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ORIGINAL ARTICLE

Developmental alterations in expression and subcellular localization of antizyme and antizyme inhibitor and their functional importance in the murine mammary gland

Y. Murakami · J. Suzuki · K. Samejima · T. Oka

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Abstract Ornithine decarboxylase (ODC), antizyme (AZ), and antizyme inhibitor (AIn) play a key role in regulation of intracellular polyamine levels by forming a regulatory circuit through their interactions. To gain insight into their functional importance in cell growth and differentiation, we systematically examined the changes of their expression, cellular polyamine contents, expression of genes related to polyamine metabolism, and β-casein gene expression during murine mammary gland development. The activity of ODC and AZ1 as well as putrescine level were low in the virgin and involuting stages, but they increased markedly during late pregnancy and early lactation when mammary cells proliferate extensively and begin to augment their differentiated function. The level of spermidine and expression of genes encoding spermidine synthase and AIn increased in a closely parallel manner with that of casein gene expression during pregnancy and lactation. On the other hand, the level of spermidine/spermine N^1-acetyltransferase (SSAT) mRNA and AZ2 mRNA decreased during those periods. Immunohistochemical analysis showed the translocation of ODC and AIn between the nucleus and cytoplasm and the continuous presence of AZ in the nucleus during gland development. Reduction of AIn by RNA interference inhibited expression of β-casein gene stimulated by lactogenic hormones in HC11 cells. In contrast, reduction of AZ by AZsiRNA resulted in the small increase of β-casein gene expression. These results suggested that AIn plays an important role in the mammary gland development by changing its expression, subcellular localization, and functional interplay with AZ.

Keywords Mammary gland development · Polyamines · Ornithine decarboxylase · Antizyme · Antizyme inhibitor · β-Casein

Y. Murakami · J. Suzuki · K. Samejima · T. Oka (✉)
Faculty of Pharmacy, Musashino University,
Nishi-Tokyo, Tokyo 202-8585, Japan
e-mail: oka_tm@wakunaga.co.jp

Y. Murakami
Department of Molecular Biology,
Jikei University School of Medicine,
Minato-ku, Tokyo 105-8461, Japan

J. Suzuki
Signaling Molecules Research Group,
Neuroscience Research Institute, National Institute of Advanced Industrial Science and Technology (AIST),
1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan

T. Oka
Health Care Research Institute,
Wakunaga Pharmaceutical Company, Ltd.,
Chiyoda-ku, Tokyo 101-0062, Japan

Abbreviations

AZ	Antizyme
AdoMetDC	*S*-adenosylmethionine decarboxylase
AIn	Antizyme inhibitor
ODC	Ornithine decarboxylase
SSAT	Spermidine/spermine N^1-acetyltransferase
cont siRNA	Control siRNA
DFMO	α-Difluoromethylornithine
siRNA	Small interfering RNA
Put	Putrescine
Spd	Spermidine
Spm	Spermine
PBS	Phosphate-buffered saline

Introduction

The polyamines putrescine, spermidine, and spermine are indispensable for cell growth and function, but they can be cytotoxic if present in excess. The cellular concentrations of polyamines are rapidly and tightly regulated by multiple factors involved in the biosynthesis and transport of polyamines. Mammalian ornithine decarboxylase (ODC) is the first and key enzyme in the polyamine biosynthetic pathway (Pegg 2006). This enzyme has a very short half-life and is highly regulated (Hayashi and Murakami 1995; Coffino 2001; Pegg 2006; Shantz and Levin 2007). ODC is induced by various growth stimuli and is degraded rapidly when polyamine levels increase (Davis et al. 1992).

Antizyme (AZ) is an inhibitory protein of ODC (Fong et al. 1976). AZ binds to ODC, inhibits, and targets it to the 26S proteasome for ubiquitin-independent degradation (Murakami et al. 1992; Zhang et al. 2004). In addition, AZ negatively regulates polyamine transport (Mitchell et al. 1994; Suzuki et al. 1994). When intracellular polyamine levels become excessive, polyamines induce the synthesis of AZ by ribosomal frameshifting (Matsufuji et al. 1995; Rom and Kahana 1994). At present, three subtypes of AZ are known in mammalian tissues. AZ1 is the most abundant and best characterized (Pegg 2006; Kahana 2009). There are 24.5 and 29 kDa forms of AZ1 due to the presence of two potential translation start sites. AZ2 is a 21-kDa protein encoded by a different gene and is expressed at much lower levels in many tissues (Ivanov et al. 1998). In contrast to AZ1 and 2, AZ3 is restricted to germ cells in the testis (Ivanov et al. 2000b, Tosaka et al. 2000) and its size is 28 kDa. Moreover, recent studies have shown that AZ also binds to several other regulatory proteins including cyclin D1, a cell cycle regulatory protein (Newman et al. 2004; Kim et al. 2006), Aurora-A, a mitosis regulatory protein (Lim and Gopalan 2007) and Smad1 (Gruendler et al. 2001), suggesting that the regulatory function of AZ is not limited to polyamine metabolism. Recently, we have shown that AZ is necessary for inducing pancreatic tumor cells to produce glucagon and that this AZ function is not mediated by alterations in the cellular contents of polyamines (Suzuki et al. 2009).

In addition to AZ, cells contain another regulatory protein, AZ inhibitor (AIn). AIn binds to AZ more strongly than ODC, releases ODC from the ODC–AZ complex, and inhibits AZ-mediated ODC degradation. In addition, AIn increases polyamine uptake. Thus, AIn functions as a positive regulator of both ODC and polyamine transport by trapping AZ in cells. Moreover, the synthesis of AIn is negatively regulated by polyamines at translational level (Ivanov et al. 2008; Murakami et al. 2009). Furthermore, AIn, like ODC and AZ, is also a short-lived protein and degraded by the proteasome. However, in contrast to ODC degradation, AZ and AIn stabilize each other (Bercovich and Kahana 2004; Kahana 2007, 2009). Recently, AIn has been shown to play a key role in regulation of centriole duplication in a polyamine-independent manner (Mangold et al. 2008). We also have found that AIn changed its subcellular location during the cell cycle and reduction of AIn by RNA interference caused the increase in the number of binucleated cells (Murakami et al. 2009). In addition, a recent study using gene knockout mouse has demonstrated an essential function of AIn during mouse development (Tang et al. 2009), while the augmented expression of AIn has been implicated in growth of cancer cells (Jung et al. 2000; Keren-Paz et al. 2006).

The growth and development of the mammary gland are regulated by synergistic actions of hormones including prolactin, glucocorticoid, insulin, and various growth factors (Borellini and Oka 1989; Oakes et al. 2008). The mammary gland of virgin mouse is developmentally dormant, but following the onset of gestation, mammary epithelial cells multiply extensively. After parturition, the gland expresses its differentiated function by synthesizing and secreting milk during lactation, and then regresses to the non-differentiated state upon weaning. Earlier studies have shown that polyamines play an important role in the growth and differentiation of the mammary epithelium (Oka et al. 1991, Rillema et al. 1977). In the present study, we analyzed the expression and intracellular localization of ODC, AZ, and AIn, and examined their inter-relationships during the development of murine mammary gland. In addition, using RNA interference, we examined the role of AIn and AZ in hormone-dependent differentiation of mammary epithelial HC11 cells in culture.

Materials and methods

Animals

Eight to 10-week-old female virgin and midpregnant DDY mice were purchased from SLC (Shizuoka, Japan). Food and tap water were given ad libitum. The day of parturition was designated as day 0 of lactation. All animal experiments were carried out in accordance with protocols approved by the experimental animal ethics committee at Musashino University.

Immunohistochemistry

Mouse mammary glands were fixed in 10% formalin for 6 h and processed for paraffin embedding. Serial 5-μm-thick sections were cut and placed on glass slides. The

tissue sections were cleared in xylenes and rehydrated through a series of graded alcohol to distilled water. Then the sections were pretreated with unmasking solution (Vector H3300; Vector Laboratorie, Burlingame, CA, USA) in an autoclave at 121°C for 20 min. The sections were then incubated for 1 h in M.O.M. mouse IgG blocking solution (Vector M.O.M. Immunodetection kit BMK-2202; Vector Laboratories, Burlingame, CA, USA) and after washing with PBS, the sections were incubated overnight at 4°C with a diluted primary antibody. After washing with PBS, the sections were incubated for 20 min with diluted anti-mouse IgG-Alexa 546 (Molecular Probes, Eugene, OR, USA) and TO-PRO-3 Iodide (Molecular Probes, Eugene, OR, USA). The optimal dilution for the mouse monoclonal anti-ODC antibody HO101 (Matsufuji et al. 1984) was 1:200 dilution (100 μg/ml); for a mouse monoclonal anti-AZ antibody 2E9 (Matsufuji et al. 1990) was 1:30 dilution (170 μg/ml); for a mouse monoclonal anti-AIn antibody (Murakami et al. 1989) was 1:500 dilution (40 μg/ml). Alexa Fluor 546-conjugated anti-mouse IgG was at 1:300 dilution. TO-PRO-3 Iodide was at 1:300 dilution. After staining, dehydration, and clearing, the sections were mounted with Fluorescence Mounting Medium (DAKO Carpinteria, CA, USA). The images were acquired with the Olympus FV1000 Laser Microscope (Fluoview, FV1000, Olympus, Tokyo) using ×40 oil-immersion objective lens (UPLFLN 40× NA:1.30). Zoom: ×3.

Cell culture

HC11 cells were grown to confluency and maintained for 3 days in RPMI 1640 medium containing, 10% fetal bovine serum, 10 ng/ml epidermal growth factor and 5 μg/ml insulin (growth medium). The cells were washed and then incubated for 2 days in the same medium containing 10% fetal bovine serum, 5 μg/ml insulin, 1 μM dexamethasone, and 5 μg/ml ovine prolactin (National Hormone and Peptid Program, National Institute of Health) to induce differentiation (differentiation medium).

Small interfering RNA knockdown

A negative control siRNA was purchased from Qiagen. An AZ1 siRNA (siRNA ID #: 155715) was purchased from Ambion (Austin, TX, USA). The sequence of AIn siRNA was AAGAUCGUGAAGAAGCACAGU which was effective in inhibiting AIn expression (Mangold et al. 2008) and was purchased from Sigma. The siRNAs (50 nM) were introduced into cells using Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturer's reverse transfection protocol. Briefly, HC11 cells were washed and suspended in the growth medium to give 50% confluency within 24 h after plating. The cells were seeded into wells of six-well plate and siRNA/Lipofectamine RNAiMAX complexes in Opti-MEM were added. The cells were cultured for 72 h and then, siRNAs were again introduced into the cells using Lipofectamine RNAiMAX according to the manufacturer's forward transfection protocol. Seventy-two hours after the second transfection, the cells were washed and incubated in the differentiation medium for 2 days. When indicated, putrescine was added to the medium at the final concentration of 50 μM 6 h after the siRNA transfection.

Real-time PCR

cDNAs were synthesized using ExScript RT reagent kit (Takara, Otsu, Japan) or PrimeScript RT reagent kit. The real-time quantitative PCR were performed using SYBR Premix Ex Taq (Takara, Otsu, Japan) in a LightCycler (Roche Diagnostics). Results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Figs. 3, 4) or β-actin (Fig. 6) mRNA levels. The primers used were as follows: ODC, forward 5′-TGATGAAAGTCGCCA GAGCA-3′ and reverse 5′-TTTGCCCGTTCCAAGA GAAG-3′; AZ1, forward 5′-GAGCAACTCCAGGCTGAC CA-3′ and reverse 5′-GGTCTCACAATCTCAAAGCCA AGAA-3′; AIn, forward 5′-ATGTACATGCCCTGTCTG ATGCTC-3′ and reverse 5′-TGAAGCCTCCACCGATG TCTAAC-3′; β-casein, forward 5′-GGATGTGCTC CAGGCTAAAGTTC-3′ and reverse 5′-TGTTTTGTGGG ACGGGATTG-3′; AdoMetDC, forward 5′-CCCAAGATC TGAATGGGATG-3′ and reverse 5′-CCCTAGCAAGCT TCAACAGG-3′; SSAT, forward 5′-TTAAGATCCGT CCAGCCACT-3′ and reverse 5′-CTATGTCCTTCAGGG GTCCA-3′; AZ2, forward 5′-ACCTCACATCGTCCAC TTCC-3′ and reverse 5′-CCCTTCCTGAAGCAGATGAA-3′; Spermidine synthase, forward 5′-GTCCTACGGGA AGTGGTGAA-3′ and reverse 5′-CGGTGATGATGAC GTCAAAG-3′; GAPDH, forward 5′-AAATGGTGAAGG TCGGTGTG-3′ and reverse 5′-TGAAGGGGTCGTT GATGG-3′; β-actin, forward 5′-AGGCGACAGCAGTTG GTTG-3′ and reverse 5′-TTGGGAGGGTGAGGGACT TC-3′.

RT-PCR

Total RNA was isolated from mouse mammary gland and liver and HC11 cells using RNA-Bee RNA isolation reagent (TEL-TEST, Friendswood, TX, USA) and then treated with DNase using DNA-free kit (Ambion, Austin TX, USA). Reverse transcription was performed according to the instructions provided with the Takara RNA PCR Kit (AMV version 3.0, Takara, Otsu, Japan). The PCR amplification employed 25–40 cycles with steps at 94°C

for 2 min, 60°C for 0.5 min, and 72°C for 1 min with TaKaRa EX Taq HS. The sequence of primers for AZ 1 was forward 5′-ATGGTGAAATCCTCCCTGCA-3′ and reverse 5′-TGTGACAAACACAGCATTCA-3′.

Homogenization of mammary gland and preparation of tissue extract

The mammary gland was homogenized in three volume of cold 25 mM Tris buffer, pH 7.4, containing 0.01% Tween 80, 1 mM DTT and a protease inhibitor cocktail (Complete Mini, Roche). After centrifugation at 10,000*g* for 10 min, the crude supernatant was again centrifuged at 100,000*g* for 1 h at 4°C. The supernatant was used for assay of ODC and AZ activity and for Western blot and protein determination.

Determination of ODC and AZ activities

The activity of ODC and AZ was measured as described (Murakami et al. 2009). We found that ODC activity in the extract of mammary gland was occasionally underestimated because of the presence of heat-stable inhibitory substance(s). We corrected the observed ODC activity by estimating the inhibitory activity in tissue extract as follows: tissue extract was heated at 80°C for 10 min, which resulted in the complete loss of ODC activity but retention of the most of ODC inhibitory activity. After cooling, the inhibitor activity was determined by adding aliquots of the heated tissue extract to the reaction mixture containing 1.5 units of crude mouse kidney ODC, 0.0625 μCi of L-[1-^{14}C] ornithine, 0.4 mM L-ornithine, 40 μM pyridoxal phosphate, 5 mM DTT, 40 mM Tris/HCl, pH7.4 and 0.01% Tween 80 in a final volume of 125 μl. The kidney ODC activity was determined in the presence (a) and absence (b) of the heated tissue extracts and the value obtained by dividing (a) by (b) was used to correct ODC activity in mammary extracts.

Measurement of cellular polyamines

The mammary gland homogenates were chilled on ice and mixed with 0.1 volume of 1 M HCl, and then 1 volume of 0.5 M $HClO_4$. The mixture was centrifuged at 12,000*g* for 10 min. The concentrations of polyamines in the supernatant solution were assayed by mass spectrometric analysis (Samejima et al. 2007).

Western blot

The supernatant solution was incubated with *Staphylococcus aureus* Cowan 1 cells which had bound anti-mouse IgG, at 4°C for 2 h and then centrifuged. The supernatant was incubated again with *S. aureus* Cowan 1 cells, at 4°C for 1 h. After centrifugation, the supernatant (approximately 80 μg proteins) was electrophoresed on SDS-PAGE (11.5% gel) and transferred to a PVDF membrane. The membrane was incubated with monoclonal anti-rat AZ1 antibody, 3H1 (2 μg/ml) or 2E9 (2 μg/ml) (Matsufuji et al. 1990) overnight at 4°C and then, after washing, incubated with a peroxidase-conjugated secondary antibody (1:200,000) (Histofine, Nichirei, Japan) for 1 h. The proteins were visualized using the ECL Advance Western Blotting Detection kit (GE Healthcare) and analyzed on LAS-3000 (Fujifilm, Tokyo, Japan) using Multi Gage V3.0 software.

Gel filtration chromatography

Lactating mice (L2) were injected with 1 nmol of putrescine/g body wt. 2 and 4 h before killing to increase the level of AZ. Mammary gland and liver tissue extracts were fractionated by gel filtration as described previously (Murakami et al. 1985). Briefly, 0.5 ml of sample was applied to a column (7.8 mm × 300 mm) of TSKgel G3000SW (TOSOH, Japan), which had been equilibrated with 25 mM Tris/HCl buffer, pH 7.2, containing 0.01% Tween 80, 1.0 M NaCl and 1 mM DTT. The column was eluted with the same buffer and the eluate was collected in 0.9 ml fractions. The flow rate was approximately 30 ml/h.

Results and discussion

AZ in mouse mammary gland

We first examined expression of AZ1 by RT-PCR (Fig. 1a) and found that AZ1 mRNAs in the mammary gland and liver were similar in size. This was confirmed by Northern blot analysis (data not shown). Gel filtration analysis showed that the sizes of AZ protein in the mammary gland and liver were similar (Fig. 1b). Furthermore, Western blot analysis with monoclonal anti-rat AZ1 antibody, 2E9 and 3H1, showed that AZ1 in the mammary gland was approximately 25 kDa (Fig. 1c). These results differed from the earlier observation that the molecular weight of AZ was 55 kDa in the mammary gland of lactating rat and 27 kDa in the liver (Hu and Brosnan 1987). The reason for the observed species difference was unclear at present. In addition to AZ1, we found that AZ2 mRNA was also expressed in mouse mammary gland (see, Fig. 4).

ODC and AZ activity during mammary development

As shown in Fig. 2, ODC activity was low in the virgin stage but increased markedly during the latter half of pregnancy when mammary cells proliferate extensively.

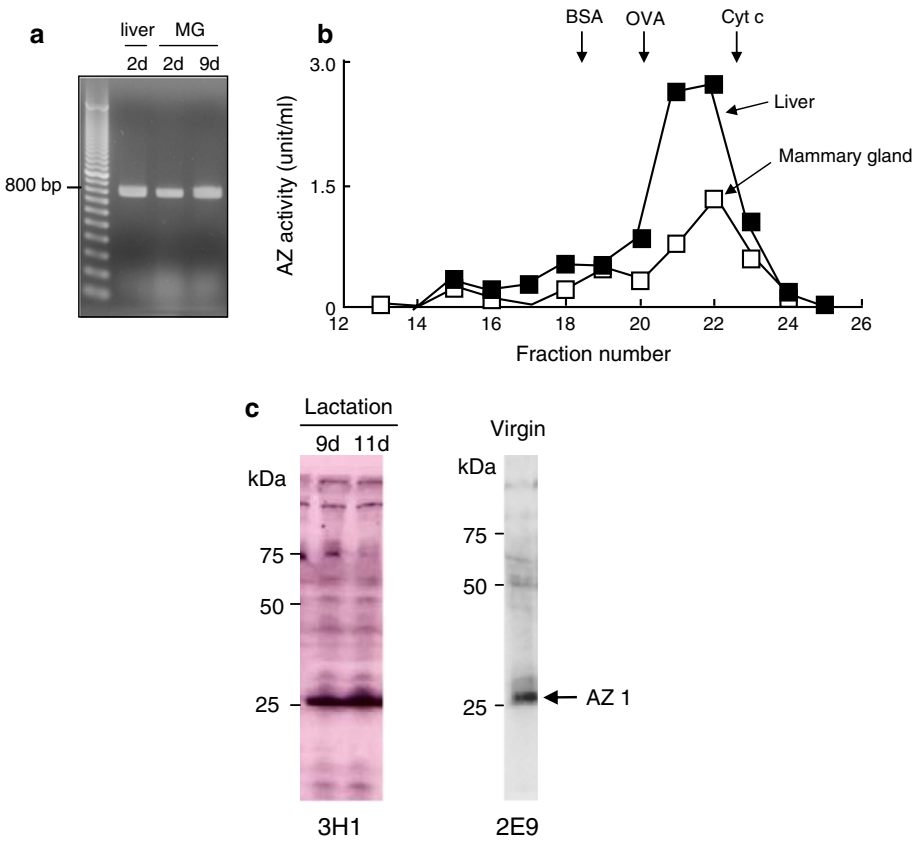

Fig. 1 AZ in mouse mammary gland**. a** RT-PCR analysis. Total RNA was isolated from the *liver* and the mammary tissue (*MG*) of day 2 and day 9 lactating mice. RT-PCR was performed on the total RNA, using AZ1-specific primers. **b** Gel filtration chromatography of AZ from mouse mammary gland and liver. Extract from mammary gland or liver was chromatographed on TSKgel G3000SW in the presence of 1 M NaCl. Fractions were assayed for AZ activity as described in the "Materials and methods". Molecular weight marker proteins were eluted under the same conditions: bovine serum albumin *BSA*, *M*r 67.5 kDa; ovalbumin *OVA*, *M*r 43 kDa; cytochrome *c Cyt c*, *M*r 12.4 kDa **c** Western blot analysis of *AZ*. Mammary gland extracts were prepared from virgin and day 9 and 11 lactating mice. Proteins were separated by SDS-PAGE and blotted to PVDF membranes. Blots were probed with monoclonal anti-rat *AZ1* antibody, *3H1* and *2E9*, as indicated

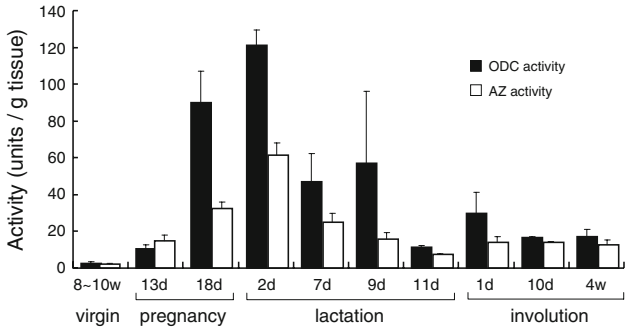

Fig. 2 Changes in *ODC* and *AZ* activity during mammary gland development. Mammary gland extracts were prepared from 8 to 10-week-old virgin mice, 13 and 18 day pregnant mice, 2, 7, 9, and 11 day lactating mice and 1, 10 day and 4 week (*w*) involuting mice. ODC and AZ activity were determined as described in the "Materials and methods". Values are the mean ± SE from three to six mice

ODC activity still remained at a high level during the early period of lactation but declined gradually to lower levels during the second half of lactation and involution. AZ activity showed the pattern of temporal change similar to that of ODC (Fig. 2). It was noted that the mammary gland contained the high level of AZ relative to ODC when compared to other tissues (Murakami et al. 1989), and the AZ/ODC ratio varied during development (Fig. 2).

β-Casein gene expression and the intracellular concentrations of polyamines in mouse mammary gland at various reproductive stages

Figure 3 showed the changes of expression of β-casein gene, a differentiation marker of the mammary gland and of the polyamine contents during the mammary gland development. In agreement with the previous report (Clarkson and Watson 2003), expression of β-casein gene began to increase during pregnancy, reached a peak during lactation and then declined to a low level during involution (Fig. 3a). The cellular level of putrescine was low in the virgin stage (Fig. 3b), but increased substantially during pregnancy following the increase of ODC activity (see

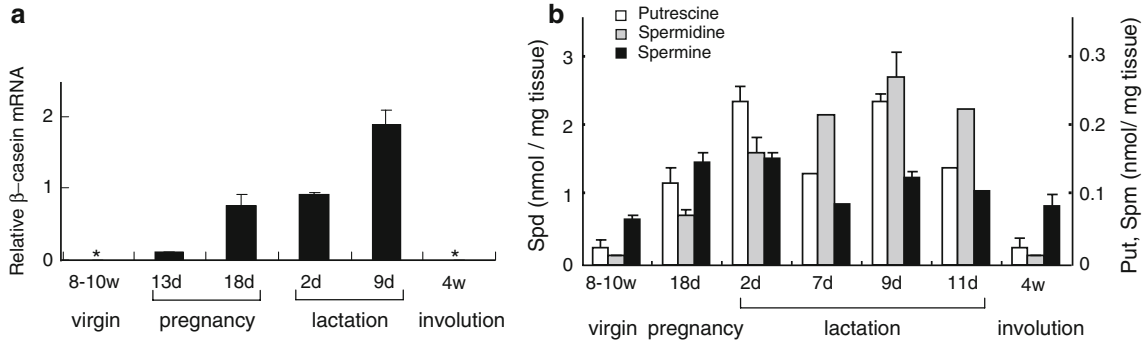

Fig. 3 β-Casein gene expression and intracellular polyamine concentrations during mammary gland development. **a** The level of β-casein mRNA. Total RNA was extracted from mammary glands at different developmental stages, as indicated. The levels of β-casein and GAPDH mRNAs were measured by real-time quantitative PCR analysis. The level of β-casein mRNA was normalized to that of GAPDH. *Undetectable level. Values are the mean ± SE from three mice. **b** Intracellular polyamine levels. The concentrations of putrescine (*Put*), spermidine (*Spd*) and spermine (*Spm*) were determined by mass spectrometric analysis as described in the "Materials and methods". Values are the mean ± SE from three mice except lactation day 7 and 11 ($n = 1$)

Fig. 2). It reached a peak during early lactation and remained relatively high until involution. In comparison with other tissues (Nishimura et al. 2006), the concentration of putrescine in the mammary gland was generally high. The level of spermidine also started to increase during pregnancy, and it continued to increase until mid-lactation. This pattern of change was similar to that of β-casein. As reported previously (Russell and Mcvicker 1972), spermidine concentration in the mammary gland was very high (about 2 mM) during lactation and the spermidine/spermine ratio was also increased, especially during the mid-lactation period. Both putrescine and spermidine returned to low levels during the regression period. Spermine changed roughly in parallel with putrescine except during the period of involution, when the level of spermine remained high. These data are in line with the view that both putrescine and spermidine are important for both mammary cell proliferations during pregnancy and functional differentiation during lactation when milk components including β-casein are massively synthesized.

Developmental changes in expression of genes related to polyamine metabolism in the mammary gland

Using real-time quantitative PCR analysis, we found that the level of ODC mRNA and spermidine synthase mRNA increased substantially during late pregnancy and lactation (Fig. 4). The temporal change of ODC mRNA expression was in parallel with that of ODC activity (Fig. 2), suggesting the transcriptional regulation of ODC gene expression. The up-regulation of ODC and spermidine synthase appeared to begin earlier than that of β-casein expression (Fig. 3a). On the other hand, AIn expression was low in the virgin stage and still remained at low levels during pregnancy when mammary epithelial cells actively proliferate. The level of AIn mRNA increased higher during lactation when milk proteins including β-casein are actively synthesized. On the other hand, the level of AZ1 mRNA did not change significantly during those reproductive stages, which was true in the case of other tissues expressing AZ1 constitutively (Ivanov et al. 2000a). Expression of SSAT, a key enzyme in polyamine catabolism, was relatively high in virgin and involuting glands and low in pregnant and lactating glands (Fig. 4). The developmental change of SSAT gene expression was inversely related with that of polyamine concentrations in the gland (cf. Fig. 3b), suggesting that polyamine catabolism was another key step in regulation of polyamine levels in the gland. Interestingly, the change in expression of AZ2 mRNA resembled that of SSAT mRNA, suggesting its regulatory role in polyamine metabolism in mammary development. AZ2 was shown to inhibit polyamine transport and promote the intracellular degradation of ODC (Zhu et al. 1999; Murai et al. 2009). We also found that overexpression of AZ2 decreased the intracellular level of polyamines (unpublished). The activity of AdoMetDC, a key enzyme in the biosynthesis of spermidine and spermine in the gland, has been reported to increase dramatically after parturition, remain elevated until the initiation of involution, and then rapidly decline (Russell and McVicker 1972). However, we found that the level of AdoMetDC mRNA was high during the virgin stage but decreased during pregnancy and lactation (Fig. 4). These data when combined together suggested that expression of genes related to polyamine metabolism including the ones for ODC, spermidine synthase, AIn, SSAT, AZ2, and AdoMetDC were regulated, at least in part, at the level of transcription during mammary gland development.

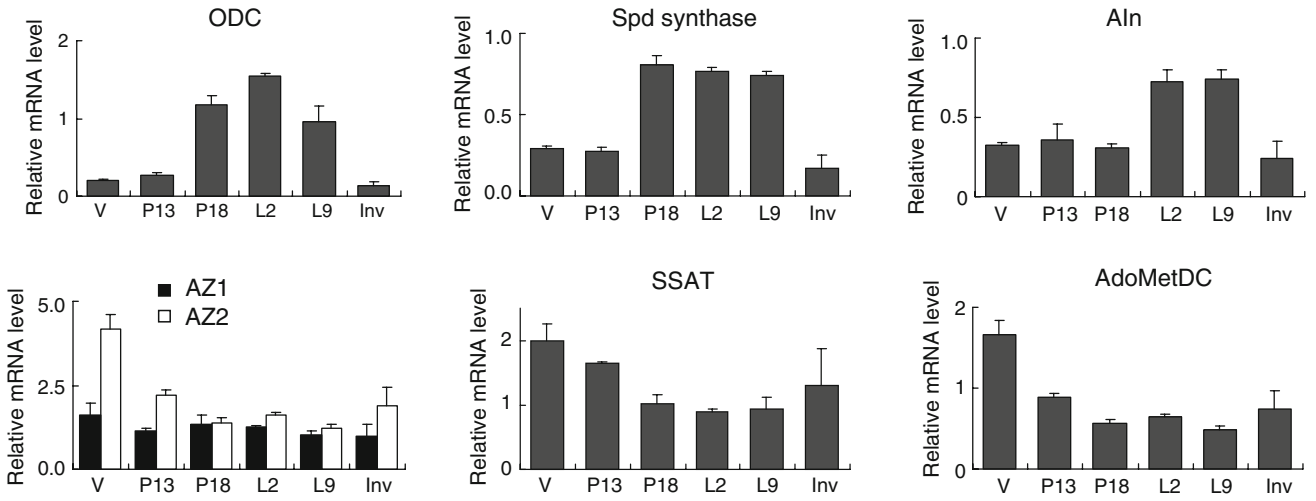

Fig. 4 Developmental changes of expression of genes related to polyamine metabolism in mammary gland. Total RNA was isolated from mammary tissue of mice at indicated stages and analyzed by real-time quantitative PCR. The stages were 8 to 10-week-old virgin *V*; pregnancy day 13 *P13*; pregnancy day 18 *P18*; lactation day 2 *L2*; lactation day 9 *L9* and involution 4 week, Inv. The expression level of each gene was normalized to that of GAPDH. Values are the mean ± SE from three mice

Subcellular localization of ODC, AZ, and AIn during the mammary gland development

The intracellular localization of ODC, AZ, and AIn was examined by immunohistochemical analysis. ODC could not be detected in virgin mammary gland possibly due to its low level. However, it was present mainly in the perinuclear region at around midgestation (P13) (not shown), and then translocated mostly to the nucleus during late pregnancy (P18) (Fig. 5a). On lactation day 2 (Fig. 5a) and day 9 (not shown), it was localized predominantly in the cytoplasm. In contrast, AZ was present predominantly in the nucleus during virgin, pregnant (P13 and P18), and early lactating (L2) periods (Fig. 5b). As shown in Fig. 5c, the subcellular localization of AIn also changed during the gland development. AIn was found in the cytoplasm and nuclear periphery on pregnancy day 13 (P13). During the late period of pregnancy (P18), AIn was present in both the nucleus and cytoplasm. During the early period of lactation (L2), AIn was present in the nucleus in some, but not all tissues examined (Fig. 5c). The changes in the subcellular localization of ODC, AZ, and AIn may reflect alterations in their interplay and function during mammary development.

The immunochemical studies together with the data in Fig. 2 indicated that ODC was present as an active form in the cytoplasm during the period from midgestation to early lactation, when the increase in the intracellular concentration of polyamines occurred to support both extensive cell proliferation and milk protein synthesis. During late pregnancy, the total level of ODC was high (Fig. 2) but the enzyme was likely to be present both as an inactive ODC–AZ complex in the nucleus and as active ODC form in the nucleus and cytoplasm. Similarly, AIn appeared to exist both as a stable AIn–AZ complex in the nucleus and as free unstable form in the cytoplasm during late pregnancy. Thereafter, AIn, although not all, remained in the nucleus during early lactation. High expression of AIn during lactation suggested its possible role in regulation of β-casein synthesis (see Figs. 3a, 4).

Effects of reducing AIn and AZ expression on the lactogenic response of mouse mammary epithelial HC11 cells

When HC11 mouse mammary epithelial cells grow to confluency, they can express differentiation function by synthesizing β-casein in response to lactogenic hormones (Ball et al. 1988). They also exhibit a unique phenotypic appearance as clusters of enlarged cells forming dome-like (mammospheres) or blister-like structures (Ball et al. 1988; Humphreys and Rosen 1997). We used this cell line to investigate whether reduction of AIn affected mammary cell differentiation. As shown in Fig. 6a and b, transfection of HC11 cells with AIn siRNA decreased AIn mRNA level by 60% as compared to control cells and markedly inhibited expression of β-casein mRNA. In agreement with the previous study (Choi et al. 2005), we found that AIn siRNA decreased the intracellular level of putrescine and spermidine by 65 and 25%, respectively. In order to determine whether inhibition of β-casein gene expression by AIn siRNA was secondary to the decrease in polyamine levels, we examined the effect of addition of putrescine on the level of β-casein mRNA in AIn siRNA-transfected cells. As shown in Fig. 6b, putrescine did not reverse the

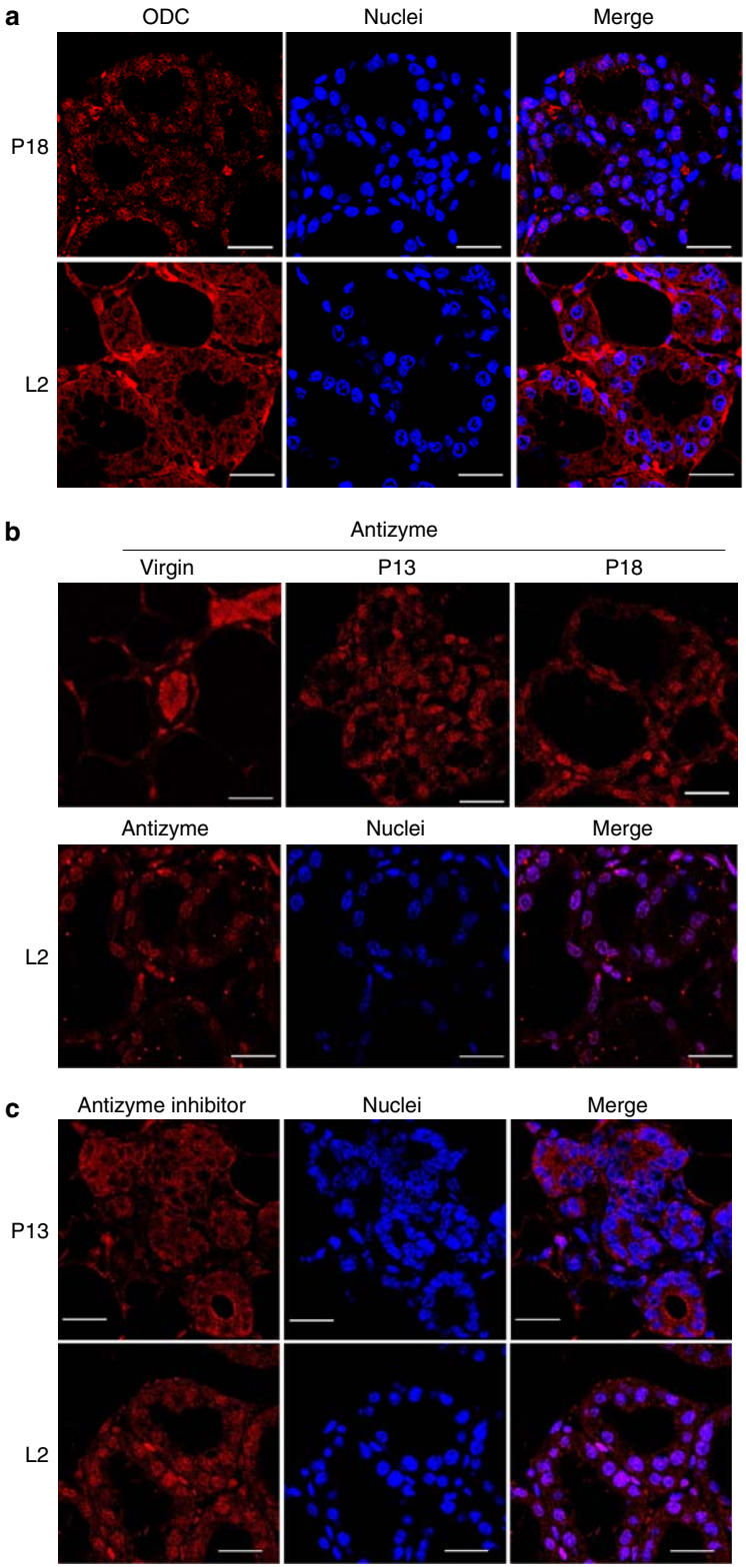

Fig. 5 Subcellular localization of *ODC*, *AZ*, and *AIn* in the mammary gland. Glands were from mice at the following stages: virgin week 8 *V*; pregnancy day 13 *P13*; pregnancy day 18 *P18*; lactation day 2 *L2*. *Nuclei* were stained with TO-PRO-3 (*blue*) whereas ODC, AZ and AIn were stained with each monoclonal antibody (*red*) as described in "Materials and methods": **a** *ODC*, **b** *AZ*, **c** *AIn*. *Bar* 20 μm. Tissue sections from at least three mice were analyzed

inhibitory effect of AIn siRNA, suggesting that the decrease in putrescine concentration was not the cause for the reduced expression of β-casein gene. These results were consistent with our observation that hormonal stimulation of β-casein gene expression in HC11 cells was little affected by depletion of putrescine and spermidine by treatment with DFMO, an irreversible inhibitor of ODC (data not shown). The results were also in accord with the

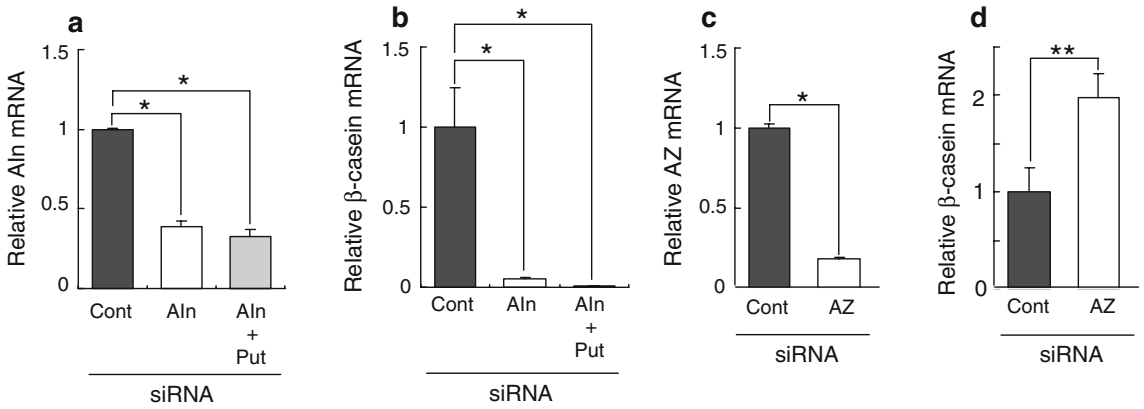

Fig. 6 Effects of reducing AIn and AZ1 expression on hormonal induction of β-casein mRNA in mouse mammary epithelial HC11cells. Cells were transfected with control siRNA, AIn siRNA or AZ1 siRNA (50 nM) and cultured in growth medium for 5 days. Cells were then induced to differentiate by culturing in the presence of dexamethasone, insulin, and prolactin for 48 h. When indicated, putrescine (*Put* 50 μM) was added to the medium at the beginning of culture and present throughout. Total cellular RNA was isolated and the levels of *β-casein*, *β-actin* and *AIn* or *AZ* mRNA were measured by real-time quantitative PCR analysis. The expression level of each gene was normalized to that of β-actin. Effect of AIn depletion on (**a**) expression level of AIn and (**b**) expression level of β-casein; effect of AZ 1 depletion on (**c**) expression level of AZ1 and (**d**) expression level of β-casein. Values are the mean ± SE from three cell culture experiments. $^{*}P < 0.0004$; $^{**}P = 0.064$

earlier observation that DFMO did not suppress the stimulatory effect of prolactin on β-casein synthesis (Rillema and Cameron 1983).

We found that the depletion of AIn also inhibited the morphological changes of HC11 cells induced by lactogenic hormones. Thus, formation of dome-like structure was reduced among AIn siRNA-transfected cells when compared with control siRNA-transfected cells. In addition, putrescine did not counteract the inhibitory effect of AIn siRNA on morphological differentiation (data not shown).

AZ siRNA reduced the level of AZ mRNA almost 80% and increased the level of β-casein mRNA approximately twofold (Fig. 6c, d). This was in contrast to the inhibitory effect of AIn siRNA. We found, however, that the extent of stimulation by AZ siRNA varied substantially among cultures, although its inhibitory effect on AZ mRNA expression was rather constant. These results clearly reflect the fact that control of β-casein gene expression is complex, and can be influenced by multiple factors including hormones and their receptors.

In the present study, we analyzed the temporal pattern of expression of ODC, AZ, AIn, and other genes related to polyamine metabolism, the cellular level of polyamines, and expression of β-casein mRNA during mammary gland development. Our findings indicated that ODC activity was increased during pregnancy and early lactation, when mammary epithelial cells proliferate extensively (Fig. 2). We also found that both AIn mRNA and spermidine increased to higher levels during lactation, when β-casein gene expression is augmented (Figs. 3, 4). Immunohistochemical studies showed the changes in the subcellular localization of ODC and AIn and the continuous presence of AZ in the nucleus during mammary development (Fig. 5). The observed patterns of localization suggested that ODC plays a role in polyamine synthesis both in the nucleus and cytoplasm during late pregnancy and that AIn protects ODC by preventing its AZ-dependent degradation in the nucleus. In addition, it was tempting to speculate that AIn protects some protein(s) associated with lactogenesis from AZ during development, since AIn depletion markedly inhibited β-casein mRNA expression (Fig. 6b). Recently, several new target proteins of AZ have been reported including cyclin D1 and aurora A (Newman et al. 2004; Kim et al. 2006; Lim and Gopalan 2007; Gruendler et al. 2001) but their involvement in control of milk protein synthesis is not clear at present. Future work is required to identify the target molecule(s) of AZ1 in lactating mammary cells.

Previous studies showed that AZ moves between the nucleus and the cytoplasm, suggesting that it is involved in the nucleocytoplasmic shuttling of ODC and its degradation in the nucleus (Gritli-Linde et al. 2001; Murai et al. 2003; Schipper et al. 2004). Moreover, two nuclear export signal (NES) sequences of AZ1 have been identified (Murai et al. 2003). Gritli-Linde et al. systematically analyzed the expression of ODC and AZ in cells of developing mouse embryo. They found that in certain cell types, AZ protein translocates to the nucleus in a developmentally regulated manner and a proteasome inhibitor caused ODC accumulation in the nucleus and suggested that the nuclear translocation of AZ was linked to proteasome-mediated ODC degradation in the nucleus. In the present study, however, we found by immunohistochemical analysis that not only ODC and AZ but also AIn was present in the nucleus of mammary gland during late pregnancy. This

pattern of localization, together with high-ODC activity during late pregnancy (Fig. 2) suggested the complex interplay of ODC, AZ, and AIn during the mammary development. In the recent study, we found that AIn and AZ were colocalized during mitosis but they were separately present during interphase and that the half-life of AIn increased in mitotic cells as compared with that in cells during interphase, suggesting that their functions vary depending on the cell cycle phase (Murakami et al. 2009).

In conclusion, we demonstrated in this study that expression and intracellular localization of ODC, AZ, and AIn are developmentally regulated in the mammary gland wherein they play pivotal roles in control of cell growth and functional differentiation. These findings will form the basis for elucidating the regulatory mechanisms involved in their expression, translocation, and functional interplay at the molecular level.

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