Targeted Inhibition of Osteopontin Expression in the Mammary Gland Causes Abnormal Morphogenesis and Lactation Deficiency*

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Osteopontin (OPN) is a sialic acid-rich, adhesive, extracellular matrix (ECM) protein with Arg-Gly-Asp cell-binding sequence that interacts with several integrins, including αvβ3. Since the ECM is a key regulator of mammary gland morphogenesis, and mammary epithelial cells express OPN at elevated levels, we sought to determine whether this protein plays a role in the postnatal mammary gland development. By generating transgenic mice that express OPN antisense-RNA (AS-OPN mice) in the mammary epithelia we achieved suppression of OPN production in this organ. The pregnant AS-OPN mice displayed a lack of mammary alveolar structures, a drastic reduction in the synthesis of β-casein, whey acidic milk protein, and lactation deficiency. In agreement with these findings, we uncovered that a mammary cell line, NMuMG, which undergoes both structural and functional differentiation on ECM-coated plates, when transfected with an antisense OPN-cDNA construct, failed to undergo such differentiation. Furthermore, the results of gel-invasion assays demonstrated that these cells manifest elevated matrix metalloproteinase (MMP) activity when OPN expression is significantly reduced. The identity of this proteinase as MMP-2 is confirmed by Western blotting, zymography, and inhibition of its activity by a specific inhibitor, TIMP-2. Taken together, our results demonstrate, for the first time, an essential role of OPN in mammary gland differentiation and that the molecular mechanism(s) of its action, at least in part, involves down-regulation of MMP-2.

Osteopontin (OPN)1 is a secreted, sialic acid-rich, adhesive, glycoprophoprotein, that contains the arginine-glycine-aspartic acid (RGD) cell-binding sequence found in many extracellular matrix (ECM) proteins. Molecular cloning and characterization of a cDNA encoding rat OPN revealed that this protein is composed of 317 amino acid residues, with a predicted molecular mass of approximately 32 kDa for the peptide backbone (reviewed in Ref. 1). However, OPNs isolated from or secreted by various tissues exhibit electrophoretic mobilities consistent with a protein of apparent molecular mass between 44 and 75 kDa (reviewed in Ref. 2). This variability is most likely due to the post-translational modifications (3). The murine OPN is encoded by a single copy gene (4) that is composed of seven exons and spans more than 8 kb of nucleotide sequence (5). It maps to the ric (Rickettsia resistance gene) locus on mouse chromosome 5, and a possible allelism between the opn and ric has been reported (6). In several cell types, OPN interacts with specific integrins, namely αvβ3, αvβ6, and αvβ1, and also with the CD44 receptor in RGD-dependent manner (reviewed in Ref. 7). The OPN-integrin interaction mediates important cell functions, most of which are RGD-dependent, including the promotion of cell adhesion and spreading on substrata, chemotactic and haptotactic activities, and cellular signaling (8–13). Furthermore, the expression of OPN-mRNA and protein is suppressed in cells derived from mice generated by targeted disruption of the protooncogene, src (14). It has also been reported that src regulation of OPN production is due to the presence of a src response element in the promoter region of the OPN gene (15). Although OPN is expressed in several organs, the epithelium of the mammary gland overexpresses this protein specifically during pregnancy and lactation, and is abundantly present in milk (16). It has now been well established that ECM at the stromal-epithelial interface plays a pivotal role in growth, survival, and differentiation of mammary epithelial cells, and unlike most tissues and organs, a major part of the mammary gland development occurs post-natally (17). Since OPN is an ECM protein, and mammary epithelial cells express this protein at elevated levels (18), we sought to determine whether this protein plays a role in the post-natal development of the mammary gland, including ductal branching, lobuloalveolar structure formation, and lactation. We rationalized that a lack of or significantly reduced levels of OPN synthesis may impair such processes. To test this hypothesis, we generated transgenic mice expressing OPN antisense RNA, specifically in the mammary epithelia, under the regulation of MMTV-LTR promoter/enhancer. This promoter was chosen because it directs mammary tissue-specific expression of transgenes at the onset of puberty without exogenous hormone supplement (19). We show here that suppression of OPN synthesis caused a virtual absence of lobuloalveolar structures, drastic reduction of β-casein and whey acidic protein (WAP) synthesis in mammary epithelia, and lactation deficiency. Similarly, in in vitro exper-
ments, a mammary epithelial cell line, NMuMG, that under-
goes structural and functional differentiation in culture, in-
cluding the formation of lobuloalveolar structures and
synthesis of milk proteins, failed to do so when they were
transfected with an OPN antisense cDNA construct. Fur-
thermore, these transfected cells showed enhanced gel
invasion, and synthesized elevated levels of metalloproteinase-2 (MMP-
2). Taken together, these results define an essential role
of OPN in mammary gland development and differentiation,
and raise the possibility that the molecular mechanism(s) of OPN
action, at least in part, involves the down-regulation of MMP-2.

EXPERIMENTAL PROCEDURES

Production and Characterization of Transgenic Mice—Mice were
housed under 10-h dark and 14-h light cycles, and were handled
in accordance with approved protocols. To generate the transgene
construct, a full-length murine OPN cDNA (20) was ligated in the anti-
sense orientation into SalI site of the eukaryotic expression vector
pMAmneo (CLONTECH). The linearized 7.4-kb NdeI-Apal (AS-OPN)
fragment was injected into single-cell embryos of B6XSJL mice, which
were then introduced into the oviducts of pseudopregnant CD1 mice
(DNX Inc.). Transgenic mice were identified by Southern blot analysis of
BamHI-digested genomic DNA from tail biopsy using mouse OPN
cDNA, gene-specific primers (Fig 1B, bottom), and for instances, mice were also genotyped by PCR amplification
using murine OPN cDNA-specific primers (MOP-L, 5'-TCA CCA TTC GGA TGA GTC 3’; MOP-R, 5’-ACT TGT GGC TCT GAT GTT CC-3’).

Whole-mount and Histological Analysis of the Mammary Glands—
For whole-mount analysis (21), the second inguinal mammary glands
were removed, kept flat in tissue cassettes, and fixed overnight in 4%
buffered paraformaldehyde at 4 °C. The tissues were then dehydrated,
delipidated, stained with iron hematoxylin, cleared in xylene, and
photographed. For histological analyses, 5–7-μm sections of paraffin
embedded tissues were stained with hematoxylin/eosin and photographed.

RNA Extraction and Analysis—Total RNA was purified from freshly
isolated mouse tissues and from actively growing cultured cells as
described (22). RNA (10 μg/ml) was electrophoresed on a 1.2% agarose-
formaldehyde gels, transferred to nylon membranes, and hybridized to
32P-labeled sense or antisense OPN riboprobes transcribed from
the pGEM4–2ar plasmid (20) using T7/SP6 RNA labeling kit following the
description (22). RNA (10 μg/ml) was applied to the Hybond-N+
membrane from Engelbreth-Holm-Swarm tumors) was obtained from
Collaborative Biomedical Products and used to coat 35-mm culture
plates at 20 μl/dish. Matrigel was allowed to gel by incubation for 1 h at
37 °C. The cells were then transfected (5.5 × 105 cells/ml) in DMEM
containing 10% fetal calf serum, 3 μg/ml bovine prolactin (Sigma), 5
μg/ml insulin (Sigma), 1 μg/ml hydrocortisone (Sigma), and hepatocyte
growth factor at concentration of 20 ng/ml. Cells were grown for 6 days
to allow morphological differentiation to occur.

Reverse Transcription PCR—For the analysis of expression of milk
proteins, an RT-PCR kit from Promega was used. Ten μg of total RNA
was used for each reaction. For β-casein, the primers used were: CAGT-
GAGGAAATCGTTGAAA (forward primer) and GGTTTTGACGACAGA-
CATATC (reverse primer). For WAP, the primers used were: 5’-TAG
CAG CAG ATT GAA ACC ATT AGT-3’ (RT primer) and 5’-GACC
GGT ACC ATG CTT G-3’ (PCR primer). The first strand cDNA syn-
thesis was carried out with avian myeloblastosis virus RT (avian mam-
malian tumor virus reverse transcriptase) at 48 °C for 40 min. Avian
myeloblastosis virus RT inactivation was carried out by heat denatur-
ation at 94 °C for 2 min. Amplification was carried out in 40 PCR cycles
consisting of a denaturation step of 30 s at 94 °C, an annealing step of
1 min at 60 °C, and an extension step of 2 min at 68 °C.

Gel Invasion Assay—For gel invasion assay, invasion chambers con-
taining inserts coated with Matrigel (BectoClone) were used as suggested
by the manufacturer (Becton Dickinson). Cells were serum-starved for
4 h, harvested by trypsination, and plated in the upper chambers at a
density of 3.5 × 104 cells/well, in serum-free medium. Growth medium
containing 10% fetal bovine serum was placed in the lower chamber
to serve as a chemottractant. After 16 h of incubation at 37 °C, the cells
attached to the upper surface of the membrane along with the layer of
Matrigel were removed using a cotton-tipped swab. The cells that migrated through the filter and attached to the lower surface of
the membrane were fixed with 10% methanol, stained with Giemsa, and
counted under a phase contrast microscope. For invasion assays in
presence of the MMP-2 inhibitor TIMP-2, the upper surface of the
membranes of the Transwell inserts (Costar) were coated with 10 μl /
cm2 Matrigel containing 10 μg/ml human TIMP-2 (Calbiochem). After
hardening of the Matrigel layer at 37 °C, the cells were plated and the
membranes were washed twice as described above.

Zymography—For establishing the proteolytic profiles of NMuMG
cells by zymography (24), cell lysates were prepared in substrate gel
sample buffer (0.1% SDS, 4% sucrose, 0.25 mM Tris-HCl, pH 6.8, and 0.1%
bromphenol blue), cleared by centrifugation, and electrophoresed in
10% SDS-PAGE gels containing 1 mg/ml gelatin. After electrophoresis
the gels were soaked twice for 30 min each at room temperature in 2.5% Triton X-100, with gentle shaking. The gels were rinsed in distilled
water and incubated overnight at 37 °C in substrate buffer (50 mM
Tris-HCl, pH 8.0, 5 mM CaCl2, and 0.02% NaN3). After incubation, gels
were stained with 0.5% Coomassie Blue, destained with water, and
photographed. The areas of gelatinolytic activity appeared as light
translucent bands over a blue background.

RESULTS

Generation of AS-OPN Mice—The construct used for the generation
of AS-OPN transgenic mice contained full-length OPN cDNA in the antisense orientation downstream from the MMTV-LTR promoter of the eukaryotic expression vector pM-
Aveo (Fig 1A). The basic transcription and biological activity of this transgene con-
struct was first tested by transfecting the murine cell lines MC3T3E1 (25) and JB6 (26). Transfected cells expressed OPN antisense RNA which efficiently suppressed OPN-protein syn-
thesis (Ref. 27; data not shown). This construct was, therefore, used to
generate transgenic mice. Six founder mice were ob-
tained, two of which were used to establish two independent
transgenic mouse lines. Southern blot and PCR analyses con-
firmed the genomic integration and germline transmission of the transgene to the progeny of both founders. Southern blot

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mM KCl, 10 mM Hepes, pH 7.5, 5% glycerol, 10 mM EDTA, 1% Triton
pulverized, and homogenized in five volumes of extraction buffer (100
analysis, mammary glands were first flash-frozen in liquid nitrogen,
analysis of BamHI-digested genomic DNA of one line of AS-OPN transgenic mice using OPN cDNA and neomycin gene probes showed the presence of 1.3- and 2.6-kb bands, respectively (Fig. 1, C and B). The Southern hybridization signal patterns were found to be different for the two lines when genomic DNA from both were digested with ClalI and hybridized with the same OPN cDNA probe (data not shown), confirming different integration site of the transgene in the two mouse lines.

Expression of OPN Antisense RNA in the Mammary Glands of AS-OPN Mice—To detect transgene expression, total RNA isolated from mammary glands of 65-day-old virgin mice was subjected to RT-PCR analysis. Fig. 2A shows that the OPN antisense RNA is expressed in the mammary glands of transgenic, but not in those of the normal control mice, although OPN-mRNA is expressed in both normal and transgenic mice. To determine whether the expression of the transgene is more pronounced in mammary tissue due to the presence of MMTV-LTR promoter, total RNA was isolated from several organs including the mammary gland, kidney, spleen, and liver of the transgenic mice and analyzed by Northern blotting, using OPN antisense RNA probe. The results showed that the OPN antisense RNA expression is significantly higher in the mammary gland, compared with that of other tissues (Fig. 2B and data not shown). To examine transgene expression in different stages of mammary gland development, RNA was extracted from glands of virgin and pregnant AS-OPN mice at early and late (17 days) gestation, and Northern blot analyses were performed. As shown in Fig. 2C, OPN antisense-RNA is expressed in the mammary glands of virgin transgenic mice, and its expression is increased during pregnancy, consistent with previous reports on the pattern of MMTV-LTR promoter-driven transgene expression in the mammary gland (28). These data confirm the coexpression of endogenous OPN gene and transgene in the mammary glands of AS-OPN mice. To determine whether OPN-protein production is suppressed as a consequence of the expression of OPN antisense RNA in transgenic mice, mammary glands from both 65-day-old virgin normal and AS-OPN mice were homogenized, and protein extracts were analyzed by Western blotting using anti-OPN serum (OST-1). 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died after birth revealed the absence of milk in their stomachs. This indicated that death of pups occurred due to starvation, and suggested that either most of the AS-OPN mothers were lactation-deficient or the pups were incapable of suckling. To examine these possibilities, pups born to AS-OPN mice, whose previous litters had died after birth, were allowed to be nursed by actively lactating foster mothers. Eighty-eight percent of these pups survived and grew normally (Table I). These results clearly show that all AS-OPN mothers have lactation deficiency and a vast majority of them are clearly non-lactating.

Abnormal Development of Mammary Gland of AS-OPN Mice—To determine the possible cause of the apparent lactation deficiency in AS-OPN mice, we examined the mammary glands of post-partum animals morphologically and biochemically. The mammary glands of non-lactating AS-OPN mice were 2–5 times smaller in size than those of normal lactating mice (data not shown). Whole mount analysis of these glands showed that, although some alveolar lobules were formed (Fig. 3C), they were greatly reduced in size and number as compared with those of the lactating non-transgenic mice (Fig. 3A). Fig. 3B shows histological sections of the normal gland where the alveolar lobules almost completely filled the fat pad, leaving little stromal space. In addition, they were also arranged in well organized, tightly packed structures with large luminal spaces (Fig. 3B). In contrast, the mammary glands of non-lactating AS-OPN mice revealed only few alveolar structures, sparsely distributed in individual patches throughout the fat pad, some in collapsing state, and with little or no luminal spaces (Fig. 3D). In order to determine whether mammary glands of AS-OPN mice are capable of synthesizing milk proteins, we examined the expression of β-casein and WAP mRNAs by RT-PCR using total RNA from the mammary tissues of the AS-OPN mice (Fig. 5). When non-transfected, mock-transfected, AS6, and AS8 cells were cultured on Matrigel-coated dishes in presence of hormone, non-transfected and mock-transfected NMuMG cells formed compact spherical structures (Fig. 5, A and B). When hepatocyte growth factor was added to the cultures, many of these spheroids or tubular structures also contained a lumen, resembling the alveoli of the mammary gland (Fig. 5E). However, AS-OPN-transfected AS6 and AS8 cells failed to form such spheroids or branching tubules (Fig. 5E). Histological analysis showed that many of these spheroids or tubular structures also contained a lumen, resembling the alveoli of the mammary gland (Fig. 5F). Therefore, an in vitro system, using mammary epithelial cells, offers a unique opportunity to further investigate the effects of down-regulation of OPN synthesis in these cells on their structural and functional differentiation. In the present study, we used a normal murine mammary epithelial cell line, NMuMG, that differentiate in vitro. NMuMG cells were transfected with the same construct used to generate the AS-OPN mice. The mock-transfected (vector only) and non-transfected cells served as controls. Several stably transfected clones were isolated, and two of these clones, AS6 and AS8, expressing the lowest levels of OPN were characterized and chosen for further analysis. As shown in Fig. 4A, both AS6 and AS8 clones stably incorporated the antisense OPN cDNA, which is absent in non-transfected and mock-transfected cells. RT-PCR analysis showed that OPN antisense RNA is expressed by both of these clones (Fig. 4B). As a result, there was a drastic reduction in the levels of OPN protein, as compared with non-transfected and mock-transfected NMuMG cells (Fig. 4C). These observations show that expression of antisense OPN-RNA effectively down-regulates the synthesis of OPN in antisense-transfected NMuMG cells.

When non-transfected, mock-transfected, AS6, and AS8 cells were cultured on Matrigel-coated dishes in presence of hormones, non-transfected and mock-transfected NMuMG cells formed compact spherical structures (Fig. 5, A and B). When hepatocyte growth factor was added to the cultures, many of those spheroids formed long branching tubules (Fig. 5E). Histological analysis showed that many of these spheroids or tubular structures also contained a lumen, resembling the alveoli of the mammary gland (Fig. 5F). However, AS-OPN-transfected AS6 and AS8 cells failed to form such spheroids or branching tubules and remained as monolayer cultures (Fig. 5, C and D). Thus, NMuMG cells cultured on Matrigel-coated dishes undergo morphogenesis, including the formation of structures typical of mammary glands in vivo, but their counterparts, expressing antisense OPN RNA and showing drastically reduced OPN production, failed to undergo such differentiation. Similar results were also obtained using branching morphogenesis assays in collagen gels (data not shown).

Functional differentiation of NMuMG cells in culture was evaluated by their capacity to synthesize major milk proteins.

### Table I

| Pups nursed by | Born | Cross-fostered | Pups survived | Survival |
|----------------|------|----------------|---------------|----------|
| AS-OPN mice   | 184 (21) | 0 | 20<sup>a</sup> | ~12 |
| Pups nursed by control mice | 67 (8) | 58 | 51 | ~88 |

<sup>a</sup> The pups were counted on the morning of delivery. The numbers in parentheses indicate the number of litters.

<sup>b</sup> On the morning of delivery, pups born to non-lactating mothers were transferred to actively lactating mothers. Not all pups born were transferred; those that appeared too weak were not.

<sup>c</sup> The number of surviving pups was determined 2–4 days after birth.

<sup>d</sup> 20 surviving pups were born to 8 AS-OPN mice; the remaining 13 out of 21 were totally non-lactating.

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**Fig. 3.** Morphological and functional characterization of post-partum mammary glands of AS-OPN mice. Second inguinal mammary glands were removed from animals 24 h after parturition, then analyzed as whole mounts (A and C) or upon sectioning (B and D). A and B represent mammary glands of normal, lactating mice; C and D, mammary glands of AS-OPN mice. Note the presence of large alveolar structures in the mammary gland of normal mouse, and their absence in the transgenic mouse. Scale bar = 300 μm for A and C and 100 μm for B and D. E, expression of β-casein (CAS) and WAP in mammary glands of 17-day pregnant normal (N) and transgenic (T) mice.
It has been reported that synthesis of milk proteins such as β-casein and WAP can be induced in mammary epithelial cells in culture (29). Therefore, we examined by RT-PCR whether AS6 and AS8 cells, grown on Matrigel-coated dishes in presence of appropriate hormones, have the ability to express these milk proteins. Non-transfected and mock-transfected NMuMG cells were used as controls. As shown in Fig. 6, while non-transfected and mock-transfected control cells expressed significant amounts of both β-casein and WAP mRNAs, the AS6 and AS8 cells did not. These in vitro data, therefore, support our observations in vivo that mammary glands of AS-OPN mice, expressing significantly reduced levels of OPN, fail to form alveolar structures and do not synthesize β-casein and WAP mRNAs.

Increased Proteolytic Activity and Invasiveness of Antisense OPN cDNA-transfected NMuMG Cells—It has been well established that ECM at the stromal-epithelial interface is a key regulator of the structural and functional differentiation of the mammary epithelium (16). Extracellular matrix remodeling of the basement membrane by ECM-degrading metalloproteinases are implicated as major determinants for the loss of mammary epithelial function during involution (21). It has been shown that the mammary gland expresses several matrix proteinases and their inhibitors during different stages of its development, and a delicate balance between the expression of these proteinases and their inhibitors controls mammary gland morphogenesis (30). To investigate whether OPN may have a role in regulating metalloproteinases and whether such regulation may explain the observed abnormality of the mammary glands of AS-OPN mice, we determined the metalloproteinase activity in AS-OPN-transfected, mock-, and non-transfected cells by gelatin zymography. The results show that AS6 and AS8 cells express a protease with a molecular mass of 72 kDa, which is present at significantly reduced levels in mock-transfected and non-transfected NMuMG cells (Fig. 7A). The molecular weight of this band closely resembles that of pro-collagenase-A (MMP-2). To determine whether this protein is indeed MMP-2, cell-lysates were subjected to Western blot analysis using an anti-MMP-2 antibody. As shown in Fig. 7B, this antibody detects a protein, the molecular weight and abundance of which resemble those of the band observed in the zymogram (Fig. 7A), indicating an elevated expression of MMP-2 in both AS6 and AS8 cells as compared with mock-transfected and non-transfected cells.

Invasion of the ECM is an important property manifested during mammary gland development and differentiation. Therefore, it is possible that the abnormal in vivo and in vitro morphogenesis of mammary epithelial cells expressing low levels of OPN is due to their increased invasiveness. To test this possibility, AS-OPN cDNA-transfected, mock-, and non-transfected parental NMuMG cells were subjected to gel-invasion assays (31). Fig. 7C shows that AS6 and AS8 clonal cells are more invasive than non-transfected and mock-transfected NMuMG cells. To determine whether the MMP-2 is responsible for the invasive phenotype, the above assays were carried out in presence and absence of TIMP-2, a specific inhibitor of MMP-2. These results show that addition of TIMP-2 results in complete inhibition of invasiveness of AS6 and AS8 cells (Fig. 7C). Taken together, these results demonstrate that increased expression of a metalloproteinase observed in AS6 and AS8
endogenous OPN gene remains functional in AS-OPN mice during early stages of mammary gland development as the MMTV-LTR promoter-directed antisense transgene expression in mammary epithelium does not occur before puberty without exogenous hormone treatment. Therefore, it may be too advanced a stage for such compensatory mechanism to offset the effect of OPN deficiency in the development of the mammary gland of the transgenic mice.

Several cDNA constructs using the MMTV-LTR promoter/enhancer to target the expression of transgenes in the mammary gland have been reported (reviewed in Ref. 18). In each of these studies, transgenic expression was observed solely or predominantly in the mammary glands of mature virgin females during pregnancy and at the post-partum stage (33). Our present observations are in agreement with those of the previous studies. Although dexamethasone treatment can further enhance transgene expression in the mammary gland and induce its expression in other tissues (34), such treatment was avoided in this study because dexamethasone profoundly affects mammary epithelial cell differentiation in vivo as well as in vitro (35). Therefore, such treatment would have obscured any effect of the AS-OPN transgene on mammary gland development. The possibility that the abnormal development of the mammary gland observed in the present study may have been caused by a cis-acting effect of the MMTV-LTR promoter on mammary specific transcription factors is highly unlikely because the results of each of the studies mentioned above show that the use of the MMTV-LTR promoter results in distinct transgene-specific phenotypes. For example, the transgenic mice showing MMTV-LTR-driven expression of TGF-β had hypoplastic mammary epithelia (36), as opposed to hyperplasias or adenocarcinomas developed by the mice overexpressing TGF-α (33) or cyclin D1 gene (37), respectively, under the control of the same promoter. Finally, the possibility exists that the observed phenotype of AS-OPN mice is due to a position effect of the transgene integration, causing insertional mutagenesis or activation of adjacent gene(s) involved in mammary gland development. However, this is highly unlikely since an identical phenotype is observed in two transgenic mouse lines independently derived from two founders. These transgenic mouse lines, as well as the NMuMG cells transfected with AS-OPN cDNA, harbor the transgene at different sites in the genome. Thus, the anomalies of the mammary gland development in AS-OPN transgenic mice, and the abnormality of AS-OPN-transfected NMuMG cells observed in the present study, are most likely caused by the down-regulation of OPN synthesis in mammary epithelial cells due to transgene activity.

In the present study, we have examined the role of OPN in mammary gland morphogenesis by utilizing transgenic mice with targeted expression of OPN antisense RNA (AS-OPN) and an established normal murine mammary epithelial cell line, NMuMG, in which OPN production is suppressed by transfection with the same AS-OPN construct. The antisense RNA technology has been employed successfully to suppress the expression of various endogenous genes in both prokaryotes and eukaryotes (32). Although the versatility of the antisense RNA has been demonstrated in various studies, there are subtleties inherent to the optimal antisense RNA structure for the inhibition of specific gene expression. Our construct appears to have effectively interfered with OPN expression, specifically in the mammary epithelial cells of AS-OPN mice originating from two independent founders. The antisense-transgene/protein levels are drastically reduced in AS-OPN mice compared with those of the normal controls. Taken together, these results suggest that expression of AS-OPN RNA interferes with
the OPN mRNA translation, and not with its transcription, maturation, or stability.

One of the striking features of most of the AS-OPN mice is their non-lactating phenotype, which in most cases caused death of the newborn pups. Morphological analysis showed that the mammary glands of the non-lactating mice, 24 h post-partum, were mostly rudimentary, and normal alveolar structures were absent. A few that could be detected had a convoluted appearance, were small in size, and appeared to have no central lumen. A small proportion of the transgenic mice produced enough milk to feed only small size litters (1–4 pups) and the glands of these animals showed only a few normal sized alveoli. Variability in OPN levels were observed in glands of AS-OPN mice, which could be due to differential activity of the transgene in individual mice or due to their genetic differences. Such variation may explain why a small percentage of AS-OPN mice could support 1–4 pups.

It has been reported that coordinated expression of matrix metalloproteinases such as MMP-2 and their inhibitors regulate mammary gland development (30). In the present investigation, we observed that antisense OPN cDNA-transfected NMuMG cells have increased MMP-2 activity associated with increased Matrigel invasiveness, although our data do not exclude the role(s) of other proteases nor do they explain how reduced levels of OPN may increase cellular MMP-2 activity. One possibility is that, as MMP-2 and OPN interact with the same αβ3 integrin (vitronectin receptor) on the cell surface (39, 40), regulation of MMP-2 activity by OPN may well be achieved through competition between these two ligands for the same receptor. Since OPN antisense-transfected cells synthesize low levels of OPN, more MMP-2 may be bound to the cell surface receptor where activation of the enzyme is reported to take place (41), resulting in the observed structural abnormalities of the mammary glands in AS-OPN mice. Furthermore, higher levels of MMP-2 detected in cell lysates of transfected cells may also be due to enhanced pericellular binding of this enzyme.

It has been observed that in vitro, that down-regulation of OPN synthesis in mammary epithelia is correlated with abnormal mammary gland differentiation, and our AS-OPN mice provide a unique and valid model to further explore the role of this protein in mammary gland development, differentiation, and tumorigenesis.

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