

—Original—

# Relationship between morphological development and sex hormone receptor expression of mammary glands with age in male rats

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**Abstract:** The aim of this study is to investigate the changes with age on morphology and sex hormone receptor expression in the mammary glands of male Sprague-Dawley rats, focusing on male-specific cells, “oxyphilic cells”, observed after sexual maturity. The mammary glands of male rats at 14, 21, 35, 50, 75 and 100 days old were examined by gross observation, microscopic observation using whole mount specimens, histological and immunohistochemical sections. Grossly, mammary glands showed brown color at 50–100 days old. In whole mount specimens, terminal end buds (TEBs) were observed at 14–50 days old and the number of TEBs was highest at 35 days old. Histologically, the male mammary glands contained small epithelial cells with scanty cytoplasm at 14–35 days old while ductal and lobular epithelial cells were changed into oxyphilic cells with abundant cytoplasm at 50–100 days old. Immunohistochemically, androgen receptor (AR), estrogen receptor (ER) and progesterone receptor (PgR) expressions were found in both mammary glands found at a young age and oxyphilic cells. In oxyphilic cells, AR expression was dominant compared to ER and PgR expressions and increased with age. From these results, the development at 50–100 days old might be strongly related to AR. Ultrastructural observation of oxyphilic cells confirmed a number of lipid droplets, deformed and/or enlarged mitochondria, lysosomes and peroxisomes in their cytoplasm.

**Key words:** development, hormone receptor, male rat, mammary gland, oxyphilic cell

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## Introduction

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Breast cancer is the second most common cancer worldwide and the most frequent cancer in women [7]. Breast cancer in men is rare, with less than 1% of all breast cancer diagnoses [17], while the incidence is increasing [10, 34]. Rats have been used for the mam-

mary carcinogenesis model [13, 15, 27, 31, 36], and several investigators have reported the development of mammary glands with age in female rats [19, 20, 24, 29]. Little is known about the male mammary glands in rats except for limited information from a few reports [4, 8, 11, 21, 28]. In particular, male-specific cells in mature male rats, with abundant and eosinophilic cyto-

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plasm, were described in some reports; however, the details remain unknown. Additionally, although sex hormone receptors were strongly related to mammary gland development, only limited information is available on intact male mammary glands.

More information is necessary to understand the morphological characteristics and hormone receptor expression in mammary gland development of male rats. The two specific aims of this study are (1) to investigate morphological changes in the male mammary glands with age from preweaning to adult stage, especially focusing on male-specific cells called "oxyphilic cells" [36], and (2) to clarify the relationship between morphological changes and hormone receptor expression.

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## Materials and Methods

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### *Ethics statement*

The use of animals in this research complied with all relevant guidelines set by Kagoshima University. The research was performed according to the Institutional Guidelines for Animal Experiments and in compliance with the Japanese Law Concerning the Protection and Control of Animal (Law No. 105 and Notification No. 6), and was approved by the Ethics Committee for Animal Experimentation at Kagoshima University (approval number 2004M00434, 2005M00012, A08054, A09032).

### *Animals*

Inbred Sprague-Dawley (SD) male rats (*Rattus norvegicus*) used in the present experiments were bred by self-breeding in Kagoshima University. The animals were maintained in a filtered air laminar flow room. The room temperature was maintained at  $25 \pm 2^\circ\text{C}$  and the relative humidity at  $55\% \pm 10\%$ , with a 12 h light/dark cycle. The animals were given a commercial diet (CE-2, CLEA Inc., Tokyo, Japan) and tap water *ad libitum*.

All animals were humanely euthanized under deep anesthesia using pentobarbital sodium (intraperitoneally, Somnopentyl; Kyoritsu Seiyaku Corporation, Tokyo, Japan), exsanguinated from the abdominal aorta, and necropsied at 14, 21, 35, 50, 75 and 100 days old (14–35 days old: young age, 50–100 days old: adult age). The number of animals in each group was five. Mammary glands were grossly observed, and bilateral abdominal mammary glands were resected and fixed in 10% phosphate-buffered formalin. Additionally, one adult male rat at 75 days old was used to obtain the samples of mammary glands for

transmission electron microscopy (TEM) examination.

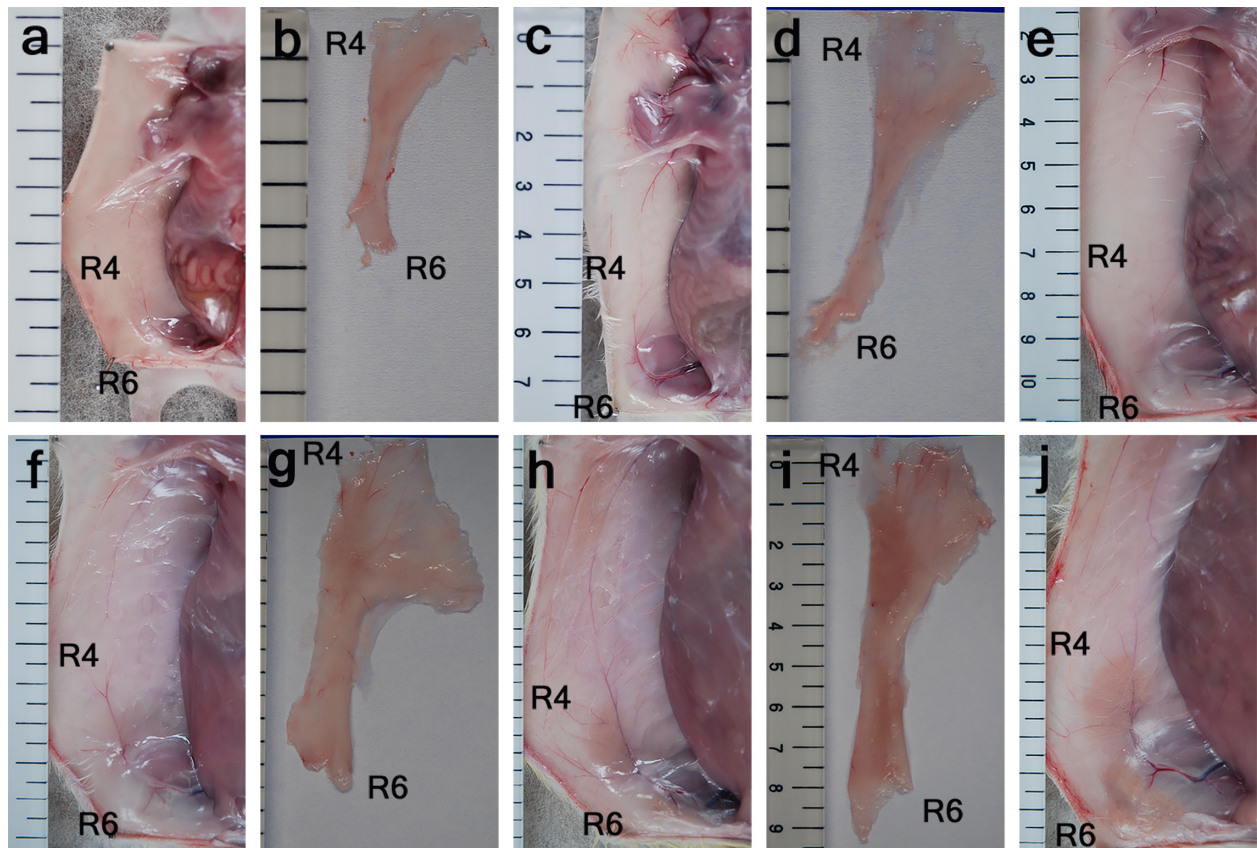
### *Whole mount specimens*

The resected right abdominal mammary glands (gland #4–6) were stained with alum carmine for 24 h and stored in cedar oil for preparation of whole mount specimens. The specimens were observed under a stereoscopic microscope, and the terminal end buds (TEBs) in the distal portions of mammary glands were counted as described previously [9, 27, 35].

### *Histological and immunohistochemical examination*

The resected left abdominal mammary glands were routinely processed for paraffin-embedded tissue sections. Five  $\mu\text{m}$  sections were stained with hematoxylin and eosin (H&E) for histological evaluation. For immunohistochemistry (IHC), the Dako Envision Polymer method was used. Paraffin slides were deparaffinized in xylene and rehydrated with graded ethanol. Antigen retrieval was performed by microwave treatment in Target Retrieval Solution, pH9.0 (Dako, Tokyo, Japan). Endogenous peroxidase was inactivated by 3% (v/v) hydrogen peroxidase-methanol solution. The sections were incubated overnight at  $4^\circ\text{C}$  with diluted primary antibodies: mouse monoclonal anti human androgen receptor (AR; Perseus Proteomics Inc., Tokyo, Japan, clone H7507, dilution 1:1,000), mouse monoclonal anti human estrogen receptor (ER; Dako, clone 1D5, dilution 1:50) and rabbit polyclonal anti human progesterone receptor (PgR; Santa Cruz, Tokyo, Japan, clone C-19, dilution 1:1,000). After rinsing with PBS, the sections were incubated with peroxidase-labelled polymer (Dako) for 60 min at room temperature and washed with PBS. Immunoreactivity was visualized with 0.05M Tris buffer containing 3,3'-diaminobenzidine tetrachloride (DAB) and 0.027% hydrogen peroxide (pH 7.5) for 2–10 min at room temperature. The sections were then washed with Tris buffer and PBS, counter-stained for 20 s in Mayer's hematoxylin, dehydrated, cleared in xylene, and mounted.

IHC examination was quantified by counting immunostained nuclei as described previously [14]. Three areas at magnification  $400\times$  per the three elements (the acini, ducts and lobules consisted of oxyphilic cells) were selected randomly. A percentage of the total epithelial cell nuclei examined was recognized as the results of IHC examination. The ducts, acini and oxyphilic cells in each microscopic field contained approximately 50–80, 70–330 and 120–580 epithelial cells, respectively.



**Fig. 1.** Gross examination. Right mammary glands in male rats at 14 (a, b), 21 (c, d), 35 (e), 50 (f, g), 75 (h, i) and 100 days old (j). Brown mammary glands were observed at 50–100 days old (f–j).

#### *TEM examination*

The specimens for TEM were prepared to observe the ultrastructure of oxyphilic cells in male rat mammary glands as previously described manner [25]. The mammary gland tissues were cut into approximately 1-mm<sup>3</sup> pieces, fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, and post-fixed in 1% osmium tetroxide. After dehydration through a graded ethanol series, the samples were embedded in resin consisted of methylnadic anhydride (MNA), Epok812, dodecenly succinic anhydride (DDSA) and 2,4,6-tris (dimethylaminomethyl) phenol (DMP-30). Ultrathin sections were prepared and stained with uranyl acetate and lead citrate, then observed using TEM (H-7000KU, Hitachi, Tokyo, Japan).

#### *Statistical analysis*

The mean differences were analyzed by Student's *t*-test. Data are presented as mean  $\pm$  SD. Dr. SPSS II program for Windows (SPSS Inc., Chicago, USA) was used

for statistical analysis. *P* values < 0.05 were considered to be statistically significant. Mean differences were analyzed by Student's *t*-test. Mean percentages of AR, ER or PgR-positive cells were evaluated by the nonparametric Mann-Whitney test.

## Results

#### *Gross examination*

Grossly, brown-colored mammary gland tissues were observed in all animals at 50–100 days old, however, they were not observed in all animals at 35 days old or younger (Fig. 1).

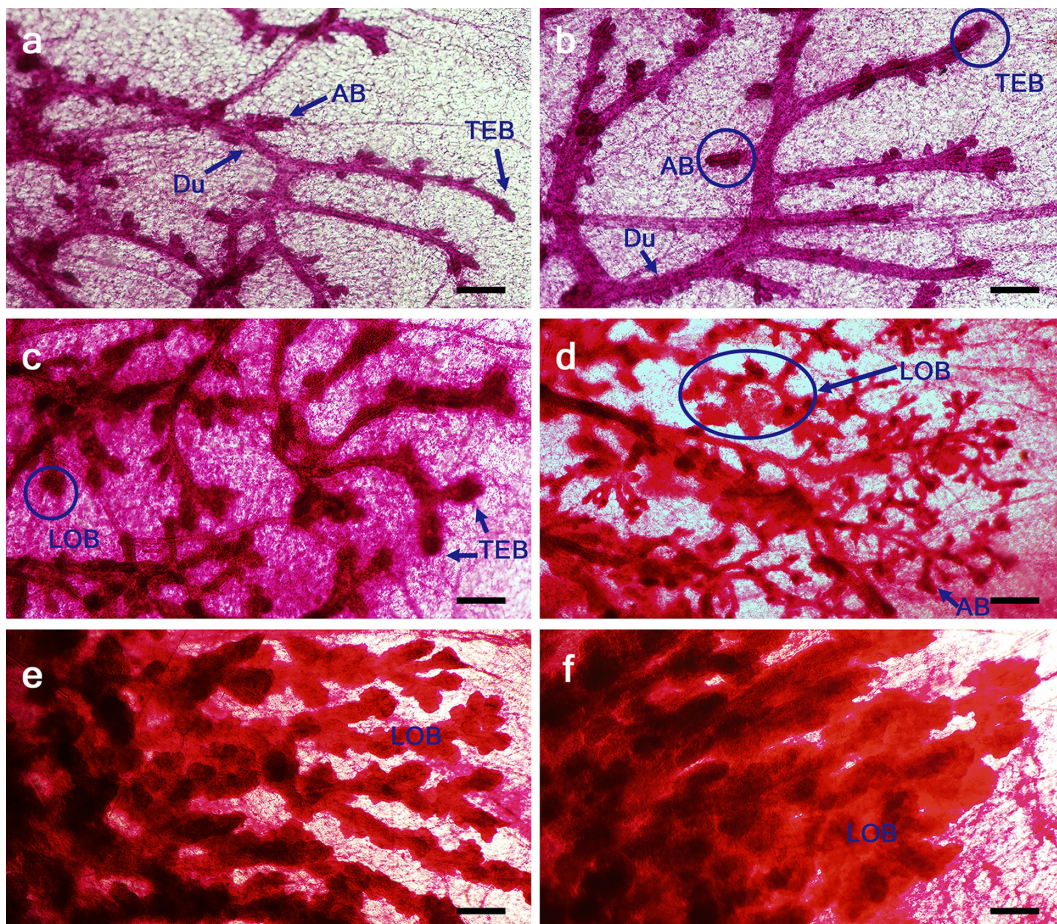
#### *Observation by stereoscopic microscope in whole mount specimens*

There observed morphological changes with age in mammary glands of male rats (Table 1). At 14 and 21 days old (Figs. 2a and b), the mammary glands were consisted of ducts (dendritically branched main struc-

**Table 1.** Morphological changes of the mammary glands in male rats (whole mount specimens)

Age (days)	Number of rats	Immature TEB	TEB	AB	Lobule	Duct	Number of TEBs
14	5	+	-	+	-	++	8.0 ± 2.3
21	5	++	-	+	-	++	16.2 ± 6.8 <sup>a)</sup>
35	5	-	++	++	+	++	98.0 ± 17.4 <sup>b)</sup>
50	5	-	+	+	++	++	21.0 ± 12.3 <sup>c)</sup>
75	5	-	-	±	+++	+	0.0 <sup>d)</sup>
100	5	-	-	±	+++	±	0.0 <sup>d)</sup>

Grade: -, negative, ±, very slight, +, slight, ++, moderate, +++; marked. a)  $P < 0.05$ : significantly different from 14 days. b)  $P < 0.01$ : significantly different from 21 days. c)  $P < 0.01$ : significantly different from 35 days. d)  $P < 0.05$ : significantly different from 50 days. TEB, terminal end bud; AB, alveolar bud.



**Fig. 2.** Whole mount specimen. Morphological changes of the mammary glands in male rats at 14 (a), 21 (b), 35 (c), 50 (d), 75 (e) and 100 days old (f). TEB: terminal end bud, AB: alveolar bud, LOB: lobule, Du: duct. Scale bars, 500  $\mu\text{m}$ .

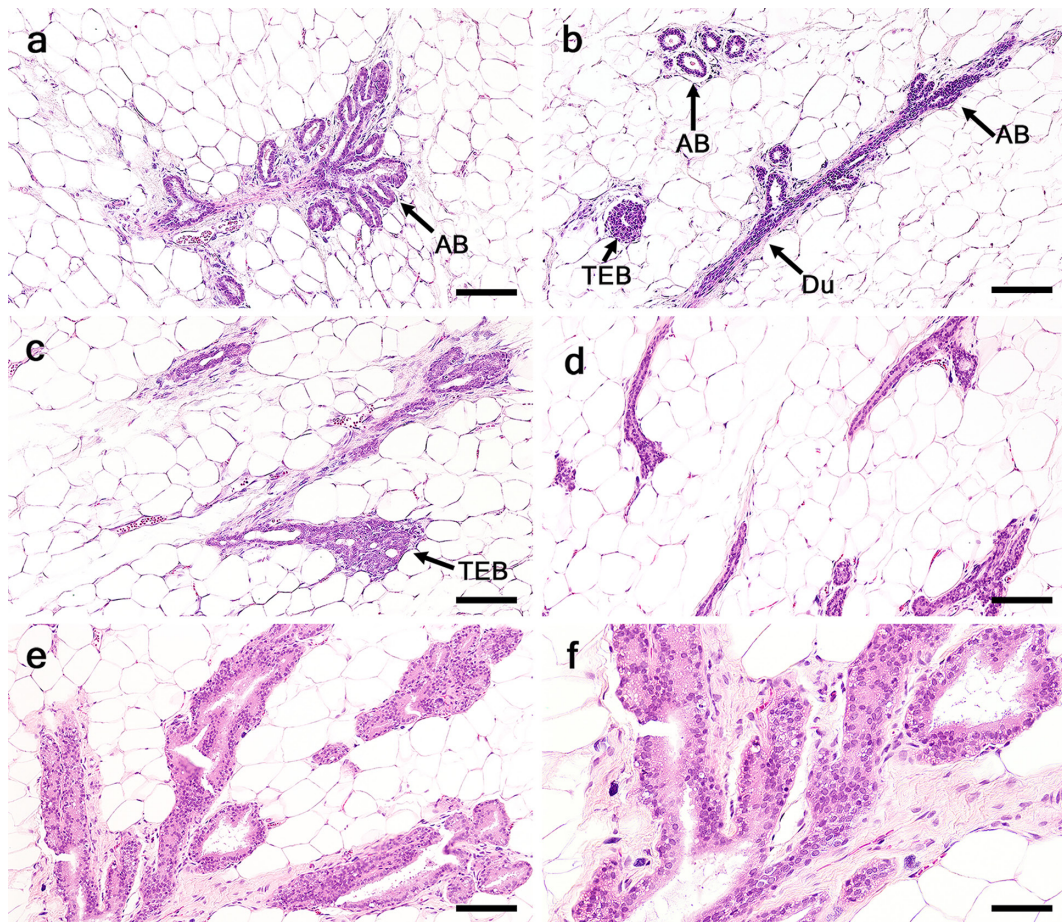
tures), immature TEBs (slightly enlarged terminal buds on a duct) and alveolar buds (ABs; structures with several projections branching from a duct). At 35 days old (Fig. 2c), TEBs (markedly enlarged terminal bud parts on a duct, shaped like teardrop) were appreciably observed and lobules (more mature clustered structures

with multiple projections) also appeared. At 50–100 days old (Figs. 2d–f), the mammary glands predominantly consisted of the lobules while ABs and TEBs tended to decrease with age. The density of mammary glands was getting higher with age mainly because of remarkable development of the lobules.

**Table 2.** Histological changes of the mammary glands in male rats (H&E stain)

Age (days)	Number of rats	Morphology at a young age					Oxyphilic cell		
		Immature TEB	TEB	AB	Lobule	Duct	AB	Lobule	Duct
14	5	++	-	+	-	++	-	-	-
21	5	++	-	+	-	++	-	-	-
35	5	-	++	++	++	++	-	-	-
50	5	-	±	±	+	+	+	++	+
75	5	-	-	-	-	±	±	++	+
100	5	-	-	-	-	±	±	+++	±

Grade: -, negative, ±, very slight, +, slight, ++, moderate, +++; marked. TEB, terminal end bud; AB, alveolar bud.

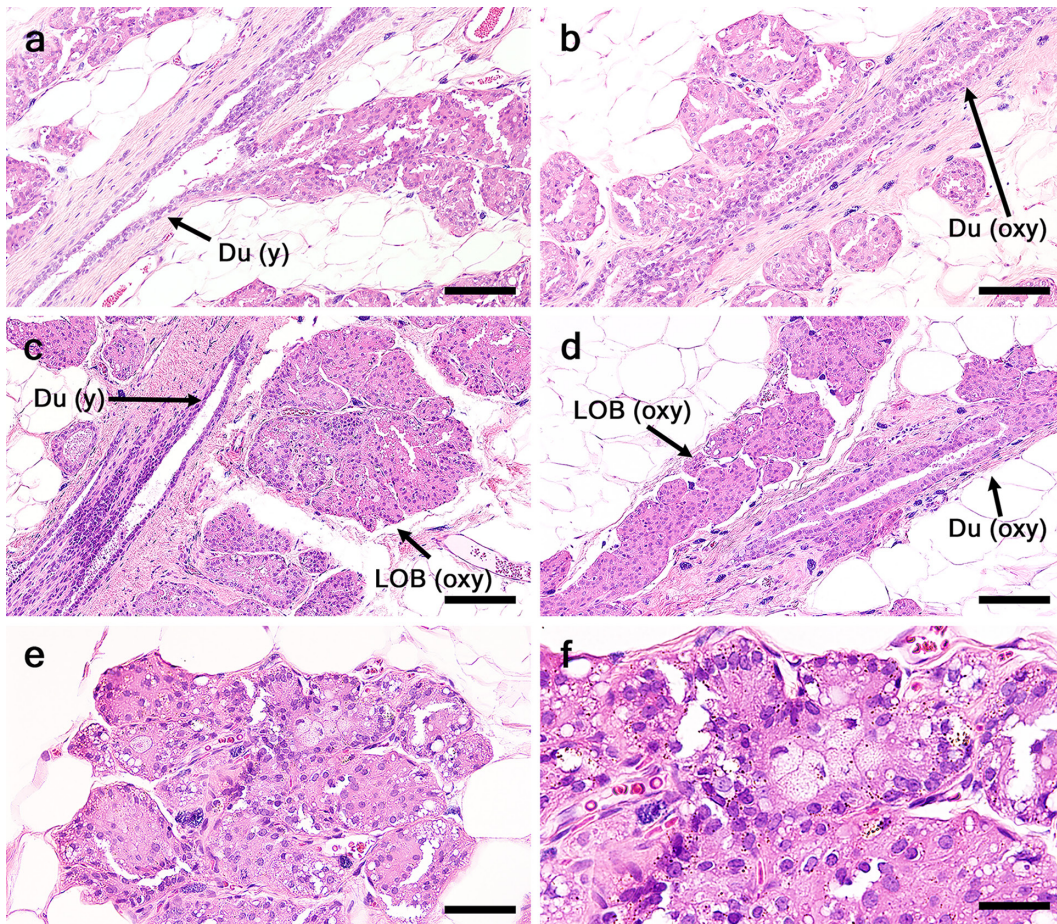


**Fig. 3.** Histological examination of the mammary glands in male rats at 14 (a), 21 (b), 35 (c) and 50 days old (d-f) (H&E stain). At 50 days old, both structures with morphology at a young age (d) and oxyphilic cells (e) were observed. (f) is the enlarged figure of structure with oxyphilic cells. TEB: terminal end bud, AB: alveolar bud, Du: duct. Scale bars, 100  $\mu\text{m}$  (a-e), 50  $\mu\text{m}$  (f).

TEBs numbers were counted in the distal portions of right mammary glands (Table 1). TEBs were observed at 14–50 days old and the number of TEBs was highest at 35 days old. The number of TEBs increased approximately 6-fold between 21 and 35 days old. At 75 and 100 days old, no TEBs were found.

#### *Histological examination*

The histological changes with age were summarized in Table 2. The male mammary glands contained small epithelial cells with scanty cytoplasm at 14–35 days old (Figs. 3a–c). The mammary glands were composed of ducts, ABs and immature TEBs at 14 and 21 days old,



**Fig. 4.** Histological examination of the mammary glands in male rats at 75 (a, b) and 100 days old (c–f) (H&E stain). (e) and (f) are the enlarged figures of a lobule consisted of oxyphilic cells. Du (y): duct with morphology at a young age, Du (oxy): duct consisted of oxyphilic cells, LOB (oxy): lobule consisted of oxyphilic cells, Scale Bars, 100  $\mu\text{m}$  (a–d), 50  $\mu\text{m}$  (e), 25  $\mu\text{m}$  (f).

and TEBs with significantly multilayered epithelium were found in addition to the abovementioned two elements (ducts and ABs) at 35 days old. At 35 days old, all TEBs were matured; no immature TEBs were observed. At 50 days old (Figs. 3d–f), oxyphilic cells with abundant eosinophilic cytoplasm appeared in the mammary glands in addition to structure at a young age (14–35 days old). No oxyphilic cells were observed at 35 days and younger. At 75 and 100 days old (Figs. 4a–f), lobules and ducts consisted of oxyphilic cells were found, and the lumens became clearly smaller in diameter. Only a few ducts which found at a young age were observed, but the elements consisted of oxyphilic cells almost completely replaced the morphology at a young age. The cytoplasm of oxyphilic cells was granular and had many fine vacuoles (Figs. 4e–f).

#### *Immunohistochemical examination*

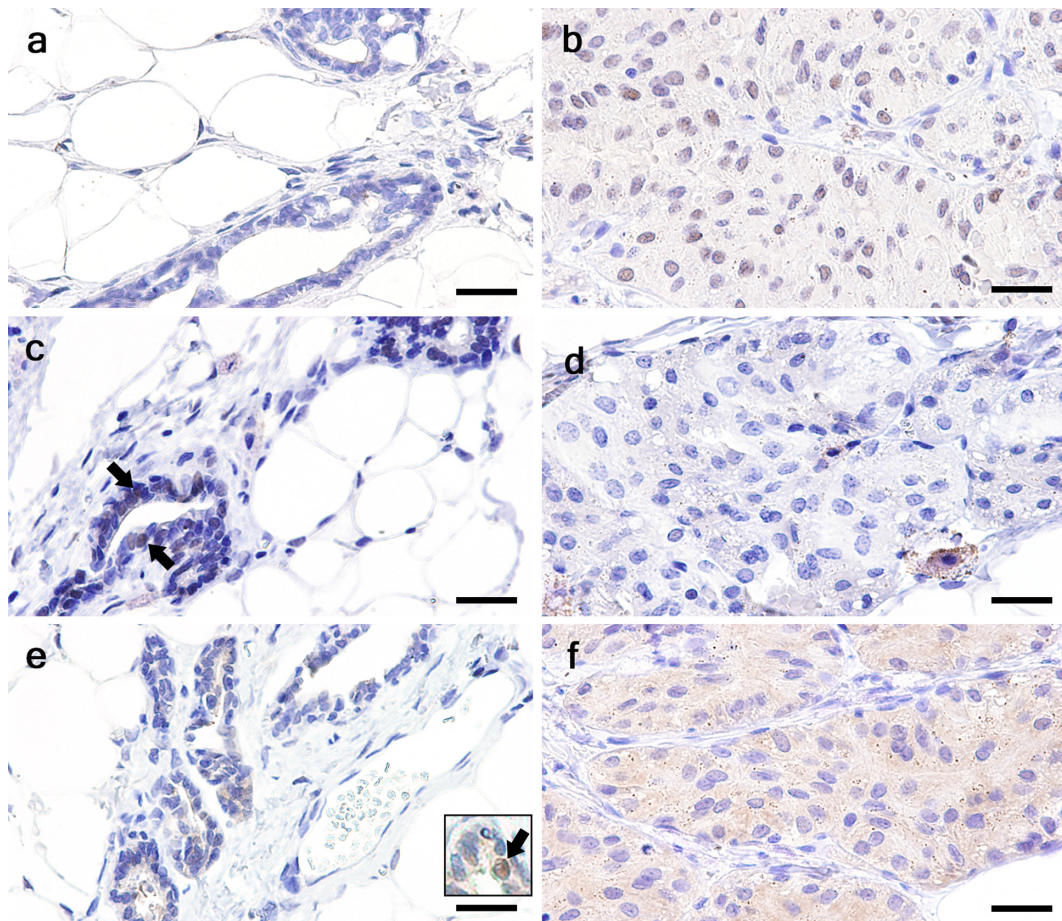
The changes of hormone receptor expression in the mammary glands with age were shown in Table 3. Representative pictures at 35 and 100 days old were shown in Fig. 5. In the mammary glands at 14–35 days old, AR expression was not found. At 50 days old, AR expression in the mammary glands was seen in the acini, ducts and oxyphilic cells. The proportion of AR expression in the oxyphilic cells increased with age from 50 days old and was higher than those in acini and ducts.

ER and PgR expressions were found in the acini and ducts at 14–50 days old and in the oxyphilic cells at 50–100 days old. The proportion of ER expression in epithelial cells of acini and ducts increased with age at 14–35 days old but decreased at 50 days old. In oxyphilic cells, the proportion of ER and PgR expressions was the highest at 50 days old while that of ER expres-

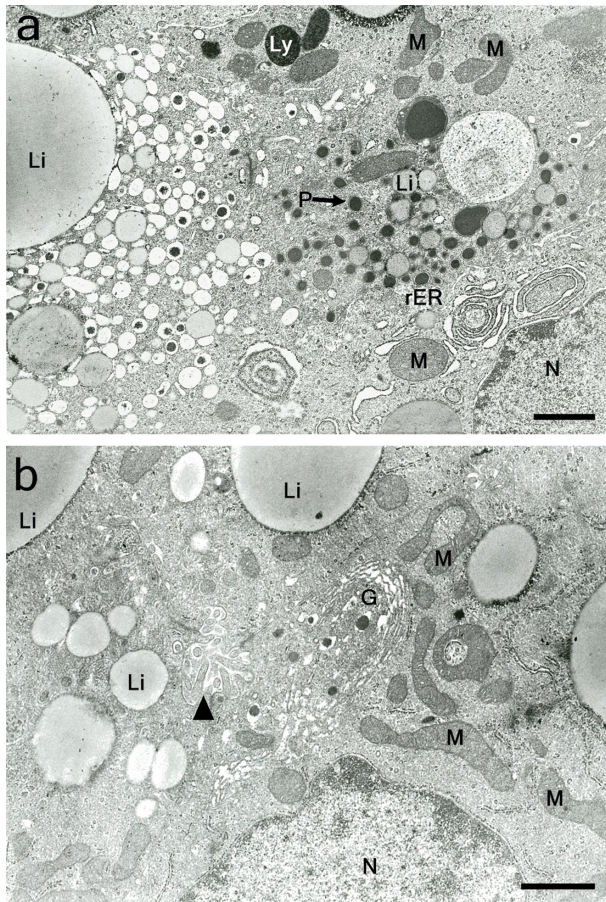
**Table 3.** Hormone receptor expression of the mammary glands in male rats

Age (days)		14	21	35	50	75	100
Number of rats		5	5	5	5	5	5
AR	Acini	0.0	0.0	0.0	11.8 ± 6.5 <sup>a)</sup>	NE	NE
	Ducts	0.0	0.0	0.0	22.9 ± 9.8 <sup>a)</sup>	NE	NE
	OCs	NA	NA	NA	50.1 ± 8.1	58.4 ± 6.2	71.3 ± 5.9 <sup>b)c)</sup>
ER	Acini	26.3 ± 2.3	29.1 ± 3.9	39.3 ± 8.3 <sup>d)</sup>	31.1 ± 12.0	NE	NE
	Ducts	16.4 ± 3.7	24.3 ± 3.5 <sup>e)</sup>	27.4 ± 7.9 <sup>d)</sup>	22.9 ± 2.2 <sup>d)</sup>	NE	NE
	OCs	NA	NA	NA	11.6 ± 2.0	2.7 ± 0.6 <sup>b)</sup>	2.2 ± 0.7 <sup>b)</sup>
PgR	Acini	2.0 ± 0.8	1.7 ± 1.2	9.7 ± 3.6 <sup>e)f)</sup>	12.6 ± 6.9 <sup>e)g)</sup>	NE	NE
	Ducts	2.5 ± 0.5	1.9 ± 2.2	5.6 ± 1.6 <sup>d)g)</sup>	17.4 ± 3.1 <sup>a)e)f)</sup>	NE	NE
	OCs	NA	NA	NA	45.9 ± 11.5	4.4 ± 2.1	1.7 ± 1.2 <sup>b)h)</sup>

Results are expressed as proportion (%) of positive cells. Percentage values are expressed as mean ± SD. a)  $P < 0.01$ : significantly different from 35 days. b)  $P < 0.01$ : significantly different from 50 days. c)  $P < 0.05$ : significantly different from 75 days. d)  $P < 0.05$ : significantly different from 14 days. e)  $P < 0.01$ : significantly different from 14 days. f)  $P < 0.01$ : significantly different from 21 days. g)  $P < 0.05$ : significantly different from 21 days. h)  $P < 0.01$ : significantly different from 75 days. AR, androgen receptor; ER, estrogen receptor; PgR, progesterone receptor; OCs, oxyphilic cells; NA, Not applicable; NE, Not evaluated.



**Fig. 5.** Immunohistochemical examination of the mammary glands in male rats. In the mammary gland at 35 days old (a, c, e), androgen receptor (AR) expression was not found (a) while estrogen receptor (ER) expression was found (c, arrows). Progesterone receptor (PgR) expression was observed in a small number of cells. The inset of (e) shows a PgR positive cell (arrow). In the oxyphilic cells at 100 days old (b, d, f), the portion of AR expression was high while the portions of ER and PgR were low. (a, b) AR, (c, d) ER, (e, f) PgR. Scale Bars, 25  $\mu$ m.



**Fig. 6.** Transmission electron microscopic examination of oxyphilic cells of the mammary glands in male rats at 75 days old. Small lumen with microvilli was found (b, arrowhead). N: nucleus, G: Golgi apparatus, rER: rough-surfaced endoplasmic reticulum, Ly: lysosome, P: peroxisome, M: mitochondria, Li: lipid drop. Scale bars, 1  $\mu$ m.

sion was lower than those of AR and PgR expressions. In addition, the proportions of ER and PgR expressions in the oxyphilic cells from 50 days old decreased with age, and the decrease of PgR expression between 50 and 75 days old was prominent. The proportions of ER and PgR expressions at 75 and 100 days old were extremely lower comparing with the proportion of AR expression.

#### TEM examination

In TEM examination of oxyphilic cells, a number of lipid droplets were found in the cytoplasm of oxyphilic cells. Lipid droplets were various in size. In addition, deformed and/or enlarged mitochondria, lysosomes and peroxisomes were observed in the cytoplasm. Small lumens with microvilli on their wall were also seen (Fig. 6).

## Discussion

We investigated the change with age about mammary gland development of male rats and evaluated the relation between the mammary gland morphological development and the hormone receptor expression, focusing on the oxyphilic cells. In the present study, grossly brown mammary glands were observed from 50 days old in male rats. Histologically, the epithelial cells composing mammary glands at 14–35 days old were different from those from 50 days old. The oxyphilic cells were observed from 50 days old, when the gross brown-colored change was detected. From whole mount specimen observation, the developments of ABs and lobules from the ducts and TEBs were seen with age. TEBs are solid or semisolid bulbous clusters of immature epithelial cells at the ends of ducts and important sites of epithelial cell proliferation during puberty [24]. The number of TEBs showed a similar tendency to the reported data about the mammary glands of female rats [8]. The number of TEBs prominently increased between 21 and 35 days old; consistent with a previous study about male rats [8].

Previous studies have demonstrated that the mammary glands in adult rats showed sexual dimorphism and male rats had male-specific cells [4, 8, 9, 11, 21, 22, 32, 33]. Sexual dimorphism in mammary glands has not been reported in other mammalian animals including mice [4]. The male-specific cells were consistent with oxyphilic cells in our study. The male-specific oxyphilic cells are considered to be derived from mammary epithelial and ductal epithelial cells by histological examination and the previous study [28]; however, they are not poorly understood in its development and ultrastructure. The TEM showed that the oxyphilic cells had numerous lipid droplets in various sizes and cytoplasm contained deformed and/or enlarged mitochondria, lysosomes and peroxisomes. Abundant cytoplasm might be caused by increased lipid droplets and large size mitochondria. In canine mammary gland tumor, the cells exhibited oncocytic metaplasia had abundant finely granular, eosinophilic and occasionally vacuolated cytoplasm, resulted from abundant mitochondria [26]. It is suggested that oxyphilic change in the male rat mammary glands is also related to enlarged mitochondria. There were some limitations to study, and we couldn't identify the biological significance of oxyphilic cells. Further studies are required to confirm their role in male rats.



In this study, the morphological changes with age are related to the expressions of sex hormone receptors. ER expression was mainly observed in the mammary glands at 14–35 days old, and AR expression was seen only from 50 days old, when oxyphilic cells appeared. Our data confirm that AR expression was found in most oxyphilic cells, thus AR is considered to be related to oxyphilic cells. Previous reports have demonstrated that the morphology of the mammary glands in rats was affected by their hormonal conditions [5, 30, 33]. In the mammary glands of castrated young male rats (about 80 days old) [33], the number of epithelial cells significantly decreased and the alveoli lumen was more obvious when compared with that of intact male rats. However, 5 $\alpha$ -dihydrotestosterone administration returned their mammary gland structure to that of the intact male rats. Conversely, the mammary glands in female rats treated with androgen were similar to those in intact male rats [5, 30, 33]. Serum testosterone concentration in male rats significantly increased from 4 days old and increased with ages at 21–160 days old, which is approximately 10 times higher than that in female rats at over 45 days old [1, 2]. In addition, sexual maturity in male rats, preputial separation, is known to occur at approximately 45–48 days old [18]. In this study, the appearance and high AR expression from 50 days old in the oxyphilic cells are considered to be associated with sexual maturity and blood androgen increased period. Therefore, it suggests that higher androgen exposure would be related to male-specific morphology of the mammary glands.

In contrast, estrogen is associated with normal mammary gland development in female rats, especially with TEB formation in the peri-pubertal period [9, 16]. Male rats exposed to ethynyl oestradiol during gestation and lactation had an increased number and density of TEBs compared with control animals [23]. These suggest that estrogen plays essential roles in mammary gland development in both male and female rats. In this study, ER expression was mainly seen at 14–35 days old; therefore, the effect of estrogen might regulate the mammary gland development during that period. However, blood estrogen level was markedly lower in male rats than in female rats up to 21 days old [2]. In addition, there was no obvious structural difference about mammary glands between wild-type and estrogen receptor knockout (ERKO) male mice while mammary glands were undeveloped in ERKO female mice compared with wild type

female mice [3]. It is reasonably assumed that the effect of estrogen via ER does not play the main role in the male mammary gland development. We also examined PgR expression. Progesterone is known to be essential in ductular and lobuloalveolar changes in prepubescent development and pregnancy [21]. Serum progesterone level had no clear difference between both sexes from birth to 21 days old in rats [6]. In male rats at 100 days old, progesterone level was lower than in female rats [12]. In this study, the PgR expression was seen at from 14 to 100 days old and the proportion of PgR expression markedly decreased from 75 days old. The progesterone might be related to male mammary gland development at young ages, not at adult ages. On the other hand, another report showed that no PgR expressions in mammary glands of male rats were observed at from 1 to 70 days old [8]. This difference on the PgR expression from our study was considered due to the differences of primary antibody used for IHC and rat strain. In addition, the other elements including prolactin and growth hormone might be related to the mammary gland development in male rats, similar to the report in female rats [29]. Further studies are required to reveal the relationship of sex hormone and other elements including prolactin and growth hormone with mammary gland development in male rats.

In conclusions, the oxyphilic cells appeared from 50 days old in mammary glands of male rats and were associated with high expression of AR. In addition, their abundant eosinophilic cytoplasm was suggested to be caused by an increased number of lipid droplets and enlarged/deformed mitochondria.

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### Conflict of Interest

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There were no conflicts of interest.

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