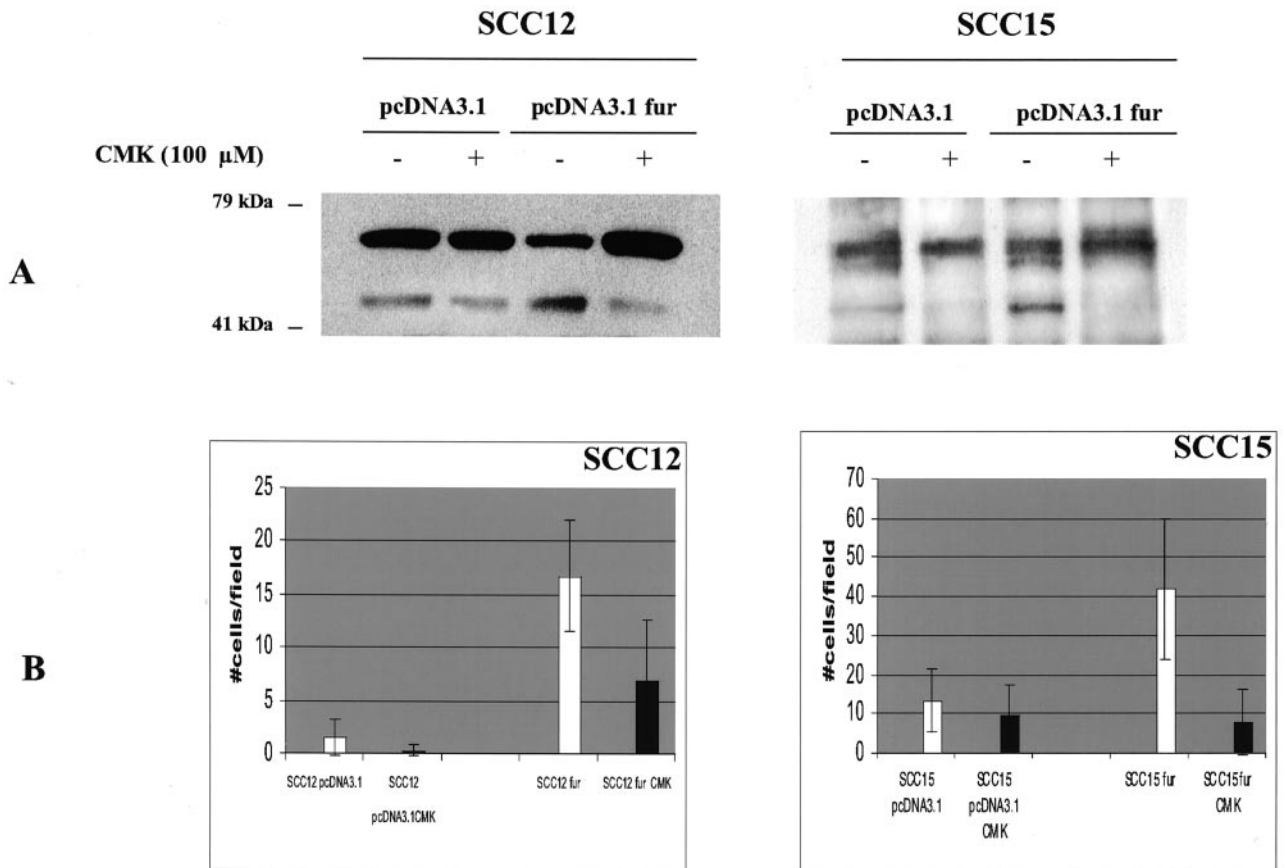
**Figure 2. A:** Gelatinase zymogram of SCC12 and SCC15 vector-alone (pcDNA3.1) and furin (pcDNA3.1 fur)-transfected cells. **B:** Histogram of the *in vitro* invasion assays performed with furin transfectants from two HNSCC lines and their respective vector-alone-transfected cells. Results are expressed as number of cells per field. Each **bar** represents a mean of three different experiments. An average of 4000 cells was counted per transfected clone. **White bars:** vector-alone-transfected cells; **black bars:** furin transfectants. One-sided *t*-test indicated statistically significant differences ( $P < 0.05$ ). Furin transfectants showed an increased ability to pass through the reconstituted ECM.

ment was similar (four- to fivefold). No significant differences in migration were observed when assayed in control inserts, ie, without ECM coating.

### CMK-Inhibition of Furin Activity Reverts the Malignant Phenotype of HNSCC

The synthetic inhibitor CMK is a lipophilic furin inhibitor capable of penetrating the plasma membrane reaching the interior of the cell<sup>23</sup> where it interacts with furin and blocks its catalytic site.<sup>24</sup> Cells were plated and incubated in the presence of 0, 50, 100, and 250  $\mu$ mol/L CMK for 2 days. No cell detachment, presence of vacuolation, or changes in the morphology was observed with concentrations as high as 100  $\mu$ mol/L. Although no vacuolation or other abnormalities were observed after incubation with 250  $\mu$ mol/L, roughly half of the cells detached. This concentration-dependent effect was observed in all cell lines, furin- or vector-alone-transfected. Trypan blue staining revealed that only 3 to 6% of the cells died when incubated at 100  $\mu$ mol/L CMK. Based on this data, a concentration of 100  $\mu$ mol/L was used in the following studies.

Inhibition of furin activity resulted in a reduction in the processing of MT1-MMP either in vector-alone or furin-transfected cells (Figure 3A). *In vitro* invasion studies pointed in the same direction. Both control cell lines displayed a low invasive ability. After incubation with the

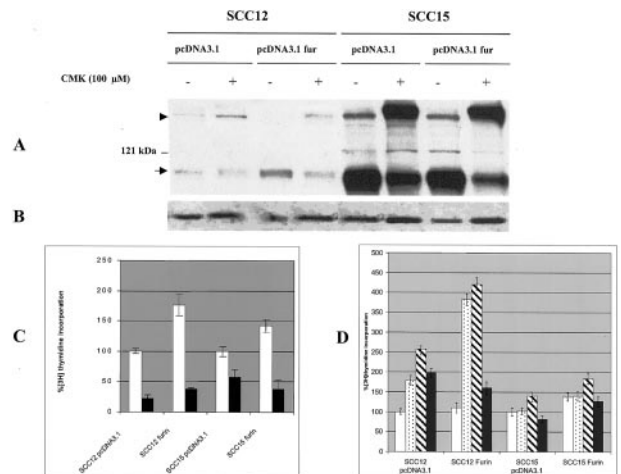


**Figure 3. A:** Western blot analysis of MT-1MMP processing by cells with or without treatment with 100  $\mu\text{mol/L}$  CMK. Inhibition of processing was observed after treatment with CMK especially in furin-transfected cells. **B:** *In vitro* invasion assays performed with furin transfectants. Assay was performed as indicated in Figure 2. The concentration of inhibitor used was 100  $\mu\text{mol/L}$  ( $P < 0.05$ ). Treatment with the inhibitor diminished ability to invade the reconstituted ECM.

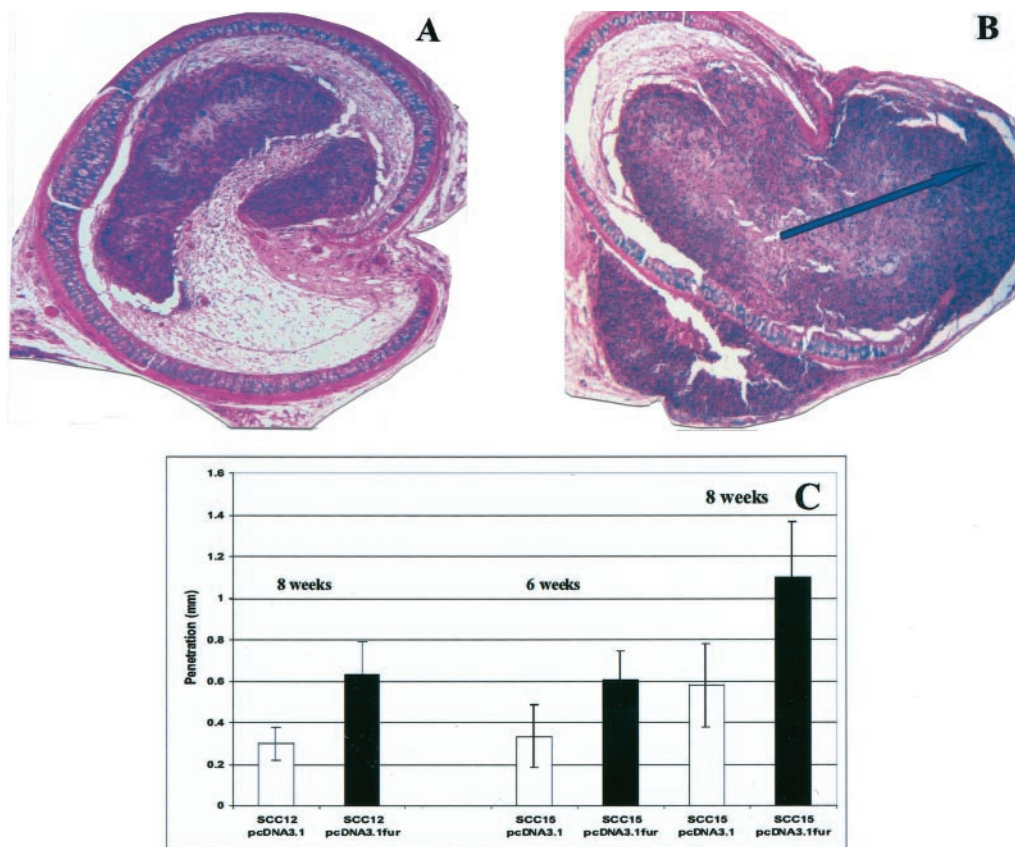
inhibitor, very few or no cells were able to invade the coated filter. When furin-transfected cells were grown in the presence of the inhibitor, a remarkably regression in invasive ability was observed when compared to the untreated counterparts (Figure 3B).

Inhibition of furin may have effects on proliferation as well. This PC is able to activate several growth factors and growth factor receptors suggesting that cell proliferation rate may be affected in furin-overexpressing cells. IGF-1R, one of these substrates,<sup>25</sup> mediates mitogenic responses in normal keratinocytes and in SCC.<sup>26</sup> To study the possible effects of furin in proliferation, IGF-1R processing was analyzed.

Both the pro-receptor and the mature form of IGF-1R (approximately 105 kD) was observed in SCC12 cells transfected with vector-alone (Figure 4A, lane 1) indicating some degree of endogenous furin activity. Overexpression of furin, resulted in a complete processing of the receptor (Figure 4A, lane 3). According to its higher levels of expression of furin,<sup>6</sup> endogenous furin activity was more evident in the more aggressive SCC15. Despite the fact that these cells expressed elevated levels of IGF-1R (Figure 4A), almost all of the receptor was cleaved in the control (vector-alone-transfected) cells. However, a slight increase in the processing was observed in the furin-transfectant cells. Measurements of [<sup>3</sup>H]-thymidine incorporation indicated that after furin transfection, a



**Figure 4. A:** Effect of furin inhibitor on IGF-1R processing. **Arrow:** Mature form corresponding to the  $\beta$  chain of IGF-1R (103 kD). **Arrowhead:** unprocessed pro-form (200 kD). CMK, 100  $\mu\text{mol/L}$ . Forty  $\mu\text{g}$  of total cell lysates were analyzed in SCC12 and SCC15, respectively. After transfection of furin, cells exhibited an increased ability to process IGF-1R. This effect was partially blocked when cells were incubated with 100  $\mu\text{mol/L}$  CMK. **B:** Membrane arrested cells with  $\alpha$ -actin antibody. **C:** [<sup>3</sup>H]thymidine incorporation assay. Growth-arrested cells (25,000) were incubated with serum for 24 hours and the last 4 hours, 0.5  $\mu\text{Ci}$  [<sup>3</sup>H] methyl thymidine/well was added.  $\square$ : 10% FBS,  $\blacksquare$ : 10% FBS + 100  $\mu\text{mol/L}$  CMK. Furin transfection increased [<sup>3</sup>H]thymidine incorporation and this was inhibited with CMK. **D:** Growth-arrested cells were incubated with the additions indicated. The last four hours, 0.5  $\mu\text{Ci}$  [<sup>3</sup>H] methyl thymidine/well was added.  $\square$ , SFM;  $\blacksquare$ , 20 ng/ml IGF-1;  $\square$ , 100 ng/ml IGF-1;  $\blacksquare$ , 100 ng/ml IGF-1 + 100  $\mu\text{mol/L}$  CMK. Furin transfectants showed an increased susceptibility to IGF-1. Note CMK inhibition of IGF-1-mediated proliferative response.



**Figure 5.** *In vivo* invasion assay. **A:** Micrograph of a tracheal transplant cross-section showing the growth pattern of vector-alone-transfected SCC 15 cells. Note that the cells remain in the luminal and interior area of the trachea. **B:** Micrograph of a tracheal transplant cross-section containing furin-transfected SCC 15 cells. Note that the cells originally placed in the lumen have grown inside the tracheal wall and have entered the peritracheal space in the direction of the **arrow**. Cells invade through the area of least resistance, ie, the pars membranacea between the opening of the tracheal cartilage ring as noted by the **arrow**. **C:** Histogram showing the level of penetration of transfected SCC15 cells. **Empty bars:** vector-alone-transfected cells; **black bars:** furin-transfected cells. One-sided *t*-test indicated statistically significant differences ( $P < 0.05$ ). **A** and **B:** Hematoxylin and eosin stain  $\times 30$ .

small increase (20 to 30%) in the proliferation rate was achieved, in agreement with the small increase of IGF-IR processing observed. Processing of IGF-IR was partially blocked after inhibition of furin activity with 100  $\mu\text{mol/L}$  CMK (Figure 4A, lines 2, 4, 6, and 8). This decrease in the processed IGF-IR resulted in a reduction in the proliferation activity of cells as noted by the lower counts in the incorporation of [ $^3\text{H}$ ]-thymidine (Figure 4C).

Incubations in the presence of IGF-1 (20 and 100 ng/ml), one of the IGF-IR ligands, resulted in a two- to fourfold increase in the incorporation of radioactivity. According to their enhanced ability to process IGF-IR, furin transfectants showed an increased incorporation of [ $^3\text{H}$ ]-thymidine when incubated with IGF-1. CMK inhibition of IGF-IR processing resulted in partial inhibition of the IGF-1-mediated stimulation of proliferation (Figure 4D).

#### Enhanced *In Vivo* Growth Patterns of Transfected HNSCC

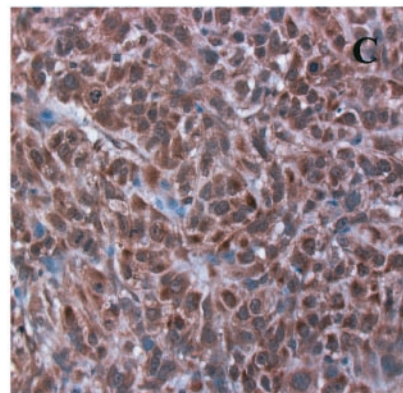
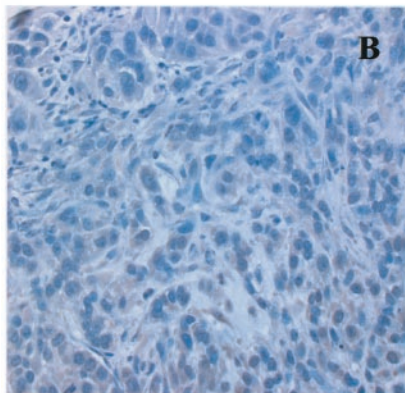
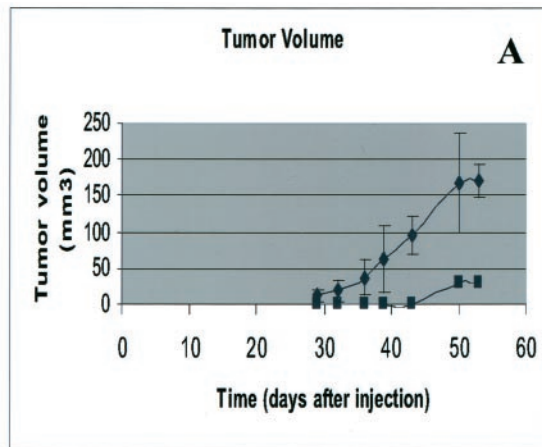
*In vivo* invasion was evaluated using the tracheal xenotransplant invasion assay. Cells were inoculated into rat tracheas and implanted s.c. in the backs of SCID mice. After either 6 or 8 weeks the tracheas were removed, sectioned, and stained. SCC15 cells transfected with the

empty vector grew in the interior of the tracheal lumen where a tumoral mass was observed. However, these cells were not able to reach the extratracheal tissues (Figure 5A). Conversely, furin transfectants were capable to breach the tracheal wall through the pars membranacea of the tracheal wall and reach the exterior of the xenotransplant (Figure 5B).

Evaluation of tumor cell penetration into the tracheal wall at 6 and 8 weeks after transplantation showed a remarkable increase in the invasive ability of furin cDNA-transfected cells. SCC15 cells grew relatively fast in tracheal transplants and the invasive ability was evaluated at 6 and 8 weeks (Figure 5C).

The less aggressive SCC12 cells were evaluated at 8 weeks after xenotransplantation. SCC12 cells, although invasive, did not penetrate as deeply at 8 weeks into the tracheal wall as SCC15 cells. Nevertheless, the increase in invasive ability after furin transfection was similar (approximately twofold) in both cell lines (Figure 5C).

Since proliferative rates of furin-transfected cells were higher than control cells (vector-alone) *in vitro*, we tested the ability of SCC15 cells to grow *in vivo* as s.c. xenotransplants. The furin transfectant cells grew faster and produced larger tumors than the vector-alone-transfected cells. The former developed tumors 15 days ear-



**Figure 6. A:** Subcutaneous tumorigenicity of SCC15 transfectant cells after inoculation in the dorsal s.c. tissue of Scid mice. **Squares**, vector-alone (pcDNA3.1)-transfected cells; **rhombus**, furin-transfected cells. One-sided *t*-test indicated statistically significant differences ( $P < 0.05$ ). Each point represents the mean and SE of measurements performed on five tumors per cell line. The latency time to tumor detection was longer in vector-alone-transfected cells than that of the furin-transfected cells. There was a two- to threefold difference between the volume attained by the tumors and the vector-alone-transfected and from furin-transfected cells. SCC15-derived tumors were removed from the animal 35 days after injection and their content of furin was evaluated by immunohistochemistry. **B:** Tumors from SCC15 transfected with vector-alone show low levels of furin immunostaining. **C:** Furin-transfected cells exhibit intense immunostaining.

lier than the vector-alone-transfected cells (Figure 6A). All of the animals injected with furin transfectant cells developed tumors. In contrast, at the time of termination of the experiment (45 days), only two of five control animals (injected with vector-alone-transfected cells) had tumors. Immunohistochemistry of tumors revealed strong expression of furin in the transfectants (Figure 6C). Weak staining was observed with SCC15 control cells (Figure 6B).

### Discussion

Activation by proteolytic cleavage is an important mechanism for controlling protein activity. De-regulation of this process can lead to a prolonged and increased activity of the substrates involved, potentially breaking the equilibrium needed in physiological processes. Degradation and synthesis of the ECM is a dynamic event that needs to be tightly controlled. Breakdown of this control mechanism by unregulated expression of enzymes involved in degradation of ECM is a hallmark of many cancer cells.

Among the participating enzymes, PCs may have a fundamental role. PCs are members of a family of serine proteases that have been implicated in several types of cancer. For example, PACE-4 is overexpressed in many cell lines derived from rodent skin cancer<sup>27</sup> and high levels of furin have been observed in human lung,<sup>4</sup> breast<sup>5</sup> and head and neck cancer.<sup>6</sup> Furin is synthesized as a proenzyme and self-activates in the ER. Once processed, it serves to activate a large variety of substrates, many of them with well-recognized roles in cancer development and progression. Overexpression of furin may activate many of these substrates in a non-regulated manner and contribute to an enhanced malignant phenotype.

We selected two human HNSCC cell lines of different degrees of aggressiveness and stably transfected them with full-length furin cDNA. After transfection with furin, both cell lines showed an increased ability to process MT1-MMP, one of the key enzymes in the cascade that leads to the degradation of ECM. This correlated with enhanced *in vitro* invasiveness. SCC15, the cell line that

displays a moderate degree of aggressiveness, showed a more malignant phenotype after furin transfection. Interestingly, SCC12, the less aggressive cell line in the SCC series, exhibited a remarkable increase in invasive ability. Although SCC12 furin transfectants were not as invasive as furin-transfected SCC15 cells, this result suggests that furin expression is sufficient to enhance the invasive ability of this low-grade cell line. The increase in the invasive ability of furin transfectants can be explained by an enhanced activation of MT1-MMP. It has been demonstrated that this membrane-type MMP is overexpressed in many SCC and that the levels found correlated positively with the overall malignancy of tumors.<sup>28</sup> Our results suggest that, at least in some cases, the primary abnormality may be the overexpression of the MT1-MMP activator (furin). MT1-MMP plays a central role in invasion and tumor progression because it is able to activate MMP-2 or collagenase IV, a major ECM degradative enzyme.<sup>7</sup> Both cell lines had low levels of MMP-2 and MMP-9. Zymographic analysis showed that collagenase IV expression and activation is enhanced in furin transfectants. This cannot entirely account for the changes observed and it is very likely that other MMP-2-independent effects are also taking place. In this context, it has been noted that for complete activation of MMP-2, several components of the ECM such as collagen are needed.<sup>29</sup> The presence of ECM components could explain the relative discrepancies observed in zymography and the *in vitro* invasion assay.

Pro-MMP-9 activity was also detected at low levels. Unexpectedly, only the active form was detected in furin transfectants, whereas only the pro-form was present in the vector-alone-transfected cells. This activity may contribute to the increased invasiveness. Although correlation between MMP-9 activation and furin activity is not clear, the minimal consensus sequence for furin cleavage (RXXR) can be recognized near but not at the carboxy-terminal part of the pro-peptide,<sup>11</sup> thus furin activity can putatively generate active MMP-9.

CMK is a potent furin inhibitor (inhibition constant,  $k_i$ , in the order of nanomolar) capable of acting intracellularly. Treatment with this inhibitor resulted in reduced cleavage of MT1-MMP and *in vitro* invasiveness in HT-1080 cells.<sup>30</sup> Inhibition studies with CMK were performed in our cell systems to provide further evidence that furin was responsible for the changes in the observed phenotype. Inhibition of furin activity resulted in an almost complete blockage of MT1-MMP processing. This result confirms that furin plays a major role in activation of MT1-MMP. This decrease in processing correlated with the marked reduction in the invasiveness *in vitro*.

### *In Vivo* Invasion Assays Confirmed the Results Obtained in Vitro

Cells were able to grow inside tracheal transplants and reach the connective tissues that bridge both ends of the cartilage (pars membranacea). SCC15 cells transfected with vector-alone remained inside the trachea even 8 weeks after the xenotransplant was performed. Despite cells being in physical contact with the pars membranacea, they did not have the capacity to degrade and

invade it. In contrast, furin-transfected cells acquired the ability to disrupt this barrier and migrate to the extratracheal compartment. *In vivo* invasive ability was also evaluated in SCC12 cells. Although these cells displayed very low invasive potential, a clear difference between vector-alone *versus* furin-transfected cells was observed. Furin can process and activate other cancer-associated substrates not directly involved in invasion or degradation of ECM such as growth factors and growth factor receptors.<sup>31-34</sup> Inhibition of furin by PDX, a specific inhibitor of this PC, resulted in a reduction in proliferation rate in HT-29, a colon carcinoma cell line.<sup>17</sup> To elucidate if overexpression of furin in HNSCC was related to enhanced proliferation, [<sup>3</sup>H]-thymidine incorporation experiments were performed. Furin transfectants showed moderate increase [<sup>3</sup>H]-thymidine incorporation (~30%) when compared to vector-alone-transfected cells. The enhanced processing of the receptor, resulting in higher concentration of the active receptor in the cell surface may enhance the response to IGF-1 in more aggressive cells leading to increase the proliferative rates. As already noted in other systems,<sup>35</sup> more dramatic effects were achieved after blocking the activity or the expression of IGF-1R. Treatment with CMK resulted in reduction of the relative amounts of the processed receptor and inhibition of the proliferative ability. Inhibition of incorporation of radioactivity was observed not only in furin-transfected but also in vector-alone-transfected SCC15 cells, suggesting that some degree of inhibition of endogenous furin had occurred. In SCC12 cells, proliferation was almost abolished.

IGF-1, one of the IGF-1R ligands, induced a dose-dependent proliferative response. Furin-transfected cells showed higher levels of proliferation than their vector-alone counterparts. This effect was more striking in SCC12 cells, in agreement with the increased ability of furin transfectants to process IGF-1R (Figure 4A). Treatment with the furin inhibitor, CMK, significantly reduced the IGF-1-mediated proliferative response. Taken together, these results demonstrate that the proliferation rate is moderately affected by the overexpression of furin but inhibition of this PC can reduce or even abolish proliferation in these cell lines.

When SCC15 cells were injected s.c. in mice, furin transfectants grew faster than the vector-alone counterparts and tumors were detected earlier. These tumors were three to four times larger than tumors derived from control-transfected cells. Early onset of tumor derived from furin-overexpressing cells may be explained, in part, by their increased sensitivity toward IGF-1.

Conversely, furin overexpression may provide a better adaptation to a relative unfavorable environment through processing of yet unknown substrates. This advantage may help malignant cells to grow or survive in adverse conditions that they may have to circumvent to colonize at distant organs. SCC12 cells grew poorly when injected s.c. in mice. Nevertheless, small tumors were obtained 3 months after injection of furin-transfected cells. On the other hand, control cells were unable to grow.

In summary, furin overexpression resulted in a remarkable increase in the invasive potential of cells. Prolifera-

tion rates were moderately higher. After inhibition of furin activity, both proliferation and invasive ability decreased due to impairment in the processing of a variety of furin substrates. These features make furin a potential target for future therapeutic efforts. One might speculate that a combined use of MMP inhibitors together with furin inhibition could improve the perspectives of targeting these tumor progression-related enzymes.<sup>36,37</sup>

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