Expression and Localization of S-Adenosylhomocysteine-Hydrolase in the Rat Kidney Following Carbon Monoxide Induced Hypoxia

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Key Words

S-Adenosylhomocysteine Hydrolase • Erythropoietin • Immunohistochemistry • Confocal LSM • Nucleus

Abstract

Background/Aims: Tissue hypoxia induces a variety of functional changes including enhanced transcriptional activity associated with high transmethylation activity (e.g. mRNA cap methylation) in the nucleus. It is well known that the kidney responds to hypoxia with enhanced transcription of erythropoietin (EPO) in the interstitial cells. Since S-adenosylhomocysteine (AdoHcy)-hydrolase regulates most S-adenosylmethionine (AdoMet) dependent transmethylation reactions by hydrolyzing the potent feedback inhibitor AdoHcy to adenosine and homocysteine we studied the effect of hypoxia by carbon monoxide (CO) inhalation (1200ppm) on

AdoHcy-hydrolase gene expression and its localization in rat kidneys. Results: CO lowered renal AdoHcy-hydrolase mRNA expression by 64% whereas AdoHcy-hydrolase activity was not changed during 4h of CO exposure 0.7 ± 0.04 mU/mg (control) vs. 0.75 ± 0.06 mU/mg protein. Using two-channel immunofluorescence confocal laser scanning microscope AdoHcy-hydrolase was visualized in different cells of the hypoxic rat kidney. A very bright immunofluorescence of AdoHcy-hydrolase was observed in the nuclei of single interstitial cells of renal cortex and outer medulla which respond to hypoxia with increased EPO secretion indicating translocation of AdoHcy-hydrolase from the cytosol to the nucleus. Conclusions: These data suggest that AdoHcy-hydrolase accumulation in the nucleus of adult mammalian cells is involved in maintaining efficient transmethylation reactions in transcriptionally active cells by removing the product inhibitor AdoHcy.

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Introduction

In mammals, S-adenosylhomocysteine-(AdoHcy)-hydrolase (EC 3.3.1.1) is the only known enzyme that catalyzes the reversible breakdown of AdoHcy to adenosine and homocysteine [1]. AdoHcy is the product of all S-adenosylmethionine-(AdoMet)-dependent transmethylation reactions. These reactions have a wide range of products, and are common in all facets of biometabolism. As a product inhibitor, AdoHcy efficiently suppresses AdoMet-dependent transmethylation reactions [2]. A sensitive indicator affecting the activity of transmethylation is the AdoMet/AdoHcy ratio, also termed as “methylation potential” (MP) [3]. Normally, the relative levels of AdoMet and AdoHcy are tightly regulated in the cells. The maintenance of the correct MP is achieved by the hydrolysis of AdoHcy to adenosine and homocysteine via AdoHcy-hydrolase. Therefore, AdoHcy-hydrolase serves physiologically not only to sustain the flux of methionine sulfur toward cysteine, but is also an essential enzyme playing a key role in the regulation of biological methylation [4, 5].

AdoHcy-hydrolase is a highly conserved enzyme and its function has been shown to be essential for development since mice lacking the AdoHcy-hydrolase gene die in early embryogenesis [6]. However, in the meantime three proven cases of human AdoHcy-hydrolase deficiency have been reported [7-9].

AdoHcy-hydrolase is widespread among procaryotic and eucaryotic cells and ubiquitously distributed in all animal tissues. In the rat, highest activity was found in the liver and pancreas, followed by kidney, adrenal gland and brain. Low enzyme activity was reported for heart, muscle, prostate, spleen and lung [10, 11]. As previously shown, AdoHcy-hydrolase appeared to be relatively uniformly distributed in the rat kidney [12].

Although AdoMet-dependent methylation reactions are likely to occur in all compartments of the cell, AdoHcy-hydrolase has been described previously as a cytoplasmic enzyme [4, 13]. However, it has been shown previously that during embryogenesis of *Xenopus laevis* AdoHcy-hydrolase is highly concentrated in the nuclei of transcriptionally active cells [14]. The nuclear localization of AdoHcy-hydrolase which coincides with high rates of mRNA synthesis may reflect the necessity to remove AdoHcy to maintain the efficient mRNA cap methylation during embryogenesis [14, 15].

The aim of our study was to investigate whether AdoHcy-hydrolase becomes detectable also in

transcriptionally active nuclei of adult mammalian cells. As an experimental model for transcriptionally active nuclei we chose EPO secretion by the rat kidney since EPO is not stored in vesicles but is produced on demand following carbon monoxide (CO) exposure.

Materials and Methods

The following materials were purchased from the different sources indicated: Sytox green nucleic acid stain (Mobitec, Germany), anti-rabbit antibodies labeled with Cy 5 (Dianova, Germany), Fluorsave Reagent (Calbiochem), normal donkey serum (NDS) (Jackson, USA), anti-AdoHcy-hydrolase antibody [12], bovine serum albumin (BSA), Tris-(hydroxymethyl)-aminomethan (Tris), AdoMet, AdoHcy, adenosine, methyladenosine, AMP, ADP, ATP, nucleoside phosphorylase (Sigma), adenosine deaminase, xanthine oxidase (Roche), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Serva), HPLC grade methanol and acetonitrile (Merck).

Animal preparation

The animal experimentation described was conducted in accordance to the German Law for Animal Protection. Male Sprague-Dawley rats (280-350g) were obtained from Charles River, UK and fed with standard laboratory rat chow and tap water *ad libitum*.

To induce hypoxia rats were placed into a cage with an air flow between 6-10l/min. CO was mixed with room air to generate the CO concentration of 1200ppm. The actual CO concentration was monitored by a CO sensor (Testo, Reutlingen). The deleterious effects of high CO concentrations in the inspired air on humans and animals have been reviewed and described by many authors [16-18]. After 4h of CO exposure rats were anesthetized with thiopental 90mg/kg i.p. (Trapanal® Byk Gulden, Germany). The left kidney was exposed by flank incision and placed on a Lucite holder and immediately snap-frozen with clamps precooled to the temperature of liquid nitrogen. After that blood samples were collected and stored at -80°C until analysis of EPO. For immunohistochemistry and for protein determination, the right kidney was removed subsequently and immediately fixed in liquid nitrogen to preserve the structure of the tissue.

Tissue preparation and analysis

The frozen left kidneys were prepared as described previously [19]. In brief, the frozen kidneys were pulverized under liquid nitrogen and the tissue protein was precipitated with 0.6N ice-cold perchloric acid. After centrifugation at 11,000xg for 30min at 4°C the supernatants were adjusted to a pH between 5.5 and 6.5 to measure adenosine, AdoHcy and AdoMet or to a pH of 7.8 to measure the adenine nucleotides by means of HPLC [19, 20].

Table 1. Nucleotide sequence of PCR primers used for amplification of AdoHcy-hydrolase and erythropoietin (EPO) mRNA by real-time quantitative RT-PCR

Gene	Primer Sequences (F, Forward; R, Reverse)	Product Size (bp)
AdoHcy-hydrolase	F: CTG AGG AGA CCA CGA CTG R: TGC CCA CAT CAC CAT AGC	216
EPO	F: CAC GAA GCC ATG AAG ACA GA R: GGC TGT TGC CAG TGG TAT TT	100
18S rRNA	F: CGG CTA CCA CAT CCA AGG AA R: GCT GGA ATT ACC GCG GCT	187

Real-time reverse transcription-polymerase chain reaction

RNA of non-hypoxic and hypoxic kidney tissue (4-7 animals/group) was isolated to analyze mRNA expression of AdoHcy-hydrolase and EPO. Briefly, total RNA was extracted from an aliquot of pulverized frozen kidney with Tri-Reagent/Trizol (PeqLab, Erlangen, Germany) by the method of Chomczynski and Sacchi [21]. cDNA was synthesized with AMV reverse transcriptase (PeqLab, Erlangen, Germany) using random hexamers (Promega, Mannheim, Germany).

Real-time-PCR analysis was performed on the Light Cycler instrument with FastStart DNA Master SYBR Green reagents (Roche, Mannheim, Germany) as described previously [5]. The relative amount of the specific mRNA of interest was normalized to 18S rRNA. Gene expression was calculated according to a mathematical model for relative quantification established by Pfaffl [22]. Primer sequences are depicted in Table 1.

Activity of renal AdoHcy-hydrolase and Western blots

The enzymatic activity of AdoHcy-hydrolase was measured in total renal homogenate in the hydrolytic direction as described previously [13]. This method is based on the reduction of MTT to formazan at λ 578nm with the following components: adenosine deaminase (1U), nucleoside phosphorylase and xanthine oxidase (0.5U each), gelatine (0.14%) and MTT (1mmol/l). The reaction was started by addition of AdoHcy (80 μ mol/l).

Hundred micrograms of protein from total kidney homogenate, determined by the Bradford method [23], were separated on 7-19% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAG) and electrophoretically transferred to a Protran B 85 nitrocellulose membrane (Schleicher and Schuell, Duesseldorf, Germany) at 400mA. The membranes were labeled with AdoHcy-hydrolase-specific primary polyclonal antibody (1:250) raised in rabbits against the native bovine AdoHcy-hydrolase [12], followed by horseradish-peroxidase-linked secondary goat anti-rabbit IgG (DAKO, Denmark). The staining as performed using 500 μ l *o*-toluidine [saturated in 7% (v/v) acetic acid], 100 μ l 4-chloro-1-naphthol [2% (w/v) in diethylene glycol], and 500 μ l H₂O₂ (3% v/v) in 50ml PBS.

Erythropoietin assay

Serum EPO concentrations were quantified using a commercial enzyme-linked immunosorbent assay (ELISA) kit

(Medac, Wedel, Germany). Serum samples from hypoxic rats were diluted 1:10.

Immunohistochemistry

Immunohistochemistry was carried out on 10 μ m cryostat renal sections using the double-immunofluorescence technique. The immunostaining protocol was employed as described previously [12]. In brief, renal sections were air dried, and fixed in acetone for 10min at room temperature (RT) and again air-dried. The sections were washed for 30min in TBS (150mM NaCl, 50mM Tris/HCl, pH 7.6). Non-specific background staining was reduced by preincubating sections for one hour with 5% normal donkey serum at RT. The sections were incubated with the polyclonal antibody against AdoHcy-hydrolase (1:100 dilution) overnight at RT. After rinsing, the sections were incubated with indocarbocyanin (Cy 5) conjugated anti-rabbit IgG (1:300 dilution) for one hour at RT. The nucleic acid stain Sytox green (1:100 000 dilution) was added to the sections for 20min.

The sections were examined with two-channel fluorescence confocal laser scanning microscopy (Zeiss, Oberkochen, Germany) LSM 410 Invert/ Axiovert 135M. Fluorescent signals after double staining were recorded simultaneously at one scan using a dual-wavelength channel at 488 and 633nm, no cross-talk was observed. All images were processed using Adobe PhotoShop (Adobe Systems, Edinburgh, United Kingdom).

In situ hybridization

Kidneys of rats obtained after 4h CO exposure at 1200ppm were fixed in 4% paraformaldehyde/0.1M sodium phosphate buffer (pH 7.2) over night and embedded in paraffin. Five μ m tissue sections were dewaxed and hybridized as previously described [24]. Hybridization probes at a length of 502bp were generated by *in vitro* transcription of a mouse EPO cDNA cloned into pGEM-3Z using T7 polymerase (antisense). The hybridization mixture (10mM Tris/HCl, pH 7.4, 50% (vol/vol) deionized formamide, 600mM NaCl, 1 mM EDTA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.05% BSA, 10% dextrane sulfate, 10mM dithiothreitol, 200 μ g/ml denatured sonicated salmon sperm DNA, 100 μ g/ml rabbit liver tRNA) contained either the ³⁵S-labeled RNA antisense EPO or an unrelated plasmid vector control RNA probe at a concentration of 500ng/ml. Hybridization with RNA probes proceeded at 42°C for 18h. Following washing steps, the slide preparations were dipped in NTB2 emulsion (Kodak, Rochester, NY) and exposed at 4°C for 3 weeks. After development the slides were stained with

Table 2. Data characterizing the tissue content of adenosine, AdoHcy, AdoMet, adenine nucleotides, AMP, ADP, ATP, and EPO mRNA expression in the rat kidneys after CO exposure. Data are mean $\pm$ SEM for n determinations. * $p < 0.05$ versus control.

	Control (n=4)	Hypoxia (n=7)
adenosine (nmol/g wet weight)	4.5 $\pm$ 0.3	7.6 $\pm$ 0.5*
AdoHcy (nmol/g wet weight)	0.65 $\pm$ 0.07	0.54 $\pm$ 0.06
AdoMet (nmol/g wet weight)	35.4 $\pm$ 2.7	24.3 $\pm$ 0.08*
AdoMet/AdoHcy ratio	54.5 $\pm$ 3.0	47.1 $\pm$ 5.0
AMP (nmol/g wet weight)	170.5 $\pm$ 3.0	108.0 $\pm$ 13.0
ADP (nmol/g wet weight)	352.4 $\pm$ 11.0	284.4 $\pm$ 22.0
ATP (nmol/g wet weight)	2176.5 $\pm$ 70.0	1388.1 $\pm$ 75.0*
Energy charge (EC)	0.87 $\pm$ 0.01	0.86 $\pm$ 0.01
EPO mRNA expression	1.68 $\pm$ 0.4	15.7 $\pm$ 2.1

hematoxylin/eosin, and photographed with a light microscope (Nikon Eclipse TE 2000-U).

Data calculations and statistics

The energy charge of the adenylate system was calculated according to Atkinson and Walton [25], defined as:

$$\frac{\text{ATP} + \frac{1}{2} \text{ADP}}{\text{ATP} + \text{ADP} + \text{AMP}}$$

The data are expressed as mean $\pm$ SEM. Statistical analysis was performed using the unpaired Student's *t*-test. Values of $P < 0.05$ were considered statistically significant.

Results

Determination of erythropoietin

In order to characterize our model of systemic CO exposure, we first determined the EPO serum concentration and mRNA expression in the rat kidneys. Within 4h of CO exposure (1200 ppm), EPO serum level was significantly elevated from 10.2 $\pm$ 0.7 (control) to 1838.5 $\pm$ 218.0 mIU/ml. EPO serum levels increased in parallel to mRNA which is 9fold up-regulated in rat kidneys (Table 2).

Effect of CO exposure on methylation potential (MP) and energy charge (EC)

To investigate whether exposure to CO changes the AdoMet/AdoHcy ratio in the kidney we measured AdoHcy and AdoMet tissue levels. Tissue level of AdoHcy in the whole kidney is not reduced significantly under CO exposure. The lowered AdoMet/AdoHcy ratio (MP) from 54.5 $\pm$ 3 (control) to 47.1 $\pm$ 5 is the result of a decreased tissue content of AdoMet. Furthermore, CO intoxication significantly increases the adenosine tissue level (Table 2). The energy charge (EC) of the rat kidney is not affected by CO exposure (Table 2).

Effect of CO exposure on AdoHcy-hydrolase expression and activity

After 4h of CO exposure global AdoHcy-hydrolase mRNA expression in the rat kidney was decreased by 64%, whereas the specific activity of AdoHcy-hydrolase (Fig. 1A) and the protein expression was unchanged (Fig. 1B).

Immunohistochemistry

The immunohistochemistry was performed on cryostat sections of the rat kidney. The specificity of the anti-AdoHcy-hydrolase antibody used was examined by West-

Fig. 1. (A) Enzyme activity and mRNA expression of AdoHcy-hydrolase in rat kidneys after CO exposure. Data are mean $\pm$ SEM for n determinations. * $p < 0.05$ versus control. (B) Western blot of AdoHcy-hydrolase from rat kidneys. Kidney homogenate (100 μ g/lane) was subjected to SDS-PAGE followed by immunoblotting with a specific polyclonal antibody. Lane 1: molecular weight markers used; Lanes 2 and 3: AdoHcy-hydrolase from control kidneys; Lanes 4 and 5: AdoHcy-hydrolase from kidneys following CO exposure (4h at 1200 ppm).

ern Blot analysis [12]. To differentiate between AdoHcy-hydrolase and nuclei staining, double labeling immunofluorescence was used with the polyclonal antibody against AdoHcy-hydrolase and the nucleic acid stain Sytox green.

The immunostaining in control kidneys (Fig. 2A) confirmed the ubiquitous distribution of AdoHcy-hydrolase in glomeruli, proximal and distal tubules of the cortex and medulla as previously reported by Kloor et al. [12]. The distribution of AdoHcy-hydrolase in rat kidneys after CO exposure when EPO secretion is enhanced showed a prominent intense staining of interstitial cells compared with the surrounding tubules (Fig. 2B). We assumed those cells as interstitial which surround tubules and capillaries. The majority of interstitial cells are fibroblasts as shown by Kaissling et al. [26]. A specific function of renal interstitial cells is the production of EPO. Compared to the control kidneys CO exposure does not affect the staining of AdoHcy-hydrolase in the tubular cells. In addition to the relatively uniform cytosolic stain-

ing of the enzyme in the control and hypoxic rat kidney, after CO exposure a very bright immunofluorescence was observed in some nuclei of interstitial cells of renal outer medulla (Fig. 3A-C). This nuclear staining was confirmed by the detection in different levels of the section under investigation. In contrast to the co-localization of AdoHcy-hydrolase with nuclei in the hypoxic kidney, the nuclear staining of AdoHcy-hydrolase in control kidneys is a very rare event (Fig. 3D-E). We therefore speculate that the co-localization of AdoHcy-hydrolase with the nucleus is in a ratio of 1 (control) to 7-9 (hypoxia).

In situ hybridization

In order to localize the cell types in kidneys which express EPO mRNA following CO exposure we performed *in situ* hybridization experiments. As shown in Fig. 4, EPO mRNA is detected exclusively in single cells of the peritubular space mainly located in the deep cortical juxtamedullary region. No autoradiographic signals

Fig. 2. Indirect immunofluorescence micrographs showing the immunohistochemical localization of AdoHcy-hydrolase in the rat kidney (A) control (B) after CO exposure. Sections are labeled with the polyclonal antibody against bovine AdoHcy-hydrolase (Cy 5, red) and with Sytox green against nucleic acid. Using two-channel immunofluorescence confocal LSM double, immunofluorescence staining of cells and nuclei are simultaneously visualized. Bar 25μm.

Fig. 3. Indirect immunofluorescence micrographs showing interstitial cells of renal medulla after hypoxia (A-C) and control (D-F). Using double immunofluorescence staining, the nuclei of the interstitial cells are simultaneously visualized and marked by arrows [(A,D) magnification x600, (B,E) magnification x1200 and (C,F) magnification x3000]. AdoHcy-hydrolase is labeled with the polyclonal antibody against bovine AdoHcy-hydrolase (red) whereas the nucleic acid is labeled with Sytox green (green).

Fig. 4. Light micrographs illustrating the *in situ* hybridization of EPO mRNA in kidneys after 4h CO exposure to 1200ppm. Interstitial cells containing EPO mRNA are located in the peritubular interstitial space mainly in the deep cortical juxtamedullary region of the kidney. Original magnification x400.

were detected in tissue specimens of the kidney when hybridized with the ^{35}S -labeled plasmid control RNA probe (Fig. 4B).

Discussion

AdoHcy-hydrolase is essential not only in the cytoplasm to maintain the AdoHcy tissue content at normal, low levels [19], but also in the nuclei of transcriptionally active cells to maintain an efficient transfer of the methyl group from AdoMet to pre-mRNA by cap methyltransferase [14]. Since during embryogenesis of *Xenopus laevis* nuclear localization of AdoHcy-hydrolase correlates with high transcriptional activity, we examined in the present study whether AdoHcy-hydrolase becomes detectable also in transcriptionally active nuclei of adult mammalian cells. As an experimental model for transcriptional active nuclei we choose erythropoietin (EPO) secretion by the rat kidney. EPO is not stored in vesicles but is produced on demand following carbon monoxide (CO) exposure, resulting in extensive EPO mRNA expression.

Using the model of CO exposure we observed that the methylation potential (MP, AdoMet/AdoHcy ratio) of

the kidney was not significantly reduced compared to control, and that the energy charge (EC) of 0.86 was within the physiological range [19]. These observations indicated that intoxication with CO does not affect transmethylation reactions and EC in the kidney cells. However, compared to control, AdoMet and ATP tissue contents are significantly lowered under CO exposure. The reduced AdoMet level within the kidney cells could be the result of a decreased specific activity of methionine adenosyltransferase (MAT) (EC 2.5.1.6) and/or of a reduced ATP and methionine tissue content and/or an increased methylation activity. As recently shown by various investigators under hypoxia MAT enzyme activity and mRNA levels were reduced in rat liver and hepatocytes resulting in subnormal AdoMet levels [27-29].

Increased transmethylation activities within the cells could also lower the AdoMet tissue content and might be responsible for high AdoHcy tissue levels since the pool of AdoHcy in the kidney, as in any other organ, is the sum of all methyltransferase reactions and the rate of synthesis and/or hydrolysis by AdoHcy-hydrolase. However, after 4 hours of CO exposure neither the AdoHcy tissue levels nor the specific activity of AdoHcy-hydrolase were significantly changed suggesting that the methylation activities were unchanged, too.

Moreover, reduction of ATP tissue content was accompanied by significantly increased adenosine tissue content under CO exposure (Table 2). Adenine nucleotide degradation accompanied by an accumulation of adenosine has been observed also in ischemic rat kidney [19]. The function of adenine nucleotide breakdown during hypoxia might be that adenosine serves as a vasoconstrictor in the kidney [30].

Since it was previously shown that hypoxia decreases AdoHcy-hydrolase expression in HepG2 cells [5,31] we also examined in this study the effect of hypoxia on the AdoHcy-hydrolase mRNA content in rat kidneys. CO exposure decreased the mRNA content of AdoHcy-hydrolase in the rat kidney by 64%. It is unclear whether decreased AdoHcy-hydrolase gene expression represents a direct response to hypoxia or whether it occurs secondarily to hypoxic suppression of an unknown, constitutively present stimulus for AdoHcy-hydrolase expression. In contrast, AdoHcy-hydrolase protein expression and enzyme activity were not altered after CO exposure for 4h. However, decreased AdoHcy-hydrolase expression might be of importance under physiological and pathophysiological conditions when CO concentration is elevated for longer time periods.

It is well known that under hypoxic or anaemic conditions, when arterial pO_2 is lowered, the EPO production increases in the renal tissue [18,32-36]. Our data are in accordance with these previous studies and show that under CO exposure rat kidney cells can serve as experimental model for transcriptionally active nuclei.

Using the specific AdoHcy-hydrolase antibody [12] and two-channel immunofluorescence confocal laser scanning microscope, AdoHcy-hydrolase was visualized in glomeruli and interstitial endothelial cells of control kidneys as well as in interstitial cells other than endothelial cells of hypoxic rat kidneys. To discriminate more precisely between cytosol and nucleus, double immunofluorescence staining was used, and it was possible to show that AdoHcy-hydrolase is localized in some nuclei of the interstitial cells of renal outer medulla (Fig. 3). The immunohistological detection of AdoHcy-hydrolase in the nu-

clei of interstitial cells of renal medulla following CO exposure is associated with the number of EPO producing cells in the kidney (Fig. 4). Characteristic for fibroblasts is the expression of 5'-ecto-nucleotidase [26,37]. Unfortunately, the immunostaining on our acetone-fixed cryostat sections exhibits no 5'-ecto-nucleotidase indicating that a paraformaldehyde fixation is necessary for the epitope recognition by the employed antibody. Accumulation of AdoHcy-hydrolase in the nucleus may be required for efficient cap methylation in transcriptionally active cells. In addition, nuclear proteins could be targets of AdoMet-dependent methylation processes [14,38]. Therefore, Radomski et al. [15] suggest a close association of AdoHcy-hydrolase to the transcriptional elongation complex to prevent product inhibition of RNA guanine-7-methyltransferase in *Xenopus leavis*.

A quantitative determination of AdoHcy-hydrolase protein expression by Western-Blot or determination of AdoHcy-hydrolase activity in the nuclei of renal cells was not possible, probably due to the very little amount of the enzyme in these nuclei. The protein determination after the purification from nuclei shows a sufficient amount of protein. This supports the assumption, that the amount of AdoHcy-hydrolase protein in these nuclei is below the detection limit of Western-Blot-analysis.

In summary, our results show that CO exposure for 4h does not affect AdoHcy-hydrolase activity, MP and EC in rat kidney. However, hypoxia results in nuclear localization of AdoHcy-hydrolase of interstitial cells, which might be required for efficient cap methylation in transcriptionally active cells.

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