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Angiogenin-stimulated Ribosomal RNA Transcription Is Essential for Initiation and Survival of AKT-induced Prostate Intraepithelial Neoplasia

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Abstract

Angiogenin (ANG), originally identified as an angiogenic ribonuclease, has recently been shown to play a direct role in prostate cancer cell proliferation by mediating ribosomal RNA (rRNA) transcription. ANG is upregulated in human prostate cancer and is the most significantly upregulated gene in AKT-driven prostate intraepithelial neoplasia (PIN) in mice. Enhanced cell proliferation in the PIN lesions requires increased ribosome biogenesis, a multistep process involving an orchestrated production of ribosomal proteins and rRNA. AKT is known to enhance ribosomal protein production through the mammalian target of rapamycin (mTOR) pathway. However, it was unknown how rRNA is proportionally increased. Here, we report that ANG is essential for AKT-driven PIN formation and survival. We showed that upregulation of ANG in the AKT over-expressing mouse prostates is an early and lasting event. It occurs before PIN initiation and lasts beyond PIN is fully developed. Knocking-down ANG expression by intraprostate injection of lentivirus-mediated ANG-specific siRNA prevents AKT-induced PIN formation without affecting AKT expression and its signaling through the mTOR pathway. Neomycin, an aminoglycoside that blocks nuclear translocation of ANG, and N65828, a small-molecule enzymatic inhibitor of the ribonucleolytic activity of ANG, both prevent AKT-induced PIN formation and reverse established PIN. They also decrease nucleolar organizer region (NOR), restore cell size, and normalize luminal architectures of the prostate despite continuous activation of AKT. All three types of the ANG inhibitor suppress rRNA transcription of the prostate luminal epithelial cells and inhibit AKT-induced PIN indicating an essential role of ANG in AKT-mediated cell proliferation and survival.

Keywords

AKT; angiogenin; prostate intraepithelial neoplasia; ribosome biogenesis; rRNA transcription

Introduction

Angiogenin (ANG) is a 14 kDa ribonuclease originally identified as an angiogenic protein (1) that has been shown to play an important role in tumor angiogenesis (2,3). ANG undergoes nuclear translocation in endothelial cells where it stimulates rRNA transcription (4,5).

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Recently, ANG has been shown to be progressively upregulated in prostate cancer (6–8) and to play a direct role in cancer cell proliferation by constitutive nuclear translocation in cancer cells where it also stimulates rRNA transcription (8,9). Knocking-down ANG expression in PC-3 human prostate cancer cells decreases rRNA transcription, ribosome biogenesis, cell proliferation, and tumorigenicity in a xenograft athymic mouse model (8). rRNA transcription is essential for ribosome biogenesis, protein translation and therefore cell growth and proliferation (10). Abnormal ribosomal biogenesis has been shown to be associated with cancer pathogenesis (11). Therefore, ANG has been proposed to play a dual role in prostate cancer progression by stimulating both angiogenesis and cancer cell proliferation (8). However, because these observations were from studies of cell culture and xenograft tumor models, it is unknown whether ANG plays a role in prostate cancer initiation from its orthotopic origins and whether it is involved in prostate cancer progression in the natural environment.

Mouse *ANG* is the most significantly up-regulated gene in the prostate during prostate intraepithelial neoplasia (PIN) development in murine prostate-restricted AKT transgenic (MPAKT) mice (7). In these mice, expression of AKT in the ventral prostate results in activation of the p70^{S6K} pathway and induction of PIN similar in character to that observed in *PTEN*^{+/-} mice (12). PTEN has been shown to regulate cell size in association with its ability to regulate ribosome biogenesis (13,14). PTEN is a phosphatase that down-regulates the PI3K pathway by dephosphorylating the lipid phosphatidylinositol-3,4,5-trisphosphate to phosphatidylinositol-4,5-bisphosphate (15,16). Inactivating somatic mutation of *PTEN* or loss of the PTEN protein are common in prostate cancer cell lines and in primary and metastatic tumor specimens (17–19). Mutation of *PTEN* leads to deregulated PI3K signaling, resulting in constitutive activation of downstream targets including the AKT kinase family. AKT kinase activity is frequently elevated in prostate cancers (20). AKT is activated through phosphorylation on Ser-473 and Thr-308. Activated AKT promotes both cell growth and cell survival.

mTOR plays an important role in PI3K- and AKT-dependent oncogenesis, especially in the pathogenesis of prostate cancer (7,21). Transformation by PI3K or AKT directly correlates with activation of mTOR and its downstream target S6K (22). S6 phosphorylation has been associated with translation of a specific class of mRNA termed TOP (a terminal oligopyrimidine track in the 5' untranslated region) mRNA (23). This class of mRNAs includes ribosomal proteins, elongation factors 1A1 and 1A2, and several other proteins involved in ribosome biogenesis or in translation control (24). Thus, AKT activation will enhance ribosomal protein production. However, a missing link from AKT overexpression to enhanced ribosome biogenesis is how transcription of rRNA, which needs to be incorporated in an equimolar ratio, is proportionally elevated. We tested the hypothesis that ANG is upregulated in the prostate of MPAKT mice to fulfill this growth requirement. Our results show that inhibition of ANG expression and/or activities by various mechanisms prevents and reverses PIN in MPAKT mice, accompanied with suppression of rRNA transcription but without affecting AKT phosphorylation.

Results

Upregulation of ANG expression in AKT-driven PIN is an early and lasting event

Mouse *ANG* is the highest upregulated gene in the PIN lesion in MPAKT mice (7). However, the role of ANG in the development and maintenance of PIN was unknown (7). It was also unknown when upregulation of *ANG* starts and how long it lasts. We therefore first used immunohistochemistry (IHC) with an affinity-purified anti-mouse ANG polyclonal antibody (R163) to show that the ANG protein levels are higher in the ventral prostate of MPAKT mice than in that of the wild type (WT) littermates across the age ranging from 4 to 12 weeks (Fig. 1A). R163 has been previously used to detect mouse *ANG* expression during development and

has been shown to be specific to mouse ANG. No IHC signals were detected if the primary antibody was omitted or if the incubation was carried out in the presence of mouse ANG protein (1 µg/ml). Therefore upregulation of ANG in the prostate of MPAKT mice is an early and lasting event. Since it is known that PIN starts to develop at week 6 in MPAKT mice and has been fully developed at week 12 (7), these results suggest that ANG may play a role in PIN initiation as well as the survival and maintenance of established PIN in these mice. ANG was detected in the extracellular matrix (indicated by white arrows), consistent with its established role in stimulating angiogenesis. Strong nuclear staining of ANG was observed in the prostate luminal epithelial cells of MPAKT mice (Fig. 1A, indicated by black arrows). More importantly, higher magnification images revealed prominent nucleolar accumulation of ANG (Fig. 1B, indicated by arrows), suggesting that ANG plays a role in ribosome biogenesis, growth and proliferation of prostate luminal epithelial cells.

Knocking-down ANG expression prevented PIN formation

To understand the role of ANG in original cell growth and proliferation in the PIN lesion, we examined the effect of knocking-down ANG expression on PIN formation. Mouse *ANG1* is the predominant form among the 6 isoforms and is the ortholog of human ANG (human has only 1 ANG gene) (25). *ANG1* (hereafter labeled as ANG) was therefore targeted with a lentivirus-based siRNA method (26). Lentiviral vector-mediated mouse ANG-specific siRNA targeting at different regions of the ANG mRNA were obtained from Open Biosystems and their efficacy in knocking down ANG expression was determined in Lewis Lung carcinoma cells by RT-PCR (Fig. 2A, left panel), and confirmed by real time quantitative RT-PCR (Fig. 2A, right panel). Four-week-old mice were treated by a single intraprostate injection of lentivirus containing ANG-specific siRNA and a nonspecific control shRNA. The mice were sacrificed when they were 8-weeks-old and examined for PIN formation by H & E staining (Fig. 2B to D). PIN was formed in the mice that were treated with the control shRNA (Fig. 2B). However, in the mice treated with ANG-specific siRNA, PIN formation was inhibited (Fig. 2C). The percentage of the glands having PIN decreased from $52 \pm 12\%$ to $16 \pm 9\%$, indicating a 64% inhibition. ANG expression was examined by IHC with anti-ANG antibody R163 (Fig. 2E to G). Prominent nuclear ANG protein staining was detected in the ventral prostate epithelial cells of the MPAKT mice treated with control shRNA (Fig. 2E), which was markedly decreased after treatment with ANG-specific siRNA (Fig. 2F). The glandular structure and ANG expression level in ANG siRNA-treated prostate (Fig. 2C and F) were not significantly different from that of the WT littermates (Fig. 2D and G). It is notable that in the ANG siRNA-treated ventral prostates, ANG protein level was higher in the 20% or so PIN-born glands than in the PIN-free glands, indicating that ANG expression is inversely correlated to PIN formation in these animals. Thus, ANG-specific siRNA successfully knocked-down ANG expression and prevented PIN formation in MPAKT mice, indicating an essential role of ANG in AKT-induced prostate luminal epithelial cell proliferation and PIN formation.

Knocking-down ANG expression did not inhibit AKT activity

It is known that AKT overexpression in the prostate of MPAKT mice induces PIN formation through the mTOR-S6K-S6P signaling pathway (7,21). In order to know whether ANG siRNA-mediated inhibition of PIN formation was a result of diminished AKT transgene expression or of interrupted signal transduction pathway from AKT to S6P, phosphorylation status of AKT and S6P in the prostate luminal epithelial cells was examined by IHC. Staining with a phosphorylated AKT (p-AKT)-specific antibody showed no difference in AKT phosphorylation between the control shRNA- and the ANG siRNA-treated prostates (Fig. 2H and I), indicating that AKT transgene expression and phosphorylation were not affected by ANG siRNA. Ribosomal protein S6 (S6RP) is a down-stream target of AKT and its phosphorylation is known to enhance ribosomal protein production (27). IHC with an anti-phosphorylated-S6RP (p-S6RP) antibody showed that S6RP phosphorylation was not inhibited

by *ANG* siRNA (Fig. 2K and L), confirming that the signal transduction pathway of AKT-S6K-S6RP was not affected. Together, these results demonstrated that down-regulation of *ANG* expression did not inhibit AKT transgene expression and did not affect signaling transduction from AKT to S6P. Thus, ribosomal protein production was not affected by *ANG* siRNA.

Knocking-down *ANG* expression inhibited rRNA transcription

Next we examined *ANG* siRNA-induced changes in rRNA transcription in the prostate luminal epithelial cells of MPAKT mice. *In situ* hybridization (ISH) with a probe specific to the initiation site of 47S rRNA showed that rRNA transcription was dramatically increased in the prostate luminal epithelial cells of MPAKT mice (Fig. 2N), as compared to that of the WT littermates (Fig. 2P). Knocking-down *ANG* expression completely abolished AKT-induced increase in rRNA transcription (Fig. 2O), suggesting that rRNA transcription in AKT-induced PIN is mediated by *ANG*.

rRNA transcription is essential for ribosome biogenesis (28,29). To confirm that ribosome biogenesis was indeed decreased after *ANG* expression was knocked down, we examined the effect of *ANG* siRNA on nucleolar organizer region (NOR) of the prostate epithelial cells. NOR are loops of rDNA that are actively being transcribed (30). NOR are associated with argyrophilic proteins and can be visualized by silver staining. Both the numbers and size of the NOR reflect the degree of ribosome biogenesis (31). Fig. 3A shows that treatment of *ANG*-specific siRNA decreased the average number of NOR per cell from 2.83 to 1.52, indicating a significant decrease in ribosome biogenesis. A slowdown in ribosome biogenesis will result in a decrease in both cell growth and proliferation. Consistent with the suppression of rRNA transcription, knocking-down *ANG* expression decreased cell size as well as cell proliferation. The diameters of the prostate luminal epithelial cells from MPAKT mice treated with the control shRNA and *ANG*-specific siRNA, and that from the WT animals are 15 ± 2 , 10 ± 2 , 10 ± 1 μm , respectively (Fig. 3B). Treatment with *ANG* siRNA also decreased Ki-67 positive cells from 57.2 ± 4.9 to $20.8 \pm 11.7\%$ (Fig. 3C). Together, these results demonstrated that knocking-down *ANG* expression suppressed rRNA transcription thereby inhibiting cell growth and proliferation and preventing PIN formation.

Prevention of PIN formation by small-molecule *ANG* inhibitors

Next, we examined the effect of neomycin and N65828, two small-molecule inhibitors that abolish *ANG* activity by different mechanisms, on AKT-induced PIN formation. Previous results have demonstrated that both nuclear translocation of *ANG* and the ribonucleolytic activity of *ANG* are essential for its biological activity (5,32). Nuclear translocation of *ANG* can be blocked by aminoglycoside antibiotic neomycin (4), whereas the ribonucleolytic activity of *ANG* can be inhibited by NCI compound N65828 (8-amino-5-(4'-hydroxybiphenyl-4-ylazo) naphthalene-2-sulfonate) (33). *ANG* has a unique ribonucleolytic activity that is several orders of magnitude lower than that of the pancreatic RNase A (32). Extensive studies on site-directed mutagenesis have shown that *ANG* variants with reduced enzymatic activity also have reduced angiogenic activity (34–40). Thus, both neomycin and N65828 have been shown to inhibit xenograft growth of PC-3 human prostate cancer cells in nude mice (8,33). These two molecules were used here to examine the effect of *ANG* inhibition on AKT-driven PIN formation. Treatment with neomycin and N65828 both prevented PIN formation (Fig. 4A to C) but accompanied with a different pattern of nuclear translocation of *ANG* (Fig. 4D to F). Under neomycin treatment, the localization of *ANG* was extracellular (Fig. 4E), consistent with neomycin being a nuclear translocation blocker. However, *ANG* remained strongly in the nucleus in N65828-treated specimen (Fig. 4F). In both cases, AKT phosphorylation (Fig. 4G to I) was not altered. However, 47S rRNA transcription was inhibited as shown by ISH (Fig. 4J to L). The mechanism by which N65828 suppresses rRNA transcription is not clear at

present. One possibility is that the ribonucleolytic activity of ANG is involved in pre-rRNA processing and its inhibition by N65828 will result in a negative feedback of rRNA transcription. Although such a negative feedback mechanism has been described for other RNA polymerase I-dependent RNase activities (41,42), it is unknown whether it is applicable to ANG before we firmly establish that ANG plays a role in rRNA processing. Nevertheless, we have shown that three different types of ANG inhibitors (ANG-specific siRNA, neomycin, and N65828) all prevented AKT-driven PIN formation. In all three cases, rRNA transcription was inhibited but AKT phosphorylation was not affected.

ANG inhibitors reversed established PIN

Upregulation of ANG in the PIN of MPAKT mice is a lasting event (Fig. 1A), suggesting that ANG is important not only for the initial cell proliferation that leads to PIN formation but also for cell survival in the established PIN. To determine whether ANG inhibition reverses PIN, 12-week-old MPAKT mice with fully developed PIN were treated with neomycin or N65828 for 4 weeks. Gross examination of the genitourinary tracts showed that the size of the ventral prostate decreased after neomycin treatment (Fig. 5A). The sizes of the ventral prostates of a representative mouse from the control and neomycin-treated groups were 69.8 and 44.3 mm³, respectively, indicating shrinkage of the PIN after neomycin treatment. Neomycin treatment also restored cell size to normal (Fig. 5B). H&E staining showed that the PIN phenotype (Fig. 6A to C) was reversed after both neomycin and N65828 treatments. AKT phosphorylation (Fig. 6D to 6F) in both treated groups was not different from that of the control group. However, 47S rRNA level (Fig. 6G to I) was dramatically decreased in both neomycin- and N65828-treated animals, indicating suppression of rRNA transcription. Apoptosis of the prostate luminal cells would have to occur for a phenotypic reversal of the established PIN. This was indeed the case as shown by TUNEL staining (Fig. 6J to L). Apoptotic index in the control, neomycin- and N65828-treated prostate was 0.95 ± 0.11 , 1.95 ± 0.19 , and 2.02 ± 0.21 (caspase 3 positive cells per duct), respectively. These results suggested that ANG inhibitors reversed the established PIN probably due to an inhibition of rRNA transcription that eventually led to cell apoptosis.

Actinomycin D inhibited PIN in MPAKT mice

Actinomycin D is a cyclic polypeptide-containing antibiotic that binds to DNA and inhibits RNA synthesis. It interferes with the elongation of growing RNA chains catalyzed by RNA polymerases and is particularly sensitive to that mediated by RNA polymerase I (43). Therefore, at low concentration, actinomycin D selectively suppresses rRNA transcription (44). In order to confirm that increased rRNA transcription is functionally relevant to AKT-induced PIN, we treated 12 week-old MPAKT mice with daily i.p. injection of 0.1 mg/kg body weight for 4 weeks. H & E staining showed that an average of $47 \pm 9\%$ glands had PIN in control group (Fig. 7A and C), but only $14 \pm 7\%$ of the glands had PIN in actinomycin-treated group (Fig. 7B and C), indicating that actinomycin D reversed the PIN phenotype in these animals. ANG expression (Fig. 7D and E) and AKT phosphorylation (Fig. 7F and G) were not altered by treatment of actinomycin D, but rRNA transcription was markedly decreased in treated animals (Fig. 7I) as compared to the untreated ones (Fig. 7H).

Discussion

The angiogenic activity of ANG has been well established (1,45). Biochemical and cell biological studies have shown that the angiogenic activity of ANG is related to its ability to stimulate rRNA transcription (46,47), which relies on translocation of ANG to the nucleus and exertion of its ribonucleolytic activity presumably in the nucleolus (48). ANG antagonists including its monoclonal antibody (2), a soluble binding protein (2), antisense oligos (3), a nuclear translocation blocker (8) and an enzymatic inhibitor (33) have all been shown to inhibit

xenograft growth of human tumor cells in athymic mice. However, the role of ANG in the growth and progression of tumors arising in a more nature environment has not been studied.

AKT over-expression in the murine prostate induces PIN, accompanied by a dramatic increase of ANG expression (7), which provides a suitable experimental model for us to study the role of ANG in prostate cancer initiation and progression in the orthotopic site. We took an approach combining siRNA and small-molecule inhibitors to examine the effect of ANG inhibition on AKT-induced PIN formation and survival. First, we injected lentivirus-mediated ANG-specific siRNA into the prostate of MPAKT mice and showed that this approach knocked-down ANG expression in the prostate and prevented PIN formation. We have also shown that knocking-down prostatic expression of ANG suppressed rRNA transcription and cell proliferation. However, phosphorylation of AKT and S6RP, a well-defined down-stream target of AKT involved in ribosomal protein production (27), was not affected by manipulating ANG expression. The finding that ANG siRNA prevented PIN formation, despite continuous expression of AKT transgene and activation of its down-stream targets, demonstrated that proliferation and growth of the prostate intraluminal cells driven by AKT requires the participation of ANG. These results suggest that upregulation of ANG stimulates transcription of rRNA that, together with the ribosomal proteins enhanced through the AKT-mTOR-S6K-S6P pathway, allows ribosome biogenesis to take place (Fig. 8). In other words, both ANG-mediated rRNA and mTOR-mediated ribosomal proteins are required for AKT-induced PIN formation.

It is of interest to note that local inhibition of ANG expression within the prostate was sufficient to prevent PIN formation. ANG is a secreted protein as the precursor contains a classic signal peptide (49) and it is normally circulating in the plasma. Therefore, ANG could potentially act through either an autocrine, a paracrine or an endocrine manner (50). The findings that knocking-down ANG expression locally in the prostate inhibited AKT-induced rRNA transcription, cell proliferation and prevented PIN formation indicate that ANG functions in a cell autonomous manner. Circulating ANG in the plasma may not have a significant contribution to PIN formation.

Nuclear translocation of ANG is essential for its biological activity (5). The function of nuclear ANG is to stimulate rRNA transcription by binding to the promoter region of rDNA (47), and for this to occur, ANG needs to be physically in the nucleus. Neomycin, an aminoglycoside antibiotic, has been shown to block nuclear translocation of ANG in both endothelial cells (4) and in prostate cancer cells (8). Here we showed that i.p. injection of neomycin blocked nuclear translocation of ANG in the prostate luminal epithelial cells of MPAKT mice, suppressed rRNA transcription in these cells and prevented PIN formation. These results not only confirmed that ANG plays a role in AKT-mediated prostate cancer, but also supported the hypothesis that blocking nuclear translocation of ANG is a therapeutic target for prostate cancer treatment (8).

ANG is a member of the pancreatic RNase superfamily. Its amino acid sequence has 35% identity and an overall of 56% homology to that of bovine pancreatic RNase A. The ribonucleolytic activity of ANG is several orders of magnitude lower than that of RNase A toward the standard RNase substrates but is essential for its biological activity (32). Its nature substrate has not been determined, but may reside in the nucleolus as it has been shown that enzymatically inactive mutant ANG proteins undergo nuclear translocation but fail to induce angiogenesis (5). Screening of 18,310 compounds from the NCI Diversity Set and the ChemBridge DIVERSet based on inhibition of the ribonucleolytic activity of ANG has identified N65828 as a lead compound that preferentially inhibited the enzymatic activity of ANG over that of RNase A and prevented xenograft growth of PC-3 human prostate cancer cells in athymic mice (33). The K_i of N65828 toward ANG is 81 μ M, a value not good enough

for drug development. However, it was a suitable agent in our experiments to show that inhibition of the ribonucleolytic activity of ANG inhibited AKT-induced PIN.

In summary, three different types of ANG inhibitors have been used in this study to ascertain that ANG inhibition inhibits PIN formation and survival. In case of *ANG* siRNA, a general decrease in *ANG* expression was observed in the prostate luminal epithelial cells. In neomycin-treated samples, luminal epithelial cells were devoid of nuclear ANG, in agreement with neomycin being a nuclear translocation blocker (8). Neither expression nor nuclear translocation of ANG was affected by N65828 that only inhibits the enzymatic activity of ANG (33). In all three cases, rRNA transcription was inhibited without any effect on AKT phosphorylation and its signaling through S6P. Although each of these inhibitors alone may have off-target or non-specific effect, the findings that three types of ANG inhibitors of different mechanisms all inhibited PIN formation strongly demonstrated that ANG is essential for AKT-driven prostate luminal epithelial cell proliferation and PIN formation in MPAKT mice.

Materials and Methods

Mouse strains and genotyping

Animal experiments were approved by IACUC of Harvard Medical School. A breeding pair of MPAKT mice was provided by Dr. W. R. Sellers of Dana Farber Cancer Institute. Genotyping was carried out as described (7). All the animals were maintained in a pathogen-free barrier facility.

Lentivirus production

Lentiviral vectors encoding *ANG1*-specific siRNA or a nonspecific control shRNA were purchased from Open Biosystems. Lentiviral particles were prepared by transient transfection in 293 cells using the ViraPower Lentiviral Expression Systems according to manufacturer's instruction (Invitrogen). Lentiviral particles were harvested after 72 h, centrifuged at $781 \times g$ for 15 min, and filtered through a $0.45 \mu\text{m}$ PVDF membrane (Millipore). The viral particles were then ultracentrifuged at $83,000 \times g$ for 1.5 h and the pellet was resuspended in PBS. The functional viral titer was determined by p24 ELISA (ZeptoMetrix) and was expressed as transducing unit per ml (TU/ml).

Intraprostate injection

Five min before the surgery, mice were given 1 ml of saline s.c. and anesthetized with i.p. injection of ketamine and xylazine at 100 and 10 mg/kg body weight, respectively. The mice (4-weeks-old) were placed on a sterile gauze covering a heating pad with an ophthalmic ointment placed on their eyes. The abdomen was wiped with betadine followed by 70 % ethanol before a middle incision was made through the linea alba. The bladder and seminal vesicle were retracted anteriorly and lentivirus was injected in a $4 \mu\text{l}$ volume (9×10^6 TU) into the ventral lobe of the prostate using a 33 gauge needle with a calibrated push-button dispensing Hamilton syringe. The incision in the fascia was then closed with 3 to 4 silk sutures (6–0) and the skin was closed with auto clips. The mice were given an additional 1 ml saline and 0.05 mg/kg buprenorphine s.c. and kept on a heating pad until fully awake. The mice received buprenorphine (0.05 mg/kg) b.i.d. for three days post operation.

Immunohistochemistry

The entire genitourinary tract was removed and fixed with 4% paraformaldehyde and embedded in paraffin. Tissue sections were hydrated, incubated for 30 min with 3% H_2O_2 in methanol at RT, washed with Milli-Q H_2O and PBS, and heated in a microwave to 95°C in 10

mM citrate buffer, pH 6.0, for 10 min (for ANG, p-S6RP and Ki-67) or in 1 mM EDTA, pH 8.0, for 15 min (for p-AKT). Sections were blocked in 5% dry milk (for mouse ANG), or in 10% FBS (for p-AKT, and p-S6RP) for 30 min and incubated with antibodies against mouse ANG (10 µg/ml, R163), p-Akt-S473 (1:100; Cell signaling), p-S6RP-S235/236 (1:200; Cell Signaling) in 1% BSA at 4°C for 16 h. For detection of Ki67, the sections were blocked in the M.O.M.TM mouse Ig blocking reagent for 60 min and incubated with anti-Ki-67 antibody (1:100; Vector Laboratories) in the M.O.M.TM diluent at 25°C for 1 h. The slides were washed with PBS, and incubated with HRP-labeled second antibody and visualized with the DakoCytomation EnVision System. Staining for PCNA, CD31, and human ANG in the xenograft PC-3 tumor tissues were carried as described previously (46,51).

In situ hybridization

ISH for 47S rRNA was carried out as described by Qian et al. (52). The templates for the sense riboprobes was prepared by PCR from mouse genomic DNA with sense primer containing a T7 promoter (5'-GGGTAATAGGACTCACTATAGGGCGA). The primers for the initiation site of the 47S rRNA precursor were: forward, 5'-GCCTGTCACCTTCCTCCCTG; reverse, 5'-GCCGAAATAAGGTGGCCCTC; PCR conditions were: 5 min at 94 °C; 35 cycles (94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min) and at 72 °C for 7 min. Digoxigenin-labeled probes were generated by *in vitro* transcription from the above PCR templates using Digoxigenin RNA labeling Kit (Roche Diagnostics). Formalin-fixed, paraffin-embedded tissue sections were deparaffined with xylene and rehydrated with ethanol. After proteinase K treatment (1.5 µg/ml for 10 min at RT) and acetylation reaction (0.25% acetic anhydride in 0.1 mM Triethanolamine at RT for 20 min), the sections were washed with 4 × SSC, prehybridized at 45 °C for 1 h in 5 × SSC containing 50% formamide, 0.5 mg/ml heparin, and 0.1 mg/ml salmon sperm DNA. Hybridization was carried out in the same buffer as prehybridization but containing 800 ng/ml digoxigenin labeled probe at 45 °C for 16 h. After successive washing in 4 × SSC (1 min at RT), 50% formamide in 2 × SSC/(1 h at 45 °C), 0.1 × SSC (2 h at 45 °C), TTBS(5 min at RT), the hybridization signal was visualized using an alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Applied Science) with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as the substrate.

TUNEL assay

Paraformaldehyde-fixed tissue sections were deparaffinized in xylene, rehydrated in ethanol and incubated with proteinase K (0.02mg/ml) for 20 min at RT. TUNEL staining was carried out using the Fluorescein-FragEL DNA Fragmentation Detection kit (Calbiochem) per the manufacturer's instructions. TUNEL-positive luminal epithelial cells were counted in all ducts of the ventral prostate.

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FIGURE 1.

ANG protein level is elevated in the PIN tissues of MPAKT mice. **A.** Thin sections of the ventral prostates of the WT and MPAKT mice at 4, 6, 8, 10, and 12 weeks of age were stained with affinity purified anti-mouse ANG IgG R163. Pictures shown were from a representative area of the ventral prostate. ANG staining in the nucleus and in the stroma are indicated by black and white arrows, respectively. Bar, 50 μ m. **B.** Higher magnification images showing nucleolar staining of ANG. Pictures shown were from 10 week-old WT and MPAKT mice. Bar, 20 μ m.

FIGURE 2.

Effect of lentivirus-mediated ANG-specific siRNA on PIN formation in MPAKT mice. **A**. Efficacy of the ANG siRNA. Lewis lung carcinoma cells were transfected with pLKO.1 containing control shRNA or mouse ANG siRNA using lipofectamine 2000. Transfectants were selected with 3 mg/ml puromycin for 6 days. Total RNA was extracted by Trizol and real-time RT-PCR was performed. **B to P**. Lentiviral particles containing a scrambled shRNA sequence (control shRNA) or an *ANG1*-specific siRNA sequence (ANG siRNA) were injected into the exposed prostate of 4-week-old MPAKT mice. The animals and age-matched WT littermates were sacrificed when they were 8-weeks-old. Pictures shown are a representative area of the ventral prostate from 1 animal. Eight mice were used per group. Similar results were observed in every animal of the same group. **B to D**. H&E staining of the ventral prostates. PIN lesions are indicated by arrows. Percentage of glands having PIN in the ventral prostate was given under the pictures. Numbers shown are the average from all 8 animals in each group. **E to G**. IHC detection of ANG protein with affinity purified anti-mouse ANG IgG R163. Nuclear staining of ANG is indicated by arrows. **H to J**. IHC analysis of AKT phosphorylation. Arrows indicate positive signals. **K to M**. IHC analysis of S6RP phosphorylation. Arrows indicate positive signals. **N to P**. rRNA transcription was detected by ISH with a probe specific to the initiation site sequence of the 47 S rRNA. Positive signals are indicated by arrows. Pictures shown were from serial sections of one animal of each group. Bar, 100 μ m in **A to C**, 50 μ m in **E to P**.

FIGURE 3.

Knocking-down ANG expression decreased NOR, normalized prostate luminal epithelial cell size, and inhibited AKT-induced cell proliferation. Four-week-old MPAKT mice were treated by intraprostate injection of lentivirus containing *ANG*-specific siRNA or nonspecific control shRNA. **A.** Silver stained NOR of the ventral prostate epithelial cells. Data shown are the average numbers of NOR of 500 cells. Bar, 20 μm . **B.** Cell size of the ventral prostate epithelial cells was measured after H & E staining. The numbers shown are the average diameters of 500 cells from 5 microscopic areas. **C.** *ANG*-specific siRNA decreases cell proliferation in the ventral prostate of MPAKT mice. IHC with an anti-Ki67 antibody were used to show proliferative cells. The pictures shown were from a representative area. Ki-67 positive cells were counted from a total of 500 cells in each sample. Bar, 50 μm .

FIGURE 4.

Neomycin and N65828 prevented PIN formation. Four-week-old MPAKT mice were treated with daily i.p. injection of PBS control, neomycin or N65818 at a dose of 10 and 4 mg/kg body weight, respectively, for 4 weeks. The mice were sacrificed at week 8 and the ventral prostates were processed for histological examinations. **A to C.** H&E staining of the ventral prostates. PIN lesions are indicated by arrows. **D to F.** IHC examinations of nuclear translocation of ANG. Staining of nuclear ANG is indicated by arrows. **G to I.** IHC examinations of phosphorylation status of AKT. Positive signals are indicated with arrows. **J to L.** ISH analysis for rRNA transcription. Positive signals are indicated by arrows. Bar, 100 μ m in **A to C**, 50 μ m in **D to L**.

FIGURE 5.

Neomycin treatment shrunk PIN lesion and normalized luminal cell size. **A.** Gross picture of the genitourinary tracts of PBS and neomycin-treated MPAKT mice. The GU tracts were dissected *en block* and the size of ventral prostate was measured by a caliper. The ventral prostate is indicated with an arrow. **B.** Cell size of PBS and neomycin-treated MPAKT and wild type mice. A total of 500 cells (100 cells each from 5 randomly selected glands) were measured in each sample.

FIGURE 6.

Treatment with neomycin and N65818 reversed established PIN in MPAKT mice. Twelve-week-old MPAKT mice, at this age PIN has been fully developed, were treated with daily i.p. injection of PBS control, neomycin, or N65818 at a dose of 10 and 4 mg/kg body weight, respectively, for 4 weeks. The mice were sacrificed at week 16 and the ventral prostates were examined. **A to C.** H&E staining, PIN lesions are indicated with arrows. **D to F.** IHC detection of p-AKT. Positive signals are indicated by arrows. **G to I.** ISH for rRNA transcription. Positive signals are indicated by arrows. **J to L.** Apoptosis of luminal epithelial cells were examined by TUNEL staining. Apoptotic cells are indicated by arrows. Bar, 100 μ m in **A to C**, 50 μ m in **D to L**.

FIGURE 7. Actinomycin D reversed established PIN in MPAKT mice

Twelve-week-old MPAKT mice were treated with daily i.p. injection of PBS control or actinomycin D at a dose of 0.1 mg/kg body weight for 4 weeks. The mice were sacrificed at week 16 and the ventral prostates were examined. **A and B.** H&E staining. **C.** Percentage of glands having PIN in the ventral prostate. Numbers shown are the average from all 6 animals in each group. **D and E.** IHC detection of ANG. **F and G.** IHC detection of p-AKT. **H and I.** ISH for rRNA transcription. Positive signals are indicated by arrows from **D to I**. Bar, 100 μ m in **A and B**, 50 μ m in **D to I**.

FIGURE 8.

Proposed role of ANG in AKT-driven cell proliferation and survival. AKT overexpression up-regulates ANG expression. ANG then undergoes nuclear translocation and stimulates rRNA transcription. Together with the ribosomal proteins synthesized by the mTOR-S6K-S6P pathway, ribosome biogenesis occurs. Therefore, ANG is a permissive factor for AKT-drive cell proliferation and survival.