Haploinsufficiency of the *Maspin* Tumor Suppressor Gene Leads to Hyperplastic Lesions in Prostate

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Abstract

*Maspin* is a key tumor suppressor gene in prostate and breast cancers with diverse biological functions. However, how *maspin* regulates prostate tumor progression is not fully understood. In this study, we have used *maspin* heterozygous knockout mice to determine the effect of *maspin* haploinsufficiency on prostate development and tumor progression. We report that loss of one copy of *maspin* gene in M$p^{+/−}$ heterozygous knockout mice leads to the development of prostate hyperplastic lesions, and this effect was mediated through decreased level of cyclin-dependent kinase inhibitors p21 and p27. Prostate hyperplastic lesions in M$p^{+/−}$ mice also induced stromal reaction, which occurred in both aged prostate tissues and in neonatal prostates during early ductal morphogenesis. We showed that *maspin* was also expressed in prostate smooth muscle cells (PSCM), and recombinant *maspin* increased PSMC cell adhesion but inhibited cell proliferation. We also observed a defective interaction between epithelial cells and basement membrane in the prostate of M$p^{+/−}$ mice, which was accompanied with a changed pattern of matrix deposition and a loss of epithelial cell polarity. Therefore, we have identified a novel property of *maspin*, which involves the control of the proliferation in prostate epithelial and smooth muscle cells. This is the first report that a partial loss of *maspin* caused an early developmental defect of the prostate and prostate hyperplastic lesions in mice. [Cancer Res 2008;68(13):5143–51]

Introduction

*Maspin* (SERPINB5) is a tumor suppressor gene belonging to the family of serpins (serine protease inhibitors). *Maspin* is expressed in many tissues such as prostate, mammary gland, skin, stomach, and uterus (1). It possesses distinct biological functions including inhibition of prostate tumor cell migration and invasion through a cell surface function (2, 3), and inhibition of angiogenesis (4, 5) and prostate tumor metastasis (6). These functions depend in part on the ability of maspin to increase cell adhesion to extracellular matrix (ECM; refs. 7–9). Although maspin binds to the pericellular urokinase- and tissue-type plasminogen activators (urokinase-type plasminogen activator and tissue-type plasminogen activator) in prostate tumor cells (10, 11), it does not show a direct enzymatic inhibition against these proteases (12–14). Several maspin-interacting proteins have been identified in other subcellular locations (15–17), suggesting that *maspin* may act in different modes depending on its cellular locations. Without any doubt, the molecular mechanisms underlying the actions of maspin actions in prostate and other cell types are still not fully understood.

Clinical studies have correlated loss of *maspin* gene expression with human prostate cancer progression (18, 19). For example, Machtens and colleagues showed that *maspin* expression was correlated with the recurrence-free survival of prostate cancer patients (18). Additionally, loss of maspin protein was correlated with higher tumor grade stages (20). Moreover, a recent study has convincingly showed that maspin is required for the inhibition of inflammation-induced prostate cancer progression (14, 21). However, to the present, it is not clear whether loss of maspin by itself may directly contribute to prostate cancer progression in an animal model and whether *maspin* expression is important for prostate development. Previously, we generated *maspin* gene knockout mice to study *maspin* function in embryonic development (22). Because homozygous *maspin* deletion was embryonic lethal, we used *maspin* heterozygous mice to study the effect of *maspin* haploinsufficiency on prostate development and cancer progression. Here, we report our findings that loss of one copy of *maspin* in M$p^{+/−}$ heterozygous mice leads to the development of prostate hyperplastic lesions and disruption in prostate development. Our data indicate that maspin plays a novel role in prostate epithelial and smooth muscle cell proliferation.

Materials and Methods

**Mice.** *Maspin* heterozygous knockout mice were generated by gene targeting event as described previously (22). The heterozygous M$p^{+/−}$ and WT M$p^{+/+}$ male siblings were derived from the mating pairs of M$p^{−/−}$ male and M$p^{+/−}$ female and were maintained at the mixed background of C57BL/6 and 129 sv. Mouse tail genotyping protocol was described previously (22).

**Adult prostate tissue collection.** Animals were anesthetized, weighted, and prostate were excised and dissected. Various prostate lobes at different stages were used in different assays for histology, immunohistochemistry, and molecular and cellular studies. However, phenotypes were mostly characterized using dorsal (DP) and ventral (VP) prostate tissues, and confirmed with anterior prostate (AP). The VP, AR DP, and lateral lobes of the prostate were harvested and divided into three parts: part I was used for making protein extracts using protein lysis buffer with proteinase inhibitors for Western blot analysis; part II was used for extracting total RNAs with Trizol for reverse transcription-PCR (RT-PCR) analysis of *maspin* RNA expression; and part III was fixed and embedded for sectioning at 6 μm.

**Dissection of neonatal VP.** Mice at 8-d-old were sacrificed and VP samples were dissected and placed in PBS buffer for morphologic analysis. Images were captured with a digital camera and analyzed for the ductal morphogenesis by counting for the node number as described by Foster and colleagues (23). Dissected VPs were sectioned for histology and immunohistochemistry.

**RT-PCR analysis.** Total RNAs were extracted from M$p^{+/−}$ and M$p^{+/+}$ prostate tissues as stated above (three mice per group), using Trizol (Invitrogen). The cDNAs were synthesized using 5 μg total RNAs with Oligo
For the detection of maspin mRNA, forward primer (5'-TCT TGA GCC CAC TTT CTG CT 3') and reverse primer (5'-CTA CAG ACA AGT TCC CTG AG 3') were used for the amplification of a 572 bp product. A ribosomal gene product L19 was used as RT-PCR loading control. For the detection of L19, forward primer 5'-CTG AGC GTC AAA GGG AAT GTG 3' and reverse primer 5'-CGA GCA CCT GTT GAT CTC 3' were used to amplify a 194 bp product. PCR condition was as follows: denaturing, 94°C for 45 s; annealing, 58°C for 30 s; and extension, 72°C for 60 s, 28 cycles. Fifteen microtiter of PCR products were analyzed in 1.5% agarose gel by electrophoresis.

**Cell culture, cell cycle synchronization, and cell adhesion assays.**

C2N prostate tumor cells were derived from primary prostate tumors from TRAMP mouse model (24). Maspin cDNA retroviral expression vector and empty vector control were introduced into C2N prostate cell line and selected for maspin-expressing C2N-Mp and control C2N clones by the method as described previously (3, 7). The cultured control C2N or C2N-Mp cells were grown at 37°C in a humidified CO2 incubator (5% CO2) with DMEM, containing 2.5% heat inactivated fetal bovine serum (FBS), 2.5% Nu serum (Becton Dickinson), dihydrotestosterone (10⁻⁸ mol/L), and 5 μg/mL insulin (Sigma Co.).

To arrest cells in the G1-S phase, cells were treated with 500 μmol/L mimosine (Sigma, Inc.) for overnight using the method as described by Ford and colleagues (25). Synchronized cells were released by adding fresh medium to plates for 6 h. Cell cycle progression was measured by flow cytometry (25), using the Beckman cell sorter in the core facility of flow cytometry at BCM. Briefly, cells (2 × 10⁶) were collected by centrifugation at 1,000 rpm and resuspended to achieve a single-cell suspension. Cells were then fixed with 70% ethanol and stained with propidium iodide staining solution with RNase A for 30 min at room temperature before they were sorted by the Beckman flow cytometer.

Prostate smooth muscle cells (PSMC) and NIH3T3 cells were cultured in DMEM, 10% FBS. For PSMC cell adhesion assay, subconfluent cultures were trypsinized, washed with warm serum-free DMEM, and incubated with recombinant proteins (0.1, 0.2, and 0.5 μmol/L) for 10 min at 37°C. Purified recombinant glutathione S-transferase (GST) and GST-Mp proteins were dialyzed against PBS buffer. Wells were precoated with fibronectin (FN) at 1.0 μg/mL overnight, and blocked with 5% bovine serum albumin (BSA) for 1 h. In all the assays, 2 × 10⁴ cells per well were plated in triplicates and allowed to adhere for 30 min at 37°C. Wells were washed with warm serum-free DMEM, and adhered cells were fixed with 5% glutaraldehyde and stained with crystal violet. A blank value corresponding to BSA-coated wells (≤5% of maximal cell adhesion) was automatically subtracted. Dye was solubilized in 10% acetic acid and absorbance was determined at 590 nm. For PSMC cell proliferation assay, PSMC cells (2 × 10⁵ per well, 12 wells per group for each assay) were supplied with recombinant protein GST or GST-Mp at 0.5 μmol/L daily for 3 d and cells were harvested for the quantitation of cell proliferation rate with crystal violet dye at 590 nm. Experiments were repeated in triplicates, and statistical analysis was done by the Student’s t test.

**Immunohistochemical analysis.**

Paraffin-embedded samples were sectioned, and slides were dewaxed and dehydrated. Antigen retrieval was performed by heat inactivation in microwave oven using the buffer of sodium citrate [0.1 mol/L (pH 6.0)] for 30 min. Primary antibodies used against the following antigens were as follows: maspin (1:70; Ab54A), p21 (1:400; Santa Cruz), p27 (1:500; BD Bioscience), laminin 1 (LN; 1:2,000; NeoMarkers), α-smooth muscle actin or α smooth muscle α-actin [SMA; 1:500; Rat monoclonal antibody (mAb); Sigma, Inc.], cytokertin-8 (1:200; troma-1; Developmental Studies Hybridoma Bank, University of Iowa), E-cadherin (1:400; mouse mAb; BD Biosciences), ZO-1 (1:250; mAb; Chemicon; clone R047,6); and Ki-67 (1:500; mAb; Santa Cruz). The color staining was performed using the avidin-biotin peroxidase system (ABC-peroxidase). Brown positive signals (maspin, p21, p27, and ki-67) were visualized as brown precipitates using 3,3'-diaminobenzidine tetra-hydrochloride (Vector Laboratories). Hematoxylin was used for counterstaining. For immunofluorescence staining, antibodies against α-SMA, LN, K14, E-cadherin, and ZO-1 were recognized by either a Texas red–conjugated secondary antibody or a FITC-conjugated secondary antibody, and were counterstained with 4,6-diamidino-2-phenylindole (DAPI) for cell nuclei. Images were taken using microscope (Leica) equipped with a SPOT charge-coupled device digital camera. For the measurement of labeling index (Ki-67–positive cells) and p21- and p27-positive cells, positively stained epithelial cells and total epithelial cells were counted with the captured images from microscope. At least four randomly selected images from each specimen were counted, and the total numbers of cells were listed. The percentage of p21- and p27-positive cells and the Ki-67 labeling index were then determined using a t test program. A P value of <0.05 is considered as significant.

**Bistone kinase assay, immunoprecipitation, and Western blot analysis.**

Mimosine treated or untreated cells were lysed by radio-immunoprecipitation assay buffer. Cell lysates were cleared by centrifugation at 13,000 rpm for 15 min and an aliquot of 500 μg of total protein were incubated with specific Antibody (Cdk2, 1:500; UpSTATE; Cyclin E, 1:250; Santa Cruz) for coimmunoprecipitation. About 25 μL of 50% protein A–agarose bead slurry (Pharmacia) was added into the mixture, which was incubated for additional 3 h. Immune complexes were centrifuged at 500 rpm for 5 min, and the precipitates were rinsed thrice with lysis buffer and twice with kinase buffer [50 mmol/L Tris-HCl (pH 7.5), 10 mmol/L MgCl2, and 1 mMol/L DTT]. CDK kinase assays with histone H1 substrate were performed by mixing the respective immune complexes with 2.5 μg of histone H1 (Roche) and 10 μCi of [³²P]-ATP (Pelkin Elmer) in 25 μL of kinase buffer. The kinase reaction was performed at 30°C for 30 min, and terminated with 6× SDS-PAGE sample buffers. The reaction mixtures were denatured and resolved by 12% SDS-PAGE analysis and transferred to a polyvinylidene fluoride (Millipore) membrane (Bio-Rad Laboratories). Histone H1 kinase activity was determined by autoradiography for 1 to 2 d, and cyclin dependent kinase (CDK)-associated CDK inhibitors were quantitated by Western blot analysis with antibodies against p21 (1:2,000; BD Pharmingen) and p27 (1:1,000; BD Biosciences). Images of radiography from kinase assays and the blotted bands from Western blot analyses were quantitated using a NIH Image J program.

Western blot analysis was performed using a rabbit polyclonal anti-maspin antibody as described previously (7). Antiactin polyclonal antibody was used as loading control.

**Results**

**Maspin expression in prostate epithelial cells.**

Prostate lobes from WT mice were dissected. In the first study, we used VP tissues for the investigation of maspin expression in prostate at RNA and protein levels (Fig. 1). VP tissues were also embedded and sectioned, and slides were used for immunohistochemistry. Immunostaining with antimaspin antibody showed that maspin was present primarily in the cytoplasm of epithelial cells and the differentiated smooth muscle cells (Fig. 1A). Basement membrane (BM) was also stained positive for maspin, suggesting that maspin was secreted from epithelial and smooth muscle cells to the adjacent BM.

Because maspin homozygous mice were embryonic lethal, we used maspin heterozygous (Mp⁺/-) mice for the analysis of prostate phenotypes. To confirm that heterozygous Mp⁺/- mice had reduced maspin expression in prostate compared with WT mice, RT-PCR and Western blot analyses were performed using VP prostate tissues from age-matched (6-month-old) maspin WT and heterozygous (Mp⁺/-) mice (Fig. 1B and C). Quantitation of bands using NIH imaging J software showed that maspin expression in heterozygous Mp⁺/- prostate samples was reduced to ~50% of WT samples (P < 0.05).

**Development of prostate hyperplastic lesions in Mp⁺/- heterozygous mice.**

We then examined the prostate phenotypes in WT and Mp⁺/- mice comparing the dissected prostate lobes from brother-paired, 6- to 12-month-old mice. At ages 6 months, some Mp⁺/- mice developed hyperproliferative, and multiple layers
of epithelial cells in the DP (Fig. 2). In contrast, DPs from WT mice contained a single layer of columnar epithelial cells (left, WT 6 mo). The abnormal changes were most prominent in the DPs (Fig. 2) and VPs (data not shown) in Mp+/− mice, and became more severe as mice ages from 6 to 12 months. At 12 months of stage, most of Mp+/− mice analyzed contained hyperplastic lesions in DPs as illustrated in Fig. 2 (right, Mp+/− 12 mo). There were highly increased infolding of the glands and tufting. The density of nuclei was also increased, which reached the level that was associated with prostate intraepithelial neoplasia in human patient (Fig. 2). Hyperplastic lesions in epithelial cells were also observed in the AP lobes in a large percent of Mp+/− mice surveyed (data not shown).

**Modified stromal patterning in the prostate of Mp+/− mice.** One of the most significant changes in Mp+/− prostate was the

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**Figure 1.** Maspin expression in mouse prostate. A, maspin expression analyzed by immunostaining in 24-wk-old VP of Mp+/+ WT mouse. Bar, 50 μm. B, analysis of maspin expression at mRNA level by RT-PCR. VP tissues were harvested from WT Mp+/+ and maspin heterozygous mice (Mp+/−) at 24 wk. Bottom, relative signals of maspin mRNA quantitated by NIH J image software. C, IP-Western blot analysis of maspin protein level with protein extracts from various samples. Tubulin serves as a loading control. Note that maspin level was reduced at mRNA and protein levels in Mp+/− VPs (B and C). Bottom, relative signals of maspin protein quantitated by NIH J image software. ***, P < 0.01 (Student’s t test).

**Figure 2.** Development of prostate hyperplastic lesions in Mp+/− heterozygous mice. Top, representative views of the histology of DP tissues from WT and Mp+/− mice at ages 6 and 12 mo. Note the lack of significant stroma surrounding the glands in WT 6-mo-old DP. The epithelium is relatively flat with few infoldings. DPs from Mp+/− mice showed the presence of hyperproliferative, multiple layers of epithelial cells, with the thickened stroma surrounding the gland and the epithelial hyperplasia. The incipient stromal proliferation inside the epithelial folding became more prominent in Mp+/− 12-mo DP. Bottom, immunostaining of sections of the top panels with anti-SMA. Note the increased multilayers of stromal cells in 12-mo-old Mp+/− DP. Bars, 50 μm.
modification of stromal pattern. Histologic analysis showed that epithelial hyperplastic lesions in $M_{p}^{+/}$ prostate were accompanied with the stromal hyperplasia (Fig. 2). The increased stromal proliferation was observed around the glands and inside the tufts. This increase in stroma was predominantly due to an increase in the cellular component. To further analyze the abnormality in stromal patterning, we used a SMA antibody to mark the smooth muscle cells in stromal compartment. Adult $M_{p}^{+/}$ prostate showed a significantly increased number of stromal cells, which were stained positively using anti-SMA antibody (Fig. 2). However, WT prostate generally had only a single layer of SMA-positive stromal cells (Fig. 2).

Stromal patterning is dependent on epithelial cell differentiation in both early and late stages of prostate development (26). During early neonatal ductal morphogenesis, a single layer of stromal cells surrounds the epithelial ductal cells in the duct. To determine whether the observed phenotypes in adult $M_{p}^{+/}$ prostate are also present in neonatal prostate during early ductal morphogenesis, we examined the stromal patterning and the status of epithelial proliferation in $M_{p}^{+/}$ and WT neonatal prostate lobes. Due to the relative larger size of VPs comparing to other prostate lobes in the neonates, we dissected VPs from neonatal 8-day-old pups to analyze the ductal morphogenesis in WT and $m_{aspin}$ heterozygous mice. There was a significant reduction in the node number in $M_{p}^{+/}$ VPs compared with that in the WT samples, suggesting that there is a defect in early ductal morphogenesis in $M_{p}^{+/}$ mice (Fig. 3A). We embedded and sectioned neonatal VPs for histologic analysis by H&E. All of $M_{p}^{+/}$ VPs dissected showed either closed lumen or clearly reduced size of lumen and multiple layers of epithelial cells in the ducts, suggesting that there is an increased epithelial proliferation during early ductal development (Fig. 3B).

Immunostaining with an anti-SMA antibody showed that all $M_{p}^{+/}$ VPs also had a significant increase in stromal hyperplasia around the ducts (Fig. 3B). Therefore, loss of one copy of $m_{aspin}$ gene in $M_{p}^{+/}$ mice caused epithelial hyperplastic lesions and a change of stromal patterning not only in adult prostate but also in neonatal prostate during early ductal morphogenesis.

**Maspin regulates prostate cell proliferation through cell cycle inhibition.** To determine whether the hyperplastic lesions observed in $M_{p}^{+/}$ mice were due to either a change in epithelial proliferation or a reduced level of cell apoptosis, we first performed a terminal deoxynucleotidyl-transferase–mediated dUTP nick-end labeling apoptosis assay using sections from WT and $M_{p}^{+/}$ VP samples. No difference was observed in the rate of epithelial apoptosis between these two groups of samples (data not shown). We then examined the effect of maspin on cell proliferation by
Figure 4. Maspin inhibits prostate epithelia cell proliferation. A, quantitative analyses of prostate epithelial cell proliferation rate in WT and Mp+/+ VPs and APs. Samples at designated ages were stained with an anti–Ki-67 antibody. Left, AP and VP from 6- to 8-mo-old mice (n = 10 for both WT and Mp+/+ groups). Right, AP and VP from 12- to 15-mo-old mice (n = 5 for both Mp+/+ and Mp+/− groups). *, P < 0.05; **, P < 0.01. B, cell cycle analyses of prostate C2N and maspin-expressing C2N-Mp cells by flow cytometry. Cells were either asynchronous or synchronized as indicated. Cell synchronization was performed with mimosine (see Materials and Methods), and cells were released and cultured in mimosine-free medium for 6 h before being harvested for cell cycle analysis by flow cytometry. C, analysis of cyclin E–associated Cdk2 kinase activity. Cell extracts were immunoprecipitated with antibodies against cyclin E or Cdk2. Cdk2 kinase activity was assayed using histone H1 as a substrate, and kinase activities were quantitated using assay blots and NIH J image software (right). Bars represent triplicate samples. Cyclin E and Cdk2 served as IP controls, and actin served as sample loading control. D, analysis of p21 and p27 levels in C2N cells and in DP from WT and Mp+/− mice. Left, C2N and C2N-Mp cell extracts from C were immunoprecipitated with anti–cyclin E or anti-cdk2 and blotted for p21 and p27 with anti-p21 or anti-p27 antibodies. Note the increased levels of p21 and p27 in C2N-Mp cells. Cyclin E and Cdk2 served as IP controls, and actin served as sample loading control. Right, quantitation of immunohistochemical analyses for the percentage of p21- and p27-positive epithelial cells in WT and Mp+/− prostate. DPs from WT and Mp+/− mice (6- to 8-mo-old) were stained with antibodies against p21 and p27. A total of 3,000 epithelial cells were counted for each sample. Bars represent 5 mice at ages 6 to 8 mo. **, P < 0.05; ***, P < 0.01.
performing an immunostaining assay using an antibody against Ki-67, a cell proliferation marker. Ki-67–positive epithelial cells were counted using VP and AP samples isolated from WT and Mpc−/− mice. The percentages of Ki-67–positive epithelial cells among all epithelial cells counted in Mpc−/− VP and AP lobes were significantly higher than that in the WT mice (Fig. 4A). As mice became older, there was an increased proliferation index in Mpc−/− prostate epithelial cells. In 12- to 15-month-old mice, the proliferation indexes in the VP and AP lobes of Mpc−/− mice were increased over 2-fold compared with that of Mpc+/− mice at ages 6 to 8 months. The proliferation indexes in both VP and AP were increased nearly 3-fold in Mpc−/− mice compared with that in WT mice at both stages of observation (Fig. 4A).

The fact that loss of one copy of maspin in Mpc−/− mice leads to epithelial hyperproliferation suggests that maspin may play a novel role in the regulation of cell proliferation. To prove this, we examined whether epithelial-derived maspin could autonomously affect cell proliferation. Because there was no good cell line of normal mouse prostate epithelia available, we used mouse prostate–derived epithelial tumor cells for in vitro analysis. We used C2N mouse prostate epithelial cells that were isolated initially from TRAMP prostate tumors, which had lost maspin expression during tumor progression (24). We then transfected maspin expression vector into C2N prostate cells to establish clones that express maspin gene. Stable clones overexpressing maspin (C2N-Mp) and control vector transfected cells (C2N) were isolated and selected by Western blot analysis with maspin antibody for further analysis. Flow cytometry analysis using asynchronously C2N-Mp and C2N cells showed that C2N-Mp had higher percentage of cells at G1 (64.4%), compared with C2N (G1, 50.8%; Fig. 4B). These cells were then synchronized using mimosine for cell cycle analysis, which arrests cells in G1–S (see Materials and Methods). Using the synchronized cells, we showed that C2N-Mp cells released from G1 arrest for 6 hours had a significant reduced rate of cell cycle progression compared with C2N, which was reflected by the presence of a higher percentage of cells at G1 (94.1%) in C2N-Mp compared with 58.4% of cells at G1 for C2N (Fig. 4B). These data suggest the presence of maspin partially blocks cell cycle progression at G1.

In mammalian cells, G1 to S transition in cell cycle is largely controlled by certain cyclins and CDKs, especially cyclin E and CDK2 kinase (27, 28). We performed in vitro kinase assays using cyclin E or CDK2-immunoprecipitated complexes and histone H1 as substrate. C2N-Mp cells had a significant reduced activity of CDK2 kinase as measured by histone H1 activity compared with C2N cells (Fig. 4C). It is well-known that CDK activity is largely controlled by CDK inhibitors (28). Two CDK inhibitors, p21 and p27, are known to inhibit CDK2 kinase activity during G1 cell cycle progression (29, 30). To determine whether there is any change in the level of p21 and p27 between C2N-Mp and C2N cells, we performed immunoprecipitation (IP)-Western blot analysis using anti-CDK2 antibody. C2N-Mp cells also displayed a higher level of p21 and p27 compared with that of C2N cells (Fig. 4C, right).

To determine whether there is a similar change of CDK inhibitors in epithelial cells of Mpc−/− prostate samples in vivo, we performed immunostaining assay for p21 and p27 using WT and Mpc−/− DP isolated from 6- to 8-month-old mice. The p21- and p27-positive epithelial cells were counted, and percentages of p21- and p27-positive cells were determined (Fig. 4D, right). The percentages of p21- and p27-positive cells in Mpc−/− samples were significantly decreased compared with that of WT samples (Fig. 4). Cell proliferation is, in part, controlled by cell adhesion. Several reports showed that elevated cell adhesion causes an increase in cyclin-dependent kinase inhibitors (such as p27) and an inhibition
of cell cycle progression (31–33). Previously, we showed that maspin increased prostate epithelial cell adhesion to ECM matrix proteins (7). Thus, epithelial-derived maspin may inhibit epithelial cell proliferation by increasing cell adhesion and the expression of CDK inhibitor p27. We collected conditioned medium from C2N-Mp and C2N control cells for Western blot analysis. Maspin was found in the conditioned medium from C2N-Mp epithelial cells (data not shown). On the other hand, maspin derived from smooth muscle cells may also control smooth muscle cell adhesion, which in turn affects cell proliferation. As shown in Fig. 5A, we found that maspin was expressed in PSMCs. Recombinant maspin protein increased PSMC adhesion in a dose-dependent manner but inhibited cell proliferation (Fig. 5B and C).

**Changed pattern of matrix composition and cell-ECM interaction in the prostate of Mp+/− mice.** In normal prostate, the matrix proteins are secreted by both epithelial and stromal cells, and are deposited to the basement membrane, which forms a unique boundary between epithelial and stromal cells. We examined the pattern of matrix proteins in WT and Mp+/− prostates by immunostaining assay. Using an anti-LN antibody, we found that Mp+/− prostate samples had drastically changed pattern of LN deposition compared with WT samples. In WT prostate, LN staining seemed as a tightly packed, continuous thin layer in the BM (Fig. 6). However, in Mp+/− prostate, LN was deposited in a wide, thick region surrounding the epithelial cells. Stromal cells were frequently found to be present in this thick, LN-deposited region (Fig. 6). We found that another major matrix protein, FN, had a similar distribution pattern as LN in Mp+/− samples (data not shown). Overall, the pattern of matrix deposition was greatly changed, which will likely change the cell-ECM adhesion.

**Discussion**

Serpins play pleitropic functions (34, 35). Maspin is also found to possess multiple functions, depending on its subcellular locations (2, 9, 36, 37). In this study, we show that extracellular maspin plays a novel role in cell proliferation. Cell synchronization assay showed that maspin-expressing prostate epithelial cells had a partial arrest of cells at G1 (Fig. 4B). Using another pair of human prostate cancer cell lines PC3 and PC3-maspin, we confirmed the findings of inhibition of cell proliferation (data not shown). Cell proliferation is controlled by CDKs (28, 30). One CKI family contains the Cip/Kip proteins, which include p21, p27, and p57 (29). Both p21 and p27 inhibitors have been shown to inhibit the activity of both cyclinD-cdk4/6 and cyclinE-cdk2 (29, 38). Our study showed that maspin-expressing prostate epithelial cells had a decreased cdk2 kinase activity but increased levels of p21 and p27 compared with the control prostate tumors (Fig. 4). We also confirmed that prostate...
epithelial cells with one copy of maspin gene in Mp+/− mice had a decreased percent of p27- and p21-positive cells (Fig. 4D, right). Such decrease explains why Mp+/− prostate developed hyperplastic lesions. This finding is also supported by a previous study by Gao and colleagues (39), which showed that p27 heterozygous mice developed prostate hyperplastic lesions.

It is well-known that cell proliferation is largely controlled by cell adhesion. Increased cell adhesion and cell-cell contact are negative factors for cell cycle progression (31, 40, 41). In addition, increased cell adhesion to ECM is shown to cause certain cell types to arrest in G1 phase (42). In particular, it was shown that the level of p27 protein was increased in response to an increased cell-cell contact or cell-ECM adhesion (31, 42). One main function of maspin is its ability to increase epithelial cell adhesion to different matrix proteins. We showed previously that maspin inhibited prostate tumor progression through increased cell adhesion to matrices (7). Recently, we provided evidence that maspin controls cell adhesion through its interaction with integrin β1 (36). Thus, it is reasonable to expect that loss of one copy of maspin in Mp+/− mice would result in a reduced cell adhesion, which would cause a decreased p27 expression level accordingly (31, 42). Furthermore, signals from cell-ECM adhesion have been shown to be connected to cell-cell adhesion (43, 44). Therefore, it is not surprising that the pattern of cell-cell contact indicated by E-cadherin staining was also drastically changed in Mp+/− prostate (Fig. 6). Our data indicates that maspin may control cell proliferation through this cell adhesion function.

A very interesting phenomenon is that partial loss of maspin in epithelial cells in Mp+/− prostate not only causes hyperproliferation of epithelial cells but also induces adjacent stromal hyperplasia (Figs. 2–3). This happens in both adult and aged prostate and in neonatal prostate during early ductal morphogenesis. Clearly, maspin secreted from epithelial cells could act on the adjacent stromal cells to inhibit cell proliferation in a paracrine manner. On the other side, because smooth muscle cells also expressed maspin (Fig. 5), it could act on smooth muscle cells in an autocrine manner. Thus, the observed stromal cell hyperplasia in Mp+/− mice may arise from reduced maspin expression in both epithelial and smooth muscle cells in Mp+/− mice (Fig. 3). Our data confirmed that maspin regulated smooth muscle cell adhesion and proliferation (Fig. 5). Alternatively, stromal reaction may in return affect epithelial cell proliferation and differentiation (45). The finding that PSMCs express maspin and loss of one copy of maspin caused stromal hyperplasia are highly significant because it emphasizes the importance of epithelial-stromal interaction during prostate tumor progression. It is very possible that such feedback interaction between epithelial and smooth muscle cells may promote prostate cancer progression. In this regard, similar to the phenotypes observed in Mp+/− mice, loss of Foxx1 gene also caused a modified pattern of stromal cells in prostate due to a defect in epithelia cell differentiation (46). Conditional deletion of PTEN not only caused epithelial cell hyperproliferation but also induces the development of reactive stroma (47). Thus, it seems to be very common that a change in epithelial cell proliferation is often accompanied with a modification in stromal compartment.

Prostate cancer progresses from early hyperplastic lesions to adenocarcinoma, and to metastasis at a late stage. In the past few years, many animal models have been established to mimic late stages of prostate cancers. Most of them involve the overexpression of oncogenes or targeted loss of tumor suppressor genes (48). A few animal models display early prostate hyperplasia. Mouse with Nkx3.1 null or one copy of Nkx3.1 gene represents a good model for the study of prostate hyperplasia (49, 50). Our study shows that maspin heterozygous mice developed the hyperplastic lesions very similar to that were observed in Nkx3.1 null or heterozygous mice. Moreover, Luo and colleagues (6, 21) identified a new inflammation-activated signaling pathway required for prostate cancer metastasis. This pathway requires the IKKα activation and the repression of maspin gene transcription, which shows unarguably that maspin is a key tumor suppressor gene that suppresses prostate cancer progression. Because of the importance of maspin in prostate cancer progression, it is highly possible that certain prostate cancer patients may have maspin mutation or deletion or other epigenetic changes that affect maspin expression. Finally, the above results also provide direct evidence that how maspin acts as a prostate tumor suppressor gene in vivo in a mouse model. The novel property that maspin inhibits cell proliferation may be exploited for therapeutic intervention of prostate cancer in the future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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