

Colony stimulating factor 1 is required for mammary gland development during pregnancy

(osteopetrotic mouse/*op*/branching morphogenesis)

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ABSTRACT The study of colony stimulating factor 1 (CSF-1), a homodimeric serum growth factor that regulates mononuclear phagocytes and is involved in maternal–fetal interactions during pregnancy, was dramatically enhanced by the observation that the recessive mutation osteopetrosis, *op*, is an inactivating mutation in the CSF-1-encoding gene. Homozygous mutant (*op/op*) mice completely lack CSF-1, are osteopetrotic consequent to a deficiency in osteoclasts, have severely reduced numbers of macrophages, and have reduced fertility evident at the pre- and postimplantation stages of pregnancy. We show here that *op/op* females have a lactational defect, and consequently, although some are able to produce offspring, few nurture any pups and none feeds a full litter. This lactational defect is due to incomplete mammary gland ductal growth during pregnancy, a precocious development of the lobuloalveolar system, and despite expression of milk proteins, a failure to switch to a lactational state. These data show that CSF-1 has a role in the development of the mammary gland during pregnancy.

Colony stimulating factor 1 (CSF-1, also known as macrophage CSF) is a homodimeric serum glycoprotein originally identified as a growth factor regulating the proliferation, survival, and differentiation of mononuclear phagocytes (1). It exerts its action through a transmembrane tyrosine kinase receptor (CSF-1 receptor; CSF-1R), the product of the *c-fms* protooncogene (2). CSF-1 also has an additional role in maternal–fetal interactions during pregnancy. Originally this role was suggested by the sex steroid hormone-regulated synthesis of CSF-1 from an alternatively spliced transcript, which in mice, during pregnancy, is expressed exclusively in the uterine epithelium and the expression of the CSF-1R in preimplantation embryos, decidual cells, and trophoblasts (3–7). This role was confirmed, along with the role of CSF-1 in mononuclear phagocyte biology, by studies with osteopetrotic mutant mice (8). These mice have an inactivating mutation in the coding region of the CSF-1 gene and therefore are completely devoid of CSF-1 (9, 10). They exhibit impaired bone resorption associated with a paucity of osteoclasts (9, 11), are deficient in many populations of macrophages (9, 11, 12), and have seriously impaired fertility evident at the pre- and postimplantation stages of development (13).

Females homozygous for the osteopetrosis (*op*) mutation display very low fertility when mated with homozygous mutant males but when mated to wild-type males can give birth, although at a lower frequency than wild-type females, to live pups of normal size (13). In this paper we show that these pups have no milk in their stomachs and die rapidly if they are not transferred to a foster mother. This failure to feed is due to a

lactational defect in *op/op* mothers caused by the incomplete development of their mammary glands during pregnancy. These data establish a previously unsuspected role for CSF-1 in mammary gland development during pregnancy.

MATERIALS AND METHODS

Mice. Osteopetrotic (*op/op*) and littermate (+/*op*) controls were obtained from *op/+* female × *op/op* male crosses and maintained in isolated units at the Albert Einstein College of Medicine as described (13). Mice were fed ad libitum with powdered chow and infant milk formula (Enfamil). At 10 days of age, *op/op* were distinguished from +/*op* (normal) mice by the absence of incisors. Eight-week-old female mice of both genotypes were housed with normal male mice and checked daily for the presence of a copulation plug. The presence of a plug was designated as day 1 of pregnancy.

In some cases, mice were injected with 10⁶ units of CSF-1 (≈12 μg; Chiron) s.c. in 50 μl of physiological saline or with saline alone from day 3 of life until the termination of the experiment.

Histology. Twenty-six mice of each genotype were killed from virgin, pregnant day 14 to term, and lactational day 1–3 mice, such that there were at least duplicates for each day. To make whole-mount preparations of mammary glands, the right inguinal gland was dissected out intact and stretched onto a glass slide, dehydrated, defatted, and stained with Harris' hematoxylin as described (14). The left inguinal glands were dissected out from the same mice and fixed in buffered formalin. Fixed tissues were embedded in wax, and 5-μm sections were cut and either stained with hematoxylin/eosin for histological examination or immunostained using a rabbit anti-whey acidic protein (WAP) antibody (1:250 dilution). In the latter case, specific immunoreactive antibody was detected with an avidin–peroxidase detection system (Vector Laboratories) as described (14). After development to indicate the presence of the immunoreactive (brown) material, the sections were lightly stained with Gill's hematoxylin. Controls for the immunostaining were prepared either by omission of the primary or secondary antibodies or with nonimmune rabbit serum, none of which gave significant staining.

For determination of the alveolar cellular area, 5-μm wax sagittal sections were taken to encompass the lymph node and to contain the whole gland. After being stained with hematoxylin/eosin, areas adjacent to the lymph node were selected randomly using a 4× lens on a Nikon microscope, and the field was captured with a Hammamatsu NewVicom videocamera connected to a Macintosh Quadra 950 computer. The lobuloalveolar cellular area, digitized to exclude

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Abbreviations: CSF-1, colony stimulating factor 1; WAP, whey acidic protein; *op*, osteopetrosis; CSF-1R, CSF-1 receptor; TGF-β₁, transforming growth factor β₁.

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Table 1. Lactational ability of osteopetrotic mice

Treatment	Genotype	Mice feeding pups,* ratio (%)
None	+/ <i>op</i>	22/22 (100%)
None	<i>op/op</i>	4/32 (13%)
PBS	<i>op/op</i>	1/8 (13%)
CSF-1†	<i>op/op</i>	5/9 (56%)

PBS, phosphate-buffered saline.

*Mice were scored on the ability to feed at least one pup to weaning.

†CSF-1 (10^6 units) was injected s.c. from day 3 of life.

the lumen, was analyzed by using the public-domain NIH IMAGE software program written by Wayne Rastrad and available electronically by anonymous ftp (file transfer program) from zippy.nimh.nih.gov. For this area analysis 24 mice of each genotype were killed. These were either virgin, pregnant day 14 to term, or from the first 36 hr postpartum.

Analysis of RNA. RNA was extracted from thoracic mammary glands dissected out on the different days of pregnancy or lactation using the guanidium isothiocyanate method as described (4). Twenty micrograms of total RNA was separated by formaldehyde-agarose gel electrophoresis, blotted onto nylon membranes, and probed sequentially, with stripping of the blots in between, with [32 P]dCTP-labeled cDNAs to WAP, α -lactalbumin, and β -casein, using described methods (4). To control for RNA loading and transfer, blots were finally probed using a radiolabeled cDNA complementary to rRNA as described (4). Densitometric measurements of the relevant bands on the autoradiograms were made by using a Molecular Dynamics densitometer and analyzed with the supplied IMAGE QUANT software. All optical densities were corrected by the optical density for the corresponding rRNA signal.

RESULTS

Lactating Behavior of Osteopetrotic Females. Pregnancy, when it occurs in *op/op* mice, is of normal duration (≈ 20 days), and the pups are born live with normal weights and in the expected Mendelian ratio (13). However, examination of these postpartum *op/op* females showed that only 13% nurtured some pups, and none were able to feed a full litter, whereas all normal females, housed under the same condi-

tions, nurtured at least some, and usually the majority of, pups in their litters (Table 1; statistically different $P < 0.0001$, Fisher's exact test). The *op/op* mothers show the nursing behavior of licking and moving of their pups, but, in contrast to the pups from normal mothers, there was no evidence of milk in their stomachs. They therefore died rapidly unless they were fostered to a normal lactating mother when pups of both genotypes (*op/op*, +/*op*) survive. These data suggest a lactational defect in *op/op* mice.

Because *op/op* mice have no systemic CSF-1, the effects on the restoration of circulating CSF-1 concentrations on their ability to feed their young were determined. A group of female *op/op* mice was injected daily from day 3 of life until the end of the experiment with 10^6 units of CSF-1 administered s.c. in physiological saline and compared with mice injected with saline alone. This dose of CSF-1 has been previously established as sufficient to restore normal circulating CSF-1 concentrations, correct the osteopetrosis, allow growth of teeth, and induce repopulation of many tissues by macrophages (12, 15). At ≈ 8 weeks, these CSF-1-reconstituted mice and their saline-injected age-matched controls were mated, and after birth, their ability to feed their pups was scored. Fifty-six percent of the CSF-1-reconstituted *op/op* mice could feed some pups, compared with $\approx 13\%$ of control *op/op* mice (Table 1; significantly different from control groups by Fisher's exact test, $P = 0.01$). Thus, restoration of humoral CSF-1 is partially able to correct the lactational defect in *op/op* mice.

Mammary Gland Development During Pregnancy. To investigate the lactational defect, *op/op* and normal mice, for comparison, were killed from day 14 of pregnancy until the third day postpartum, and their mammary glands were examined. Sagittal sections of the inguinal mammary glands of normal postpartum mice show the classical morphology of a well-differentiated and fully functional tissue (Fig. 1B) (16). The alveoli have a secretory phenotype as indicated by the greatly enlarged, clear lumen surrounded by flat alveolar cells. The secretory cells are highly differentiated with nuclei aligned against the basal border of the cell. Alveolar epithelial cells before parturition are nonsecretory, have a cuboidal structure, and contain large cytoplasmic lipid vesicles (Fig. 1A). Functional differentiation that coincides with parturition

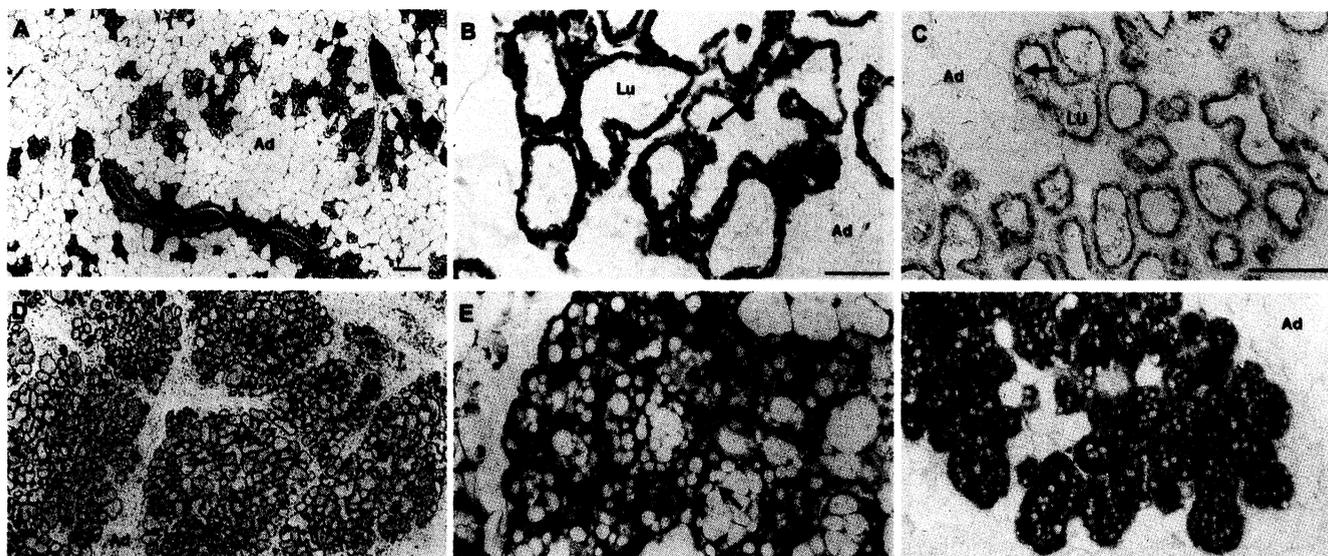


FIG. 1. Sagittal sections of +/*op* and *op/op* mammary glands. (A, B, D, and E) Sagittal sections stained with hematoxylin/eosin, showing alveolar structure at day 18 of pregnancy (A and D) and day 2 postpartum (B and E). [Bar = 300 (A and D) and 50 μ m (B and E)]. (C and F) Similar sections of day 1 postpartum mammary glands immunostained to detect WAP. The brown deposit shows immunoreactive material, and the sections were lightly counterstained with hematoxylin. Ad, adipose tissue; Lu, lumen. Arrow, secretory cell in alveolar epithelium. (A, B, and C) +/*op*. (D, E, and F) *op/op*. (Bar = 100 μ m.)

is accompanied with a loss of these vesicles, cell flattening, and an enlargement of the lumen (14, 16). This result was confirmed by electron microscopy of cells at day 1 postpartum, which indicates cells with very abundant rough endoplasmic reticulum and many secretory vesicles, some of which are being extruded from the cell (data not shown). In contrast, microscopic analysis of sagittal sections of mammary glands taken from postpartum *op/op* mothers reveals incompletely differentiated tissue with a nonsecretory phenotype (Fig. 1E). The alveolar cells are cuboidal, contain very large lipid vesicles (oil-red O positive), and the lumen is either very small or unformed. These features are reminiscent of alveolar structures of normal mice at the end of pregnancy (ref. 16; Fig. 1A), but there is substantially more cellular swelling and distortion of the cellular architecture. Furthermore, the nuclei are sometimes forced away by extremely large vesicles from their normal position apposite to the basal membrane of the cell (Fig. 1E). These features were confirmed by electron microscopy, which shows cells bulging into the lumen because of the inclusion of very large vesicles within the cell (data not shown). Thus, the alveolar epithelial cells appear unable to secrete their contents into the lumen. In addition, ductal structures were poorly developed with incomplete arborization. The major ducts are clear, which is further evidence for a failure to secrete into and from the alveolar lumen. A similar morphology is observed on day 1–3 postpartum, although by day 3 involution is already apparent.

In confirmation of this morphological analysis, immunohistochemical staining for one of the major milk proteins, WAP, in postpartum mammary glands of normal mice shows a secretory phenotype with WAP being rapidly secreted, leaving only small amounts at the apical surface of the cell (Fig. 1C). In contrast, immunostaining for WAP shows that the alveoli of postpartum *op/op* mice have failed to undergo functional differentiation. WAP accumulates throughout the cells and is neither secreted nor cleared from the duct (Fig. 1F).

Whole-mount preparations of mammary glands from *op/op* mice also show abnormal development. Normal mammary glands exhibit substantial growth and branching at midpregnancy (Fig. 2B) followed by extensive lobulo-alveolar development. By day 18, the highly branched alveolar structures have filled the mammary fat pad, and a

characteristic gland is shown in Fig. 2C. In contrast, in *op/op* mammary glands, ductal growth into the fat pad and branching is strongly reduced, although lobulo-alveolar development is premature, being established at day 14 (Fig. 2E), and exceeds that of normal mice. This reduced growth results in atrophic mammary glands without the characteristic branching patterns at any stage of pregnancy and a density of lobulo-alveolar structures in excess of that in normal mice (Fig. 2E). These observations on whole mounts are confirmed by histological observations of sagittal sections through pregnancy, which show an increased density of alveolar structures; a day 18 mammary gland of *op/op* mice compared with normal mice is shown in Fig. 1A and D. Whole-mount and sagittal sections of virgin *op/op* mammary glands, however, show similar morphologies between normal and *op/op* mice, although the ducts appear somewhat more developed in *op/op* mice than in *+/op* mice, which probably reflects the age or stage of the estrus cycle when the mice were killed (Fig. 2A and D; Fig. 3).

Although the morphological abnormalities in *op/op* mammary glands reported here do exhibit variable penetrance with respect to the severity of the phenotype, these abnormalities were observed to a greater or lesser extent in all 25 pregnant and lactating mice examined. The variations in penetrance of the mutation may be due to the segregating genetic background and probably explain the $\approx 10\%$ of *op/op* mice that can lactate, although inefficiently.

Digital analysis of the lobulo-alveolar cell area in histological sections of mammary glands taken during pregnancy and lactation also shows gross lobulo-alveolar development is more pronounced in *op/op* than in normal mice, confirming the morphological analysis. In *op/op* mice the density of the virgin glands is comparable to normal mice, but by day 14 of pregnancy, there is precocious development of the lobulo-alveolar structures, such that 24% of the mammary gland is occupied by lobulo-alveolar cells compared with 8% in normal mice. By day 16 the density of the lobulo-alveolar cells in *+/op* is maximal; however, in *op/op* mammary glands this density continues to increase until day 18 of pregnancy, when it is approximately two times (56% of total area) that of the control mice (Fig. 3; difference by ANOVA between *+/op* and *op/op* during pregnancy and lactation, $P < 0.001$). Thus, the

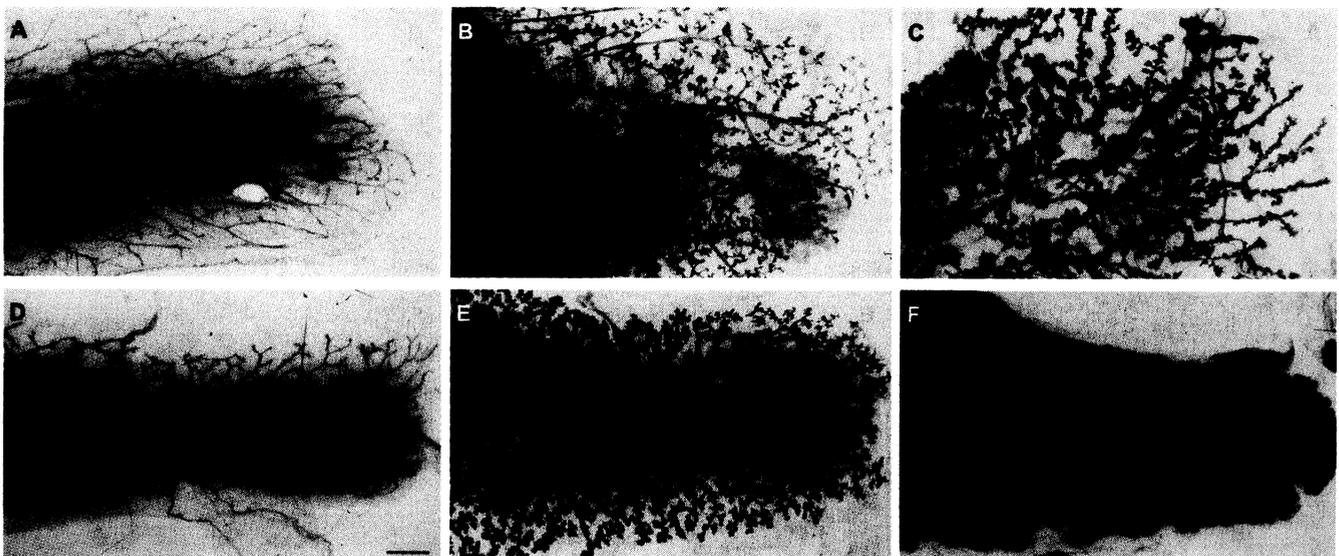


FIG. 2. Whole mounts of *+/op* and *op/op* mammary glands. Whole-mount preparations are shown for virgin (A and D), day 14 (B and E), or day 18 pregnant (C and F) mammary glands derived from *+/op* (A–C) or *op/op* (D–F) mice. The photomicrographs were taken at the same magnification of the lobe of the mammary gland distal to the lymph node. By day 18, only a small portion of the tip of the *+/op* mammary gland (C) is included, whereas almost the entire lobe of the *op/op* (F) gland is included in the photograph, showing the atrophic development in *op/op* mice. (Bar = 1 mm.)

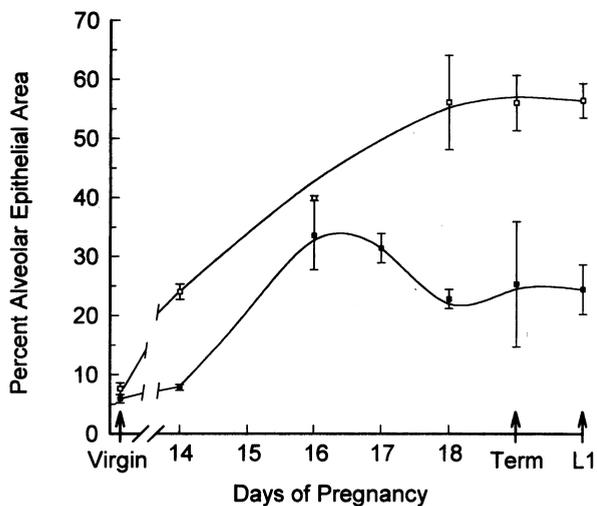


FIG. 3. Lobulo-alveolar density in *op/op* and *+/op* mice. Percentage of mammary gland taken up by lobulo-alveolar cells is shown for virgin, days 14 to term, and the first 36 hr postpartum (L1) *op/op* (□) and *op/+* (■) mice. Five-micrometer sections of left inguinal glands were made to section through the lymph node and encompass the entire gland. After being stained with hematoxylin/eosin, areas adjacent to the lymph node were selected randomly, and the area occupied by alveolar cells, digitized to exclude the lumen, was calculated as described. Points represent mean \pm SEM of at least three mice per time point.

ratio of glandular tissue to adipose tissue is significantly increased in *op/op* mice compared with control mice.

Gene Expression in the Mammary Gland. To determine whether the alveolar cells of *op/op* mothers undergo a normal developmental program of gene expression, RNA was extracted from mammary glands at different stages of development and analyzed for expression of the milk protein genes, WAP, α -lactalbumin, and β -casein (17, 18). Expression of these genes is under hormonal control and follows a characteristic pattern during mammary development. Thereby, they can be considered differentiation markers for specific developmental stages. Whereas β -casein gene expression is induced already in early pregnancy and little increase of its mRNA is seen between day 14 of pregnancy and day 1 of lactation, high levels of WAP mRNA are first seen around day 16 of pregnancy (17). Induction of the α -lactalbumin gene is confined to late pregnancy and lactation. In *op/+* mice the expression of the three milk protein genes during pregnancy followed the predicted pattern (Fig. 4). In contrast, high levels of WAP and α -lactalbumin mRNA were detected precociously in *op/op* mice (Fig. 4). These data are especially striking for α -lactalbumin mRNA, which is substantially expressed in *op/op* mammary glands at day 18, 2 days before parturition, whereas in normal mice, as in other strains (14), the major step-up in expression does not occur until after parturition (Fig. 4). WAP mRNA is significantly expressed in *op/op* mammary glands on day 14 of pregnancy at concentrations \approx 8-fold higher than *+/op* mice at the same day. In contrast, WAP mRNA expression in *+/op* mice at either day 14 or 15 is not significantly different from nonpregnant mice. By day 16 however, lobulo-alveolar development is similar between both genotypes (Fig. 3) and so is WAP mRNA expression (Fig. 4). These data are consistent with the early differentiation of the lobulo-alveolar structures in *op/op* mice seen in histological sections. Differences in steady-state levels of WAP mRNA observed between individual mice (both *op/op* and *op/+*) at day 16 of pregnancy are within the normal range of variation obtained at that day. This is because the WAP gene is induced \approx 100-fold around day 16 of pregnancy and therefore small

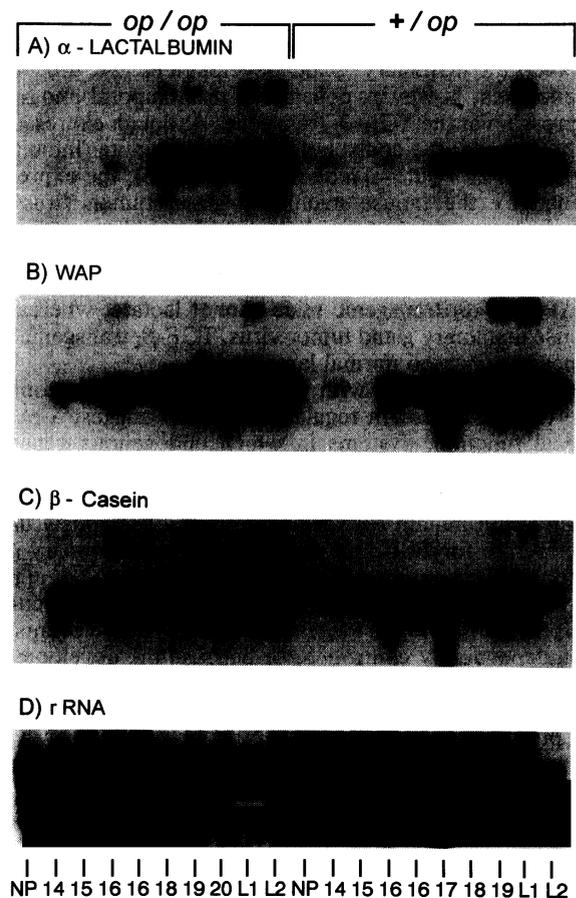


FIG. 4. Northern blots of mammary gland mRNA expression. Autoradiograms of 0.7-kb α -lactalbumin (A), 0.68-kb WAP (B), and 1.45-kb β -casein (C) mRNA expression in mammary glands derived from *op/op* (Left) or *+/op* (Right) virgin or pregnant mice as shown at bottom. L1 and L2, lactational day 1 and day 2, respectively; NP, nonpregnant. The major lower band in A and B corresponds to the specific mRNA, and because the RNA sample is total cellular RNA, the upper minor band is probably precursor nuclear RNA. (D) The 1.9-kb 18S rRNA band used to control for RNA loading and transfer. Each lane represents the RNA from a single mouse, and the analysis was repeated using different RNA samples at least once with similar results.

developmental differences can result in large differences in WAP mRNA concentration. Dependent on the time of copulation with respect to the time of plug check, animals considered 16 day pregnant could actually be between 15½ and 16½ days of pregnancy, which would also explain the differences seen in individual mice. Expression of β -casein mRNA, which normally shows little change over the last half of pregnancy, appears relatively similar from day 14 to lactational day 2 in both genotypes. At parturition all three genes show comparable levels of expression (corrected for rRNA expression) in mammary glands of both genotypes (*+/op* and *op/op*), indicating that the expression per cell reaches the same concentration by the end of gestation regardless of genotype.

DISCUSSION

Mammary gland development during pregnancy proceeds in two distinct stages, the outgrowth of the ductal trees into the fat pad and lobulo-alveolar proliferation (19). These processes are regulated by hormones of both maternal and fetal origin (20–22). In addition, locally synthesized growth factors play a critical role in mammary development (23). For example, transforming growth factor β_1 (TGF- β_1) is synthe-

sized in lobulo-alveolar structures during pregnancy, and overexpression in mammary tissue of transgenic mice results in aberrant mammary gland development (24, 25). These phenotypes, however, depend on the temporal and spatial expression of the TGF- β_1 transgene. Although expression of TGF- β_1 under the control of the WAP promoter inhibits the formation of lobulo-alveolar structures (25), the expression guided by the mouse mammary gland tumor virus long terminal repeat results in decreased branching but without alteration of the lobulo-alveolar structures (24). These different phenotypes are physiologically relevant in that the WAP/TGF- β_1 transgenic mice cannot lactate, whereas the mouse mammary gland tumor virus/TGF- β_1 transgenic mice appear to undergo normal lactation.

While experiments with transgenic mice that ectopically overexpress a growth regulator can only suggest a physiological function, systems in which endogenous genes are nonfunctional are more powerful because they clearly ascribe a role for that gene in the affected process. The current study, using a null mutant for CSF-1, defines a previously unsuspected role for CSF-1 in the regulation of mammary gland differentiation during pregnancy. This growth factor appears to regulate the branching morphogenesis that occurs at midpregnancy and the switch to lactation. These events may be independently controlled, or because the developmental defects are observed as early as day 14 of pregnancy, it may be that the failure in ductal growth and arborization allows the precocious differentiation of the lobulo-alveolar system without the supporting ductal system which, in turn, causes the failure to switch to the lactational state.

The action of CSF-1 could be within the mammary gland (organ autonomous) either by directly effecting alveolar-epithelial cell growth or through its effects on the macrophages resident in the mammary gland that are, in turn, induced by CSF-1 to secrete a trophic factor or that are involved in tissue remodeling essential for ductal growth. CSF-1R mRNA is detected at low levels in mammary gland RNA of normal but not *op/op* mice (unpublished observations), suggesting that this CSF-1R mRNA expression is restricted to macrophages. CSF-1 mRNA expression, however, if present, is below the level of sensitivity of Northern blotting (unpublished observation), suggesting that if CSF-1 has a direct action within the mammary tissue, it would be obtained from the serum rather than being synthesized in this tissue. Significantly, restoration of serum CSF-1 can partially restore the ability of *op/op* mice to feed their young.

Alternatively, the action of CSF-1 could be indirect, influencing another tissue to produce a hormone or cytokine that, in turn, regulates mammary gland development (organ nonautonomous). During pregnancy in the mouse, CSF-1 receptor mRNA is expressed at high levels in the placental giant trophoblasts, and uterine CSF-1 concentrations are elevated ≈ 1000 -fold (26) in a temporal manner that parallels the growth and differentiation of the placenta (4, 27). In other mammals, even though they may have different mechanisms of placentation, the endocrine trophoblasts (e.g., binucleate trophoblasts of the cow and syncytial trophoblasts in the human) also express CSF-1Rs (28, 29). The placenta synthesizes lactogenic hormones in these trophoblasts—for example, placental lactogen I and II in the mouse (20, 22). Therefore, another hypothesis is that uterine-synthesized CSF-1 stimulates endocrine trophoblasts to produce a hormone/growth factor that is secreted into the circulation and that regulates mammary gland development. Such a mechanism allows the synchronization between fetal development and the maternal preparation for lactation and would be consistent with observations that show mammary gland development proportional to the number of placentae (30). This explanation is also consistent with the failure to completely correct the lactational defect of *op/op* mice by

systemic administration of CSF-1, which, although restoring circulating CSF-1 concentrations, cannot fully mimic the very high concentrations found in the utero-placental unit during pregnancy.

In conclusion, regardless of the mechanism, this report shows that the *op* mutation results in aberrant structural and functional development of the mammary gland during pregnancy and demonstrates a role for CSF-1 in this process.

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