Published in final edited form as:

Biotech Histochem. 2009 December ; 84(6): 267–274. doi:10.3109/10520290902984258.

Exogenous thyroid hormone affects myoepithelium and proliferation in the developing rat parotid gland

R Ikeda^{1,2}, S Aiyama², and RS Redman³

¹ Department of Dental Hygiene, The Nippon Dental University College at Tokyo, Tokyo, Japan

² Department of Histology, School of Life Dentistry, The Nippon Dental University College at Tokyo, Tokyo, Japan

³ Oral Pathology Research Laboratory, Department of Veterans Affairs Medical Center, Washington, DC

Abstract

In the mature rat parotid gland, myoepithelial cells (MEC) invest intercalated ducts, but not acini. During postnatal development, however, these cells differentiate around both intercalated ducts and acini, then translocate to only intercalated ducts during weaning. Previously, we found that thyroxine (T₄) accelerates translocation of cells with small secretory granules from acini into intercalated ducts and the number of apoptotic cells increased tremendously with high doses. We present here additional analysis of the effects of T₄ on developing rat parotid gland, namely, the distribution of MEC and the proliferation of parenchymal cells. Beginning at age 4 days, pups were given daily subcutaneous injections of low, medium and high doses of T₄ or vehicle or no injection. At ages 4, 7, 10 and 15 days, glands were excised and processed for light microscopy. Sections were double-immunostained with antibodies against proliferating cell nuclear antigen (PCNA) and actin, and counterstained with hematoxylin. Proliferative activity was assessed via PCNA histochemistry and MEC were identified using actin histochemistry. MEC in the T₄ groups invested mostly acini at 15 days in vehicle/normal glands and mostly intercalated ducts after 10 days in the T₄ groups. The proliferative activity of acinar cells and MEC in vehicle/normal glands declined progressively with age and T₄ increased the rate of this decline in the MEC in a dose-dependent manner. We conclude that T₄ accelerates the translocation of MEC from acini to intercalated ducts, with an important mechanism being the more rapid decline in the proliferative activity of MEC than in acinar cells in the T₄ groups. Some of the decline in the proliferative activity of all cells in the high and medium dose T₄ groups after 7 days may have been due to dose-related thyroxine toxicity.

Keywords

differentiation; myoepithelial cells; parotid gland; proliferation; rat; salivary glands

Most of the growth and differentiation of the rat parotid gland occurs postnatally (Lawson 1970, Redman and Sreebny 1970a, 1971). This permits the study of factors that influence gland development via interventions applied directly to the pups, thus avoiding modifications of the effects of application via the dams. Alterations of diet and hormones have been shown to influence strongly postnatal development of rodent salivary glands (Sasaki et al. 1976, Takeuchi et al. 1977a,b 1978, Takuma et al. 1978, Kumegawa et al. 1980, Johnson 1987, Ikeda et al. 2008, Inukai et al. 2008). Serum thyroid hormone concentration is low in the perinatal

rat, increases to twice the adult level by 16 days of age, and returns to the adult level by 30 days (Clos et al. 1974, Porterfield and Hendrick 1981, Dussault et al. 1982, Porterfield 1985, Farwell and Dubord-Tomasetti 1999).

In the mature rat parotid gland, myoepithelial cells (MEC) invest intercalated ducts, but not acini (Garrett and Parsons 1973). During postnatal development, however, these cells differentiate around both intercalated ducts and acini, then translocate to only intercalated ducts during weaning (Redman et al. 1980, Tsujimura et al. 2006). Previously, we found that exogenous thyroxine (T_4) accelerates the translocation of cells with small secretory granules from acini into intercalated ducts, and apoptotic cells increased tremendously with high doses (Ikeda et al. 2008). We present here further analysis of parotid glands to determine the effects of T_4 on the distribution of MEC and proliferation of parenchymal cells in the developing rat parotid gland during the two weeks after birth.

Materials and methods

All experimental procedures were approved by the appropriate review boards of The Nippon Dental University and the Washington, DC, Veterans Affairs Medical Center.

Details of many of the methods have been published earlier (Ikeda et al. 2008). Briefly, certified pathogen-free Sprague-Dawley female rats at 14 days postconception were obtained from Harlan (Indianapolis, IN). Pups were considered 0 days old on the day of birth. Beginning at 4 days, two pups in each of four litters of 10 pups per age were given daily subcutaneous injections of 0.1, 0.5 or 5 $\mu\text{g/g}$ body weight of T_4 (low, medium and high dose groups, respectively), or vehicle injection or no injection (minimum n per group = 6). At ages 4, 7, 10 and 15 days (12 for highest dose of T_4), at 3 h postinjection, pups were deeply anesthetized with 2-bromo-2-chloro-1,1,1-trifluoroethane (Halothane; Sigma-Aldrich, St. Louis, MO), heart blood was collected for assessing serum T_4 , and the parotid glands were excised.

The serum T_4 assessment was done as described earlier (Ikeda et al. 2008). The parotid glands were fixed in 4% formaldehyde prepared from paraformaldehyde in 0.05 M phosphate buffer, pH 7.4, for 6 h, then processed for embedding in paraffin. Five-micrometer-thick paraffin sections were immersed in 0.3% H_2O_2 in methanol for 30 min to inhibit endogenous peroxidase activity, rinsed with phosphate-buffered saline (PBS) and immersed in 10% normal rabbit serum (Immuno-Biological Lab. Co., Ltd., Takasaki, Japan) for 30 min to prevent nonspecific binding. After rinsing with PBS, the sections were incubated for 1 h in a mouse monoclonal antibody against muscle actin (Nichirei, Tokyo, Japan), rinsed again with PBS, then incubated for 10 min with biotinylated rabbit anti-mouse IgG, IgA, and IgM (Nichirei). The streptavidin-horseradish peroxidase (HRP) complex (SABC) method was applied using diaminobenzidine (DAB; DOJINDO, Kumamoto, Japan, lot no. WE052; brown stain). After three rinses in 0.1 M glycine-HCl buffer at pH 2.2 for 2 h, the sections were rinsed with PBS and immunostained with a mouse monoclonal antibody against proliferating cell nuclear antigen (PCNA; DAKO, Glostrup, Denmark) overnight at room temperature using the SABC method, visualized with aminoethylcarbazol (Nichirei, product number H0709; red stain), and counterstained lightly with hematoxylin (Sigma; ScH-2). Proliferative activity was assessed using PCNA histochemistry and MEC were identified via actin histochemistry.

The total cells, the total of each cell type (acinar, myoepithelial, and intercalated and large ductal), and the cells that were PCNA-positive (total and by cell type) were counted in each sample. The PCNA-positive cells divided by the total cells $\times 100$ = percent positive cells, or labeling index (LI). We also counted the number of acini and intercalated ducts that were fully, partly or not invested with MEC, and converted these data into scores representing the predominant distribution of MEC in each section. Scores ranged from 1 (only acini invested)

to 5 (all MEC invested intercalated ducts). Only whole numbers were entered for analysis for each sample. Thus, if only a few MEC processes surrounded or nearly surrounded most acini while almost all intercalated ducts were invested with MEC, the score for that sample would be 4.0. The scoring system is described also in Table 1. The data are presented as means $\pm$ standard error (SE) and were subjected to one-way analysis of variance using the SPSS ver.14 software package (SPSS Japan, Tokyo).

Results

Because there were no differences between the vehicle-injected and non-injected pups for any measurement at any age, these values were combined into a single vehicle/normal group (minimum $n = 14$) for comparison with the groups that were injected with T_4 . The serum T_4 values and body weights of the pups were presented in our earlier paper (Ikeda et al. 2008). The former data are reprinted in Fig. 1 to facilitate comparison with the new data. In general, the serum value of T_4 was proportional to the dose administered and the gain in body weight with age was slowed in proportion to T_4 dose beginning at 7 days. Pups in the high dose group failed to thrive and one died at age 11 days. To prevent an unacceptable discomfort level and loss of data, the remaining pups in this group were euthanized at age 12 days.

Photomicrographs illustrating the changes in proliferative activity and the distribution and differentiation of the myoepithelium by age and treatment are presented in Figs. 2 (vehicle/normal and low dose T_4) and 3 (medium and high dose T_4). The proportions of MEC investing acini and intercalated ducts by age and treatment are compiled in Table 1. The proliferative activities (percent that reacted with anti-PCNA) by age and treatment of all parenchymal cells and by cell type are presented in Tables 2 and 3, respectively.

Only scattered MEC were sufficiently differentiated for reaction with anti-muscle actin in the vehicle/normal pups at age 4 days. Of these, almost all were around acini. At 7 days and older, many more MEC were seen, because most reacted with anti-muscle actin, but these still invested mostly acini at 15 days in vehicle/normal glands. The proportion of MEC investing intercalated ducts in the T_4 treatment groups increased in proportion to the increase in T_4 at 10 days and older, investing mostly intercalated ducts by 12–15 days.

It is important to note that mitosis of well differentiated acinar (Redman and Sreebny 1970b, Redman 1995) and myoepithelial (Redman 1994) cells in the developing rat parotid gland has been documented by electron microscopy. The proliferative activity of the acinar and MEC in vehicle/normal glands of pups at 4 days was high, then progressively declined with age. Administration of T_4 accelerated their rate of decline in a roughly dose-dependent manner; that of MEC declined more rapidly than that of acinar cells in the T_4 treatment groups, but not in the vehicle/normal group. There were no overt differences in the proportion of PCNA-positive MEC between those investing acini or intercalated ducts (data not shown).

Discussion

The translocation of MEC from investing mostly acini to almost entirely intercalated ducts in the rat parotid gland occurs between 14 and 25 days after birth (Redman et al. 1980). Tsujimura et al. (2006) showed that the more rapid slowing of proliferative activity of MEC than of acinar cells during this period is a major factor in this shift. The observed pace of MEC translocation and the chronology of changes in proliferative activity with age in the vehicle/normal group generally are consistent with previous findings (Redman et al. 1980, Redman 1995, Tsujimura et al. 2006).

The acinar and intercalated duct LI, however, tended to be lower than those obtained by Redman (1995) using ^3H -thymidine, especially at age 15 days. This is not unexpected given

conflicting reports on the validity of PCNA expression as a marker of proliferating cells. Some authors have noted poor correlation of PCNA expression and good correlation of Ki-67 expression with LI assessed via uptake of bromodeoxyuridine or ^3H -thymidine (Sullivan et al. 1993, Jones et al. 1995, Mushelishvili et al. 2003). Others have reported PCNA and Ki-67 to have essentially equal value in this regard (Cher et al. 1995, Hicks and Flaitz 2000). A low level of PCNA expression tends to linger long after the mitotic cycle has ended (van Dierendonck et al. 1991). Elias (1997) noted that there are several reasons for this. In addition to a longer half-life than that of Ki-67, some forms of PCNA are associated with DNA repair and other nonreplicative functions. Further, fixation for more than 30 h can affect performance of PCNA antibodies. He noted also, however, that the antibody to Ki-67 that can be used with paraffin sections is less effective than the one that is useful with fresh frozen sections.

The fixation period for our samples was uniform and much less than 30 h, and our criteria for cells being “PCNA-positive” excluded those with faintly brown nuclei. In doing this we may have over-corrected, because the foregoing suggests that PCNA LI should be higher than LI provided by uptake of DNA precursors. Nonetheless, on balance, it seems unlikely that the problems with PCNA cited above were a significant confounder of our within-study LI comparisons.

Apoptosis was rare in the vehicle/normal group in the same parotid glands that were used for our earlier report (Ikeda et al. 2008) and no apoptotic MEC cells were observed by Tsujimura et al. (2006). Thus, apoptosis was not a factor in the translocation of MEC from acini to intercalated ducts. Our results show that T_4 clearly accelerates the translocation of MEC from acini to intercalated ducts. An important mechanism for this is an even more rapid decline in proliferative activity of the MEC than of the acinar cells in the pups injected with T_4 .

We concluded previously that the rapid decline in the rate of body weight gain and a huge increase in vacuoles and apoptosis among the parotid cells indicated that thyroid toxicity had developed in the pups of the high and medium dose groups by 10 days. This makes it difficult to determine how much of the precipitous decline in the proliferative activity of all parotid parenchymal cells observed in the high and medium dose groups is another manifestation of thyroxine-induced acceleration of cytodifferentiation and how much is due to thyroid toxicity. The decline in proliferative activity in the low dose pups can be attributed more clearly to accelerated cytodifferentiation, because signs of thyroid toxicity were minimal or absent in this group. Indeed, as we noted earlier, the serum T_4 levels in the low dose pups came close to meeting our goal of causing a premature rise of thyroid hormone to the level that normally occurs 6–10 days later.

The effects of exogenous thyroid hormone on the developing rat parotid gland reported here and in our earlier paper must be considered in the context of the hormone's other known effects on rat development. These include advancing by 1–2 days the ages at which the eyelids open (14–15 days) and the incisors and first molars erupt enough to become functional (normal 8–10 and 15–17 days, respectively (Donaldson 1924, Huxley 1971, Ehrmann et al. 1984, Gamborino et al. 2001), the development of independent thermoregulation (Henning et al. 1979) and the ability to obtain fluids independent of milk from the dam (normal approximately 18 days; Kreck and Kreckova 1957). Indeed, eyelid opening, hair growth, and molar tooth eruption occurred about a day earlier in the T_4 low dose pups and even earlier in the medium and high dose pups than in the vehicle/normal pups. Together, these effects could advance the weaning process that normally occurs between 15 and 28 days, during which a significant dietary switch from mostly milk to mostly solid food occurs (Redman and Sweney 1976, Henning et al. 1979). The much greater stimulation of parotid secretion by solid food promotes parotid acinar maturation between 21 and 25 days (Redman and Sreebny 1976). Although no evidence such as stomach contents was collected, the watery vacuoles in the acini of the

medium and high dose pups at 10 days and older was taken as evidence of hypersecretion (Ikeda et al. 2008) and thus indirectly of precocious ingestion of solid food. In addition, exogenous thyroid hormone has been shown to induce precocious rises in serum corticosterone and corticosteroid-binding globulin in rat pups (D'Agostino and Henning 1982). Exogenous corticosteroids have been shown to increase the rate of other aspects of rat and mouse salivary gland differentiation (Johannessen 1965, Sasaki et al. 1976, Takeuchi et al. 1977a,b, Kumegawa et al. 1980, Johnson 1987, Ikeda et al. 2008, Inukai et al. 2008). Thus, it is possible that a T₄-induced rise in serum corticosteroids also may have been involved in the acceleration of MEC translocation and decline in parenchymal cell proliferative activity observed here.

Acknowledgments

This research was supported in part by The Nippon Dental University, Tokyo, Japan (RI), Grant DE 14995 from The National Institute of Dental and Craniofacial Research, The National Institutes of Health, Bethesda, MD (RSR), and the Research Service of the Department of Veterans Affairs Medical Center, Washington, DC. We thank Ms Sumie Satoh for her technical assistance.

References

- Cher ML, Chew K, Rosenau W, Carroll PR. Cellular proliferation in prostatic adenocarcinoma as assessed by bromodeoxyuridine uptake and Ki-67 and PCNA expression. *Prostate* 1995;26:87–93. [PubMed: 7855002]
- Clos J, Crepel F, Legrand C, Legrand J, Rabie A, Vigouroux E. Thyroid physiology during the postnatal period in the rat: a study of the development of thyroid function and of the morphogenetic effects of thyroxine with special reference to cerebellar maturation. *Gen Com Endocr* 1974;23:178–192.
- D'Agostino J, Henning SJ. Role of thyroxine in coordinate control of corticosterone and CBG in postnatal development. *Am J Physiol* 1982;242:E33–E39. [PubMed: 7058886]
- Donaldson, HH. Biology. In: Donaldson, HH., editor. *The Rat*. 2. Wistar Institute; Philadelphia: 1924. p. 15–29.
- Dussault JH, Coulombe P, Walker P. Effects of neonatal hyperthyroidism on the development of the hypothalamic-pituitary-thyroid axis in the rat. *Endocrinology* 1982;110:1037–1042. [PubMed: 6799277]
- Ehrmann O, Mansour R, Jacquart G. A micrometric and cytomorphometric study of the simultaneous development of the cerebellar cortex and dental function in the rat during the early post-natal period. *Arch Oral Biol* 1984;6:407–411. [PubMed: 6589982]
- Elias JM. Cell proliferation indexes: a biomarker in solid tumors. *Biotech & Histochem* 1997;72:78–85.
- Farwell AP, Dubord-Tomasetti SA. Thyroid hormone regulates the expression of laminin in the developing rat cerebellum. *Endocrinology* 1999;149:4221–4227. [PubMed: 10465295]
- Gamborino MJ, Sevilla-Romero E, Munoz A, Hernandez-Yago J, Renau-Piqueras J, Pinazo-Duran MD. Role of thyroid hormone in craniofacial and eye development using a rat model. *Ophthalm Res* 2001;33:283–291.
- Garrett JR, Parsons PA. Alkaline phosphatase and MEC in the parotid gland of the rat. *Histochem J* 1973;5:463–471. [PubMed: 4752918]
- Henning SJ, Chang S-SP, Gisel EG. Ontogeny of feeding controls in suckling and weanling rats. *Am J Physiol* 1979;237:R187–R191. [PubMed: 474790]
- Hicks J, Flaitz C. Mucoepidermoid carcinoma of salivary glands in children and adolescents: assessment of proliferation markers. *Oral Oncol* 2000;36:454–460. [PubMed: 10964053]
- Huxley HG. The eruption times of first and second molars in an albino strain of rat. *N Z Dent J* 1971;67:179–180. [PubMed: 5291421]
- Ikeda R, Aiyama S, Redman RS. Effects of exogenous thyroid hormone on the postnatal morphogenesis of the rat parotid gland. *Anat Rec* 2008;291:94–104.
- Inukai Y, Ikeda R, Aiyama S. Effect of glucocorticoid on the differentiation and development of terminal tubules in the fetal rat submandibular gland. *Cells Tissues Org* 2008;187:233–242.

- Johannessen LB. Effects of desiccated thyroid, propylthiouracil, vitamin B12 and caloric restriction on somatic growth, food intake and dentinogenesis in cortisone-treated immature albino rats. *Arch Oral Biol* 1965;10:819–32. [PubMed: 5226912]
- Johnson, DA. Regulation of salivary glands and their secretions by masticatory, nutritional and hormonal factors. In: Sreebny, LM., editor. *The Salivary System*. CRC Press; Boca Raton, FL: 1987. p. 135–156.
- Jones AS, Roland NJ, Caslin AW, Cooke TG, Cooke LD, Forster G. A comparison of cellular proliferation markers in squamous cell carcinoma of the head and neck. *J Laryngol Otol* 1994;108:859–864. [PubMed: 7989834]
- Kreckek J, Kreckova J. The development of the regulation of water metabolism. III The relationship between water and milk intake in infant rats. *Physiol Bohemoslov* 1957;6:26–34.
- Kumegawa M, Maeda N, Yajima T, Takuma T, Ikeda E, Minamida C. L-thyroxine, cortisol and diet affect the level of amylase in the parotid gland of developing rats. *J Endocr* 1980;87:65–71. [PubMed: 6159442]
- Lawson KA. Morphogenesis and functional differentiation of the rat parotid gland in vivo and in vitro. *J Embryol Exp Morphol* 1970;24:411–424. [PubMed: 4250039]
- Muskhelishvili L, Latendresse JR, Kodell RL, Henderson EB. Evaluation of cell proliferation in rat tissues with BrdU, PCNA, Ki-67 (MIB-5) immunohistochemistry and in situ hybridization for histone mRNA. *J Histochem Cytochem* 2003;51:1681–1688. [PubMed: 14623936]
- Porterfield SP. Prenatal exposure of the fetal rat to excessive L-thyroxine or 3,5-dimethyl-3¹-isopropyl-thyroxine produces persistent changes in the thyroid control system. *Endocrinology* 1985;108:1060–1063. [PubMed: 7460831]
- Porterfield SP, Hendrich CE. Alterations of serum thyroxine, triiodothyronine, and thyrotropin in the progeny of hypothyroid rats. *Endocrinology* 1981;108:1060–1063. [PubMed: 7460831]
- Redman RS. Myoepithelium of salivary glands. *Microsc Res Tech* 1994;27:25–45. [PubMed: 8155903]
- Redman RS. Proliferative activity by cell type in the developing rat parotid gland. *Anat Rec* 1995;241:529–540. [PubMed: 7604968]
- Redman RS, Sreebny LM. The prenatal phase of the morphosis of the rat parotid gland. *Anat Rec* 1970a; 168:127–138. [PubMed: 5506918]
- Redman RS, Sreebny LM. Proliferative behavior of differentiating cells in the developing rat parotid gland. *J Cell Biol* 1970b;46:81–87. [PubMed: 5459013]
- Redman RS, Sreebny LM. Morphologic and biochemical observations on the development of the rat parotid gland. *Dev Biol* 1971;25:248–279. [PubMed: 5562853]
- Redman RS, Sreebny LM. Changes in patterns of feeding activity, parotid secretory enzymes and plasma corticosterone in developing rats. *J Nutr* 1976;106:1291–1302.
- Redman RS, Sweney LR. Changes in diet and feeding patterns of developing rats. *J Nutr* 1976;106:616–626.
- Redman RS, Sweney LR, McLaughlin ST. Differentiation of myoepithelial cells in the developing rat parotid gland. *Am J Anat* 1980;158:299–320. [PubMed: 7446434]
- Sasaki R, Mura M, Takeuchi T, Furihata C, Matsushima T, Sugimura T. Premature induction of amylase in pancreas and parotid gland of growing rats by dexamethasone. *Biochim Biophys Acta* 1976;428:619–626. [PubMed: 179580]
- Sullivan RP, Mortimer G, Muirheartaigh IO. Cell proliferation in breast tumors: analysis of histological parameters Ki67 and PCNA expression. *Ir J Med Sci* 1993;162:343–347. [PubMed: 7903289]
- Takeuchi T, Kameye T, Tsumuraya M, Sugimura T. Development of exocrine cells of the pancreas and parotid gland in rats. *Digestion* 1978;18:266–279. [PubMed: 738543]
- Takeuchi T, Ogawa M, Furihata C, Kawachi T, Sugimura T. Perinatal changes in amylase and serum corticosterone levels in rats. *Biochim Biophys Acta* 1977a;497:657–662. [PubMed: 889881]
- Takeuchi T, Ogawa M, Sugimura T. Effects of various hormones and adrenalectomy on the levels of amylase in rat pancreas and parotid gland. *Experientia* 1977b;33:1531–1532. [PubMed: 200463]
- Takuma T, Nakanishi M, Takagi Y, Tanemura T, Kumegawa M. Precocious differentiation of mouse parotid glands and pancreas induced by hormones. *Biochim Biophys Acta* 1978;538:376–383. [PubMed: 620074]

- Tsujimura T, Ikeda R, Aiyama S. Changes in the number and distribution of MEC in the rat parotid gland during postnatal development. *Anat Embryol* 2006;211:567–574. [PubMed: 16937148]
- van Dierendonck JH, Wijsman JH, Keijzer R, van de Velde CJH, Cornelisse CJ. Cell-cycle-related staining patterns of anti-proliferating cell nuclear antigen monoclonal antibodies. Comparison with BrdU labeling and Ki-67 staining. *Am J Pathol* 1991;138:1165–1172. [PubMed: 1673821]

Fig. 1. Serum total T₄ (µg/dl) by age and treatment. The scale for the vehicle/normal (vehicle-injected, non-injected) pups is on the right multiplied $\times 40$, because the increase with age would appear barely above baseline if set to the scale of the T₄-treated pups. Reprinted from Ikeda et al. (2008).

Fig. 2.

Sections of parotid glands from vehicle/normal (column on left; A,C,E) and low T_4 -treated (column on right; B,D,F) pups at ages 7 (top row), 10 (middle row) and 15 (bottom row) days. The cytoplasm of MEC and smooth muscle cells in large blood vessels (arrows) are stained red (aminomethylcarbozol chromogen) from immunohistochemical reactions with antibodies to muscle actin. Nuclei in which reactions with antibodies to PCNA have occurred are brown (DAB chromogen). Insert to A) Higher magnification of the two labeled MEC on the right side (the tips of the arrows are retained). The nuclei of both of these MEC are surrounded by anti-muscle actin-positive (red) cytoplasm and thus clearly illustrate an essential criterion for identifying MEC in our study. The MEC on the right has a very brown nucleus, thus is clearly

PCNA-labeled, while the nucleus of the MEC on the left is not stained brown and so just as clearly is PCNA-negative. ID, intercalated ducts; LD, large (striated and excretory) ducts. Hematoxylin counterstain. For A-F (except the insert to A), scale bar = 50 μ m.

Fig. 3.

Sections of parotid glands from medium (left column; A,C,E) and high (right column; B,D,F) T_4 -treated pups at ages 7 (top row), 10 (middle row) and 15 (E) and 12 (F) days. The cell identification labels, colors resulting from immunohistochemical reactions and counterstain are the same as in Fig. 2. For A-F. Scale bar = 50 μ m.

Table 1Proportions of myoepithelial cells investing acini and intercalated ducts (mean score $\pm$ SE)

Treatment	Age (days)			
	4	7	10	15 ^a
High	1.3 $\pm$ 0.2	2.3 $\pm$ 0.3	3.6 $\pm$ 0.2 ^b	4.0 $\pm$ 0.0 ^b
Medium	1.2 $\pm$ 0.2	2.0 $\pm$ 0.0	3.0 $\pm$ 0.2 ^b	4.0 $\pm$ 0.1 ^b
Low	1.0 $\pm$ 0.0	1.2 $\pm$ 0.2	2.4 $\pm$ 0.2	3.2 $\pm$ 0.1 ^b
Vehicle/Normal	1.1 $\pm$ 0.6	2.0 $\pm$ 0.0	2.0 $\pm$ 0.0	2.5 $\pm$ 0.1

Scoring system: 1, all myoepithelial cells (MEC) invest acini; 2, most MEC invest acini, some invest intercalated ducts; 3, distribution of MEC is equal between acini and intercalated ducts; 4, most MEC invest intercalated ducts, some invest acini; 5, all MEC invest intercalated ducts.

^a High T4 pups in this column are age 12 days.

^b Significant difference from vehicle/normal group at this age.

Table 2Proportion of PCNA-positive parenchymal cells, all types combined (mean % $\pm$ SE)

Treatment	Age (days)			
	4	7	10	15 ^a
High	29.4 $\pm$ 1.4	10.5 $\pm$ 0.5 ^b	4.6 $\pm$ 0.6 ^b	1.5 $\pm$ 0.3 ^b
Medium	35.0 $\pm$ 0.8	15.8 $\pm$ 2.4	7.2 $\pm$ 0.9 ^b	2.0 $\pm$ 0.2 ^b
Low	32.5 $\pm$ 3.6	16.2 $\pm$ 1.7	12.2 $\pm$ 1.5	3.8 $\pm$ 0.4 ^b
Vehicle/Normal	31.8 $\pm$ 2.2	21.0 $\pm$ 1.3	17.1 $\pm$ 1.7	12.5 $\pm$ 1.0

^aHigh T4 pups in this column are age 12 days.^bSignificant difference from vehicle/normal group at this age.

Table 3

Proportion of PCNA-positive cells by cell type (mean % $\pm$ SE)

Age (days)	Treatment	Acinar cells	Myoepithelial cells	Intercalated duct cells	Large duct cells
4	High	37.5 $\pm$ 1.7	50.0 $\pm$ 2.6	28.4 $\pm$ 1.6	13.1 $\pm$ 2.1
	Medium	41.6 $\pm$ 1.1	49.4 $\pm$ 2.1	29.5 $\pm$ 1.1	20.4 $\pm$ 2.7
	Low	36.7 $\pm$ 5.1	51.6 $\pm$ 2.1	24.2 $\pm$ 1.4	14.9 $\pm$ 3.2
	Vehicle/Normal	39.7 $\pm$ 3.1	46.7 $\pm$ 2.8	24.3 $\pm$ 1.9	19.0 $\pm$ 3.6
7	High	16.0 $\pm$ 2.4 ^b	9.0 $\pm$ 1.0 ^b	10.0 $\pm$ 0.4 ^b	5.7 $\pm$ 2.3
	Medium	23.2 $\pm$ 2.6	17.3 $\pm$ 2.8	13.5 $\pm$ 1.6	2.8 $\pm$ 0.8 ^b
	Low	20.8 $\pm$ 1.8	16.6 $\pm$ 1.9	14.4 $\pm$ 2.3	3.5 $\pm$ 0.9 ^b
	Vehicle/Normal	27.7 $\pm$ 2.1	19.7 $\pm$ 2.0	16.8 $\pm$ 0.9	9.2 $\pm$ 1.1
10	High	9.0 $\pm$ 1.9 ^b	3.6 $\pm$ 0.6 ^b	7.3 $\pm$ 1.8	4.1 $\pm$ 0.8
	Medium	15.6 $\pm$ 1.9	4.8 $\pm$ 1.6 ^b	5.3 $\pm$ 0.6 ^b	3.3 $\pm$ 0.5
	Low	15.8 $\pm$ 2.2	10.9 $\pm$ 2.7	6.4 $\pm$ 0.8	2.9 $\pm$ 1.1
	Vehicle/Normal	19.9 $\pm$ 2.3	14.9 $\pm$ 3.1	9.6 $\pm$ 1.2	6.9 $\pm$ 2.2
15 ^a	High	1.0 $\pm$ 0.4 ^b	3.8 $\pm$ 0.5	3.7 $\pm$ 1.4	1.7 $\pm$ 0.9
	Medium	4.1 $\pm$ 0.9 ^b	1.6 $\pm$ 0.4 ^b	4.0 $\pm$ 0.7	3.3 $\pm$ 0.8
	Low	13.6 $\pm$ 4.4	1.0 $\pm$ 0.4 ^b	7.8 $\pm$ 1.5	5.0 $\pm$ 1.1
	Vehicle/Normal	15.6 $\pm$ 2.4	6.9 $\pm$ 1.0	7.5 $\pm$ 1.1	5.1 $\pm$ 1.1

^a High T4 pups in this row are age 12 days.^b Significant difference from vehicle/normal group at this age.