Part 4: Mammary Gland

Normal Physiology and Structure

The structure of the male and female rat mammary gland responds to hormonal stimulation, and can provide a sensitive indicator of endocrine disruption. The development and detailed structure of the male and female mammary gland as well as its response to endocrine active compounds has been reviewed recently by (Rudmann *et al.*, 2005; Lucas *et al.*, 2007)

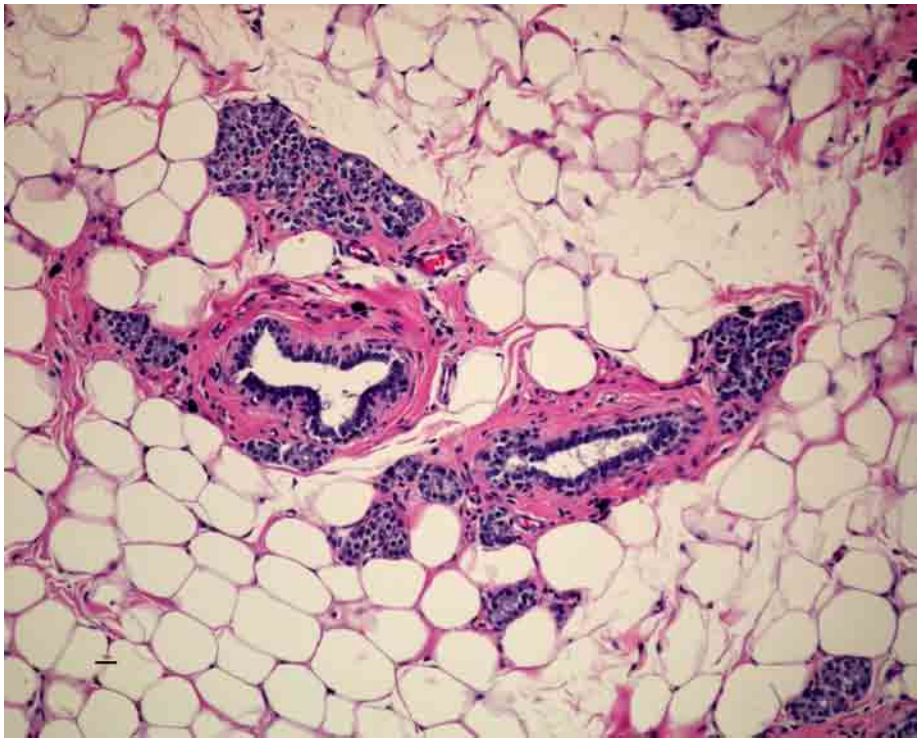
In most mammals, the male glandular tissue remains rudimentary, but the mammary gland of the rat is unique in showing marked development in the male and in showing sexual dimorphism (Cardy, 1991). Development of the mammary glands begins in utero and the overall structure is similar between males and females up until approximately post natal day 35 (beginning of male pubertal development), when the structure of the gland becomes recognizably divergent between the two sexes.

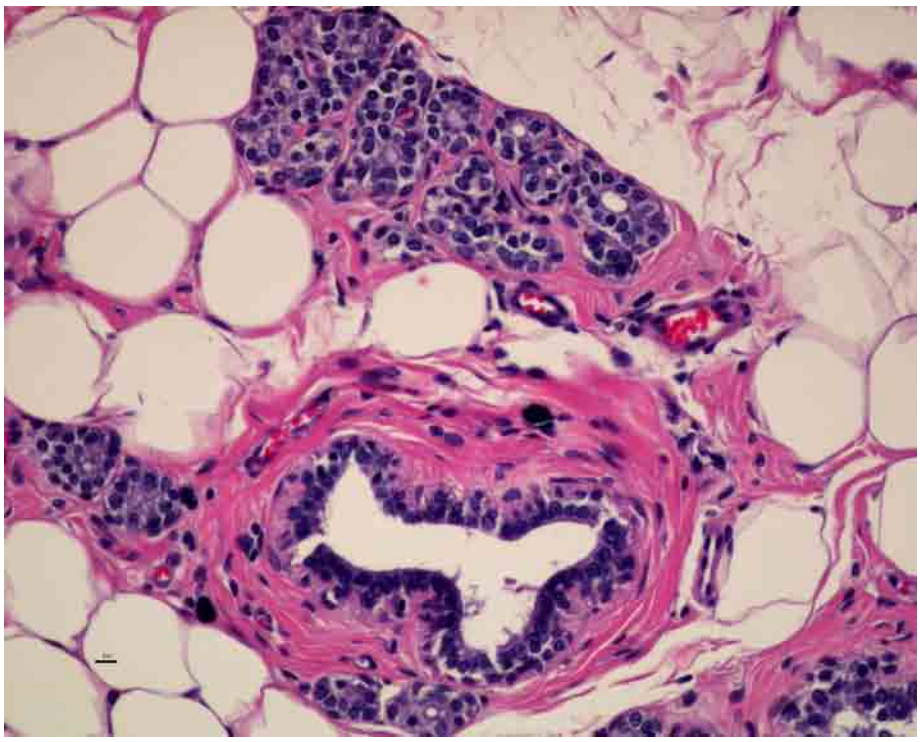
In the adult female rat, the mammary glands are made up of scattered tubular branching ducts and glandular alveoli, both lined by one to two layers of low cuboidal epithelium and both having well defined lumina. The epithelium rests on a basement membrane and is surrounded by a layer of myoepithelial cells, although these are generally difficult to distinguish in conventional H&E stained sections. The overall structure of the female gland is described as “tubuloalveolar”.

In males, the glandular tissue is greater in volume and is organized into lobules of alveoli that are made up of large, pale staining, foamy and vacuolated cells that are arranged around indistinct lumina that occasionally contain small amounts of secretory material. Although ducts are present, they are lined by multiple layers of epithelium, also have indistinct lumina and resemble alveoli. The structure in the male is termed “lobuloalveolar”.

In the virgin female, the mammary gland comprises fine branching ductules which lead to terminal end buds, which differentiate into alveolar buds and alveoli. The glandular tissue is surrounded by a connective tissue stroma and embedded in adipose tissue. Following puberty, the mammary gland of the female continues to grow in a branch like manner during each estrous cycle. Duct proliferation occurs during proestrous and estrous and regresses again during metestrous and diestrous (under the regulation of the oestrogen, progesterone and prolactin surges). In practice however, this growth and regression is not easily detectable by examination of conventional stained sections. Growth occurs only at the outer-most limits of the glandular network, where terminal end buds actively grow and differentiate, by branching and elongating into the surrounding fat pad.

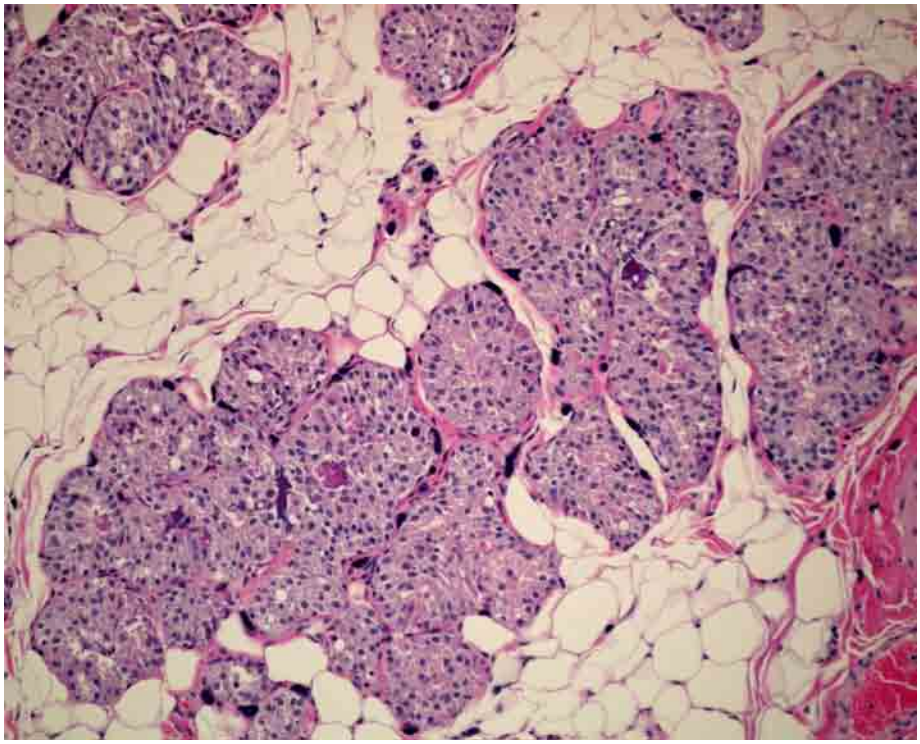
Figure 1: Normal female mammary gland



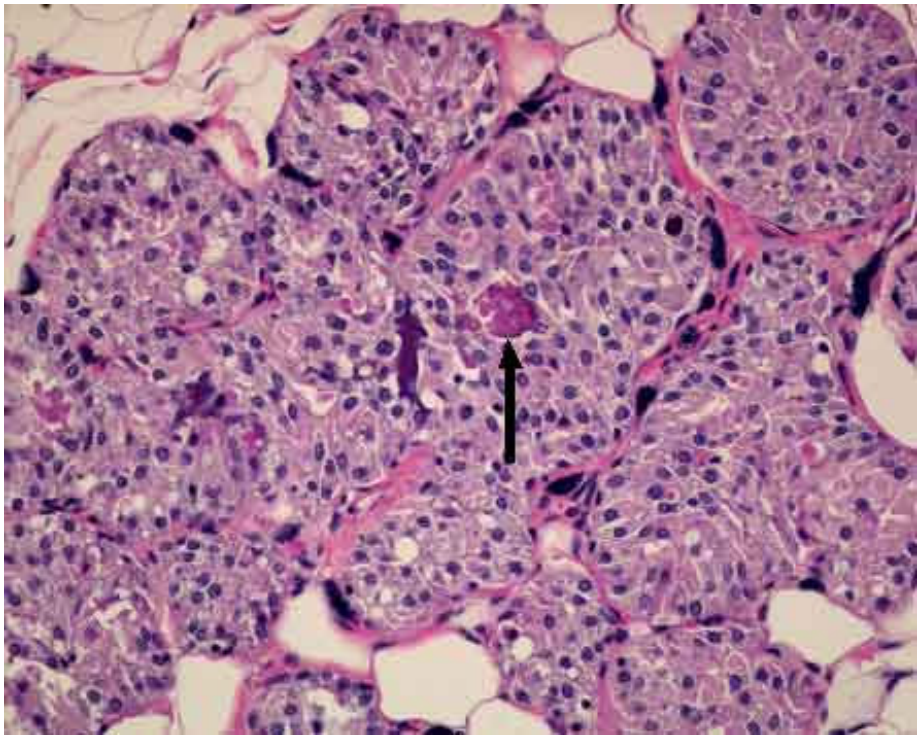


Normal <u>tubuloalveolar</u> female gland is made up of branching ducts that are surrounded by smooth muscle with small alveoli that bud off the ducts. Both ducts and alveoli have a discrete lumen.

Figure 2: Normal Male Mammary Gland



Normal lobuloalveolar male mammary gland is made up of larger lobules of large, pale staining, foamy and vacuolated cells that form alveoli with no clear lumen. Small droplets of proteinaceous secretory fluid (arrow) may be present in both



Hormonal Regulation of Glandular Function

Mammary gland development relies on the presence of growth hormone and prolactin, with oestrogen providing additional regulation of ductular development. During pregnancy, oestrogen, progesterone and prolactin act on the gland to produce a lobuloalveolar proliferation of the gland, more similar in appearance to the normal male gland.

The main modulating hormones of the mammary gland are estrogen, progesterone, prolactin and growth hormone but the mammotrophic action of most of these hormones are thought to be mediated through stroma-derived growth factors that are released locally (paracrine regulation) causing a mitogenic response in the adjacent epithelial structures rather than the hormones having a direct action on the glandular tissue. The cells of the fat pad are particularly important in this stromal paracrine interaction.

Oestrogen is important in proliferation and elongation of ductal structures from the terminal end buds while progesterone is more important for alveolar development but does also play a role in regulating ductal branching and ductal enlargement. Prolactin plays a role in mammary gland growth as well as initiating lactogenesis. The role of androgens in mammary gland development is largely undetermined but may be involved in the regulation of stem cell development in the terminal end buds of females and in the regulation of apoptosis. Androgens appear critical in producing and maintaining the male form of glandular differentiation.

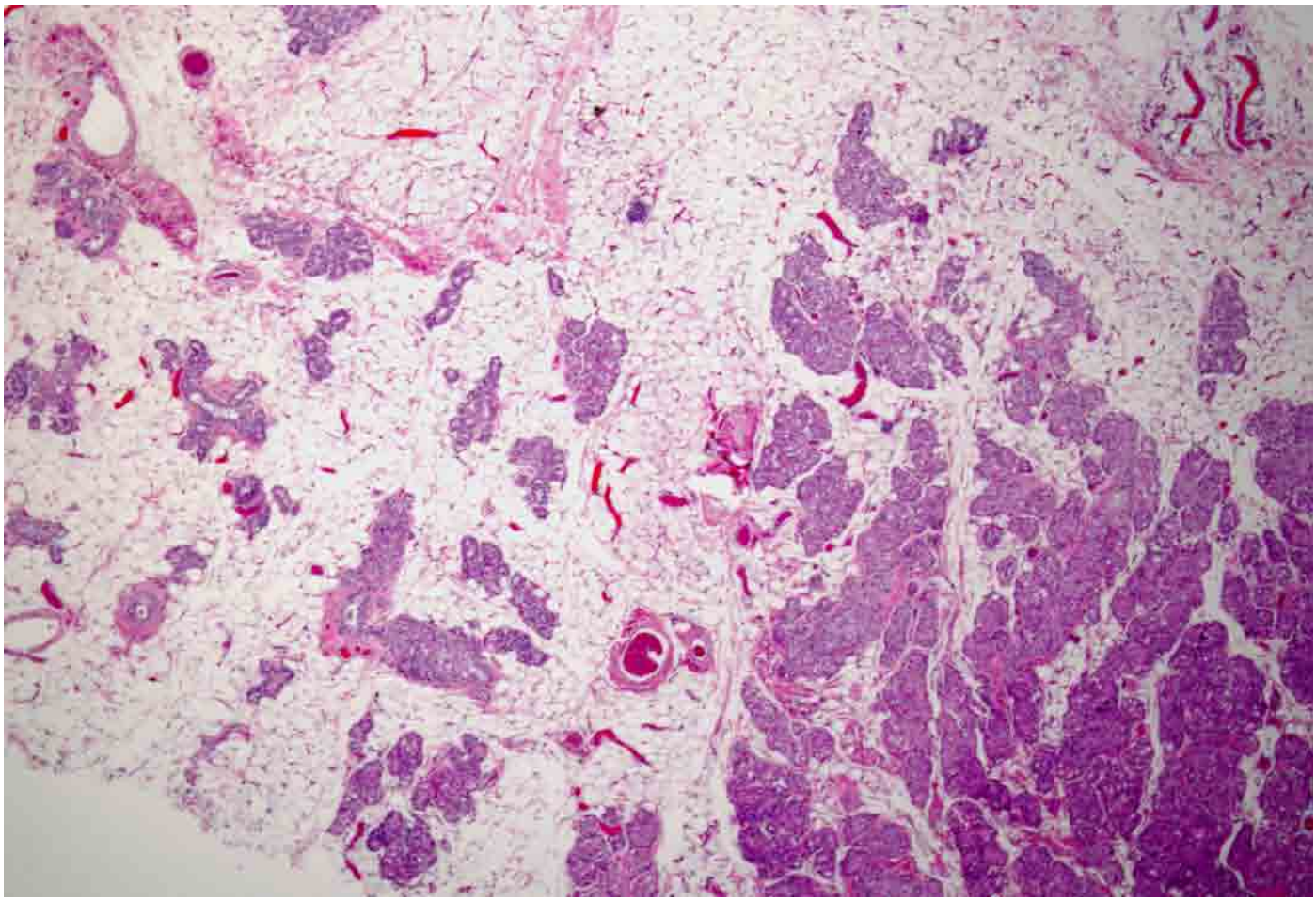
Normal Background Variation of Structure

In the growing male mammary gland, the terminal branches of the glandular tissue have a tubuloalveolar (female type) morphology before differentiating into the lobuloalveolar pattern. This can result in the presence of glandular alveoli with male and female pattern of differentiation adjacent to one another, or if the gland is sectioned only through this terminal area, it can give the appearance of male to female differentiation. Care should be taken not to confuse the newly growing terminal end buds with foci of epithelial hyperplasia (Lucas *et al.*, 2007).

Partial conversion of male to female pattern of glandular differentiation occurs as part of the normal ageing process in rats, probably due to an age related increase in prolactin secretion (Cardy, 1991). Due to the young age of rats used in the TG407 study design, this should not be a confounding factor for these studies.

Small amounts of proteinaceous eosinophilic secretion can occur as a normal finding in male and female glands, it should only be described as a finding if the amount of secretion is significantly above background levels.

Normal variation in male mammary gland structure



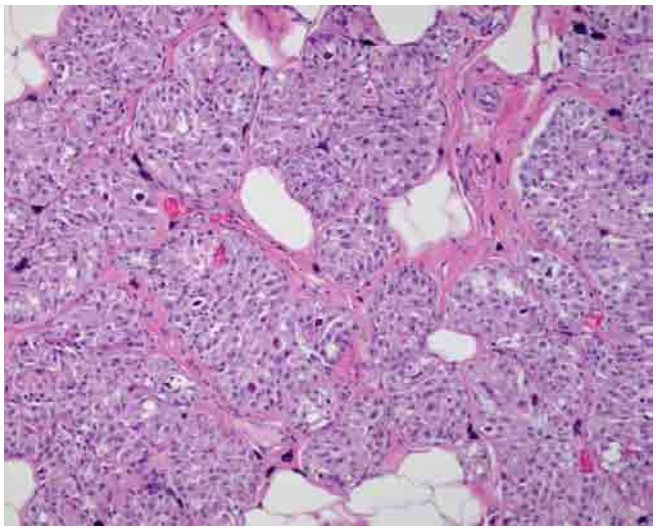
Normal male mammary gland with lobuloalveolar differentiation of the main gland (right) and tubuloalveolar differentiation of the proliferating terminal end buds (left). This is normal, but if only the terminal branches are present on a slide, the appearance may be mistaken as a test article induced change in differentiation.

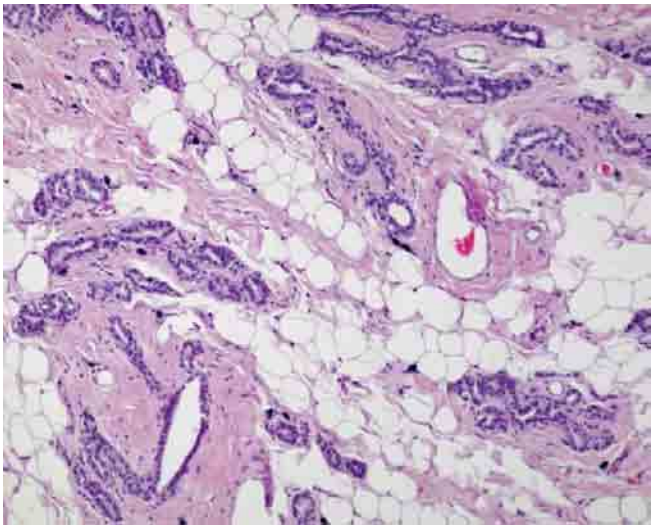
Morphologic Patterns of Hormone Disruption

Disruption of circulating levels of oestrogen, androgen, prolactin, growth hormone or their receptors has the potential to result in detectable changes in the male and/or female mammary gland. The main changes are atrophy, hyperplasia, or altered differentiation (feminization of male pattern or masculinization of female pattern). The precise change will depend on the hormones that have been disturbed. However, in most cases, disruption of one hormone generally causes secondary disruption of other hormones, so the picture can be complex. Patterns of change in the male and female gland associated with the disruption of various hormones has been recently reviewed by (Lucas *et al.*, 2007).

Male to female differentiation

In males administered estradiol, phytooestrogens, oestrogenic compounds, and hyperprolactinemic compounds, the normal large amount of lobuloalveolar glandular tissue is replaced by a small amount of tubuloalveolar tissue, typical of that seen in the female (Cardy, 1991; Biegel *et al.*, 1998; Andrews *et al.*, 2002; Wang *et al.*, 2006). The dramatic decrease in volume of the glandular tissue in affected males has also been described as "atrophy" (Okazaki *et al.*, 2001; Yamasaki *et al.*, 2002), but the important aspect of the change induced by oestrogens and prolactin, is that it represents altered differentiation from male to female acinar pattern. The oestrogenic action may be mediated through an effect on prolactin rather than a direct oestrogenic effect (Biegel *et al.*, 1998).





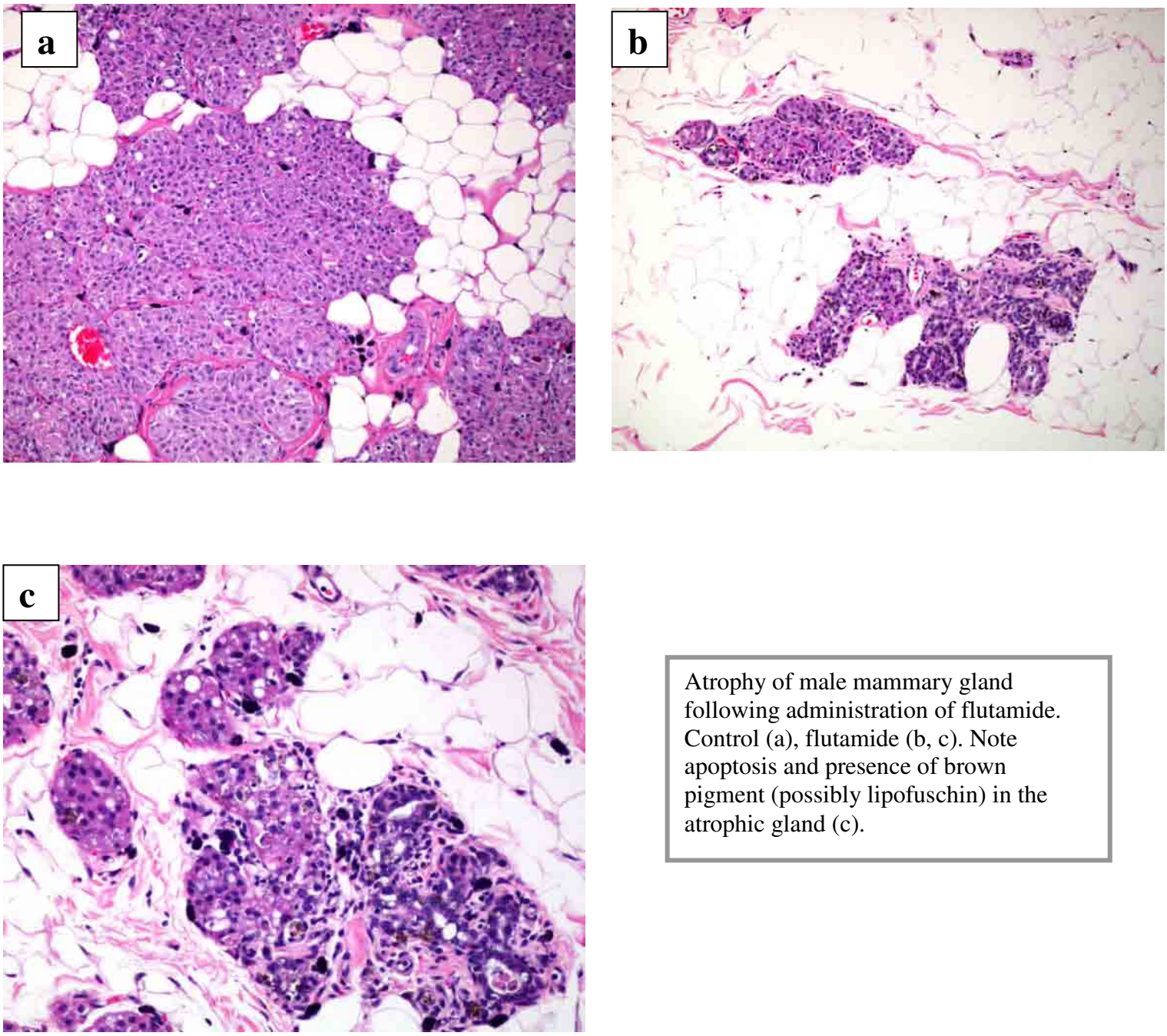
Normal lobuloalveolar male mammary gland (left) with conversion to tubuloalveolar female patter (right). This may be seen in response to estrogenic stimulation and following exposure to compounds that raise prolactin levels e.g. dopamine antagonists.

Male and female glandular atrophy

Atrophy of the glandular tissue in the male has been described in response to flutamide (Toyoda *et al.*, 2000) and has been suggested to be a likely response to any compound that causes decreased circulating testosterone levels e.g. inhibitors of testosterone biosynthesis (Rudmann *et al.*, 2005; Lucas *et al.*, 2007). However, this change has not been specifically described in the literature.

Atrophy of the male and female gland has also been described in response to potent oestrogen receptor antagonists e.g. tamoxifen and toremifene (Kennel *et al.*, 2003; Lucas *et al.*, 2007). In females, both

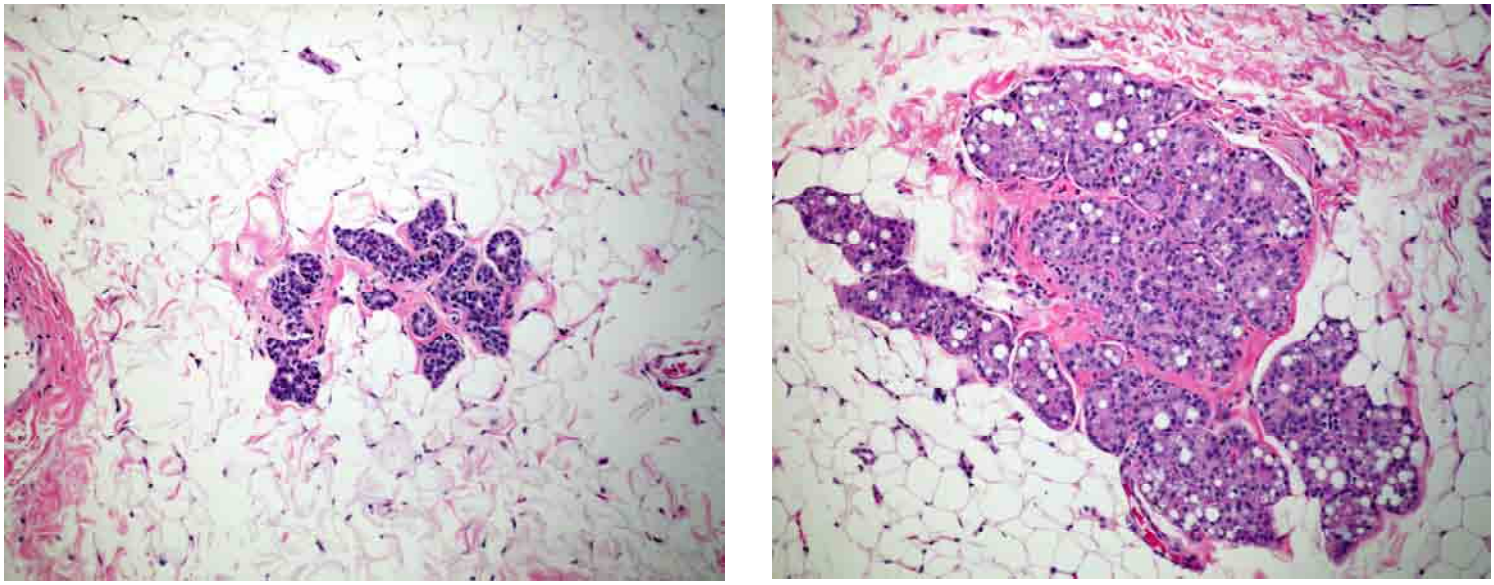
the alveoli and the ducts atrophy. However, other selective oestrogen receptor modulators (SERMs) such as LY2066948 that also cause hyperandrogenemia, result in masculinization of the female gland (see below).



Atrophy of male mammary gland following administration of flutamide. Control (a), flutamide (b, c). Note apoptosis and presence of brown pigment (possibly lipofuschin) in the atrophic gland (c).

Female to male differentiation

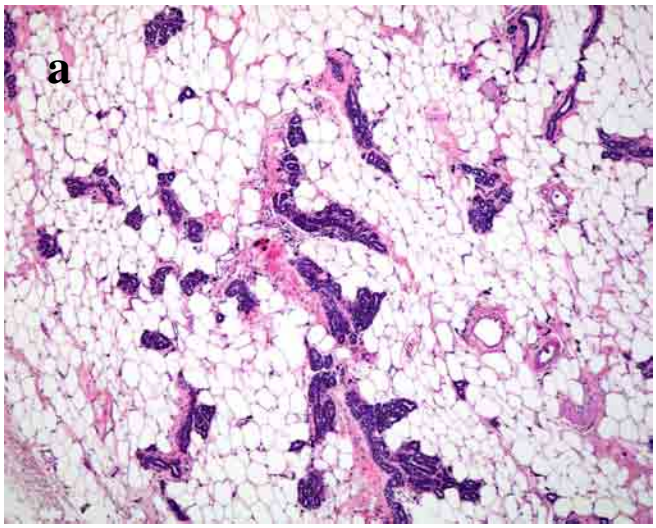
Females administered androgens (e.g. dihydrotestosterone, dehydroepiandrosterone, testosterone, 17 α methyl testosterone) develop lobuloalveolar glandular tissue replacing the normal tubuloalveolar pattern. A similar response is seen in the female gland following administration of some SERMs such as LY2066948 (Rudmann *et al.*, 2005), which are potent oestrogen receptor antagonists but also cause hyperandrogenemia. The altered glandular differentiation has been shown to be due to direct androgenic action on the mammary gland since it can be blocked using the androgen receptor blocker flutamide (Sourla *et al.*, 1998; Rudmann *et al.*, 2005). The lobuloalveolar hyperplasia seen in females in response to androgen administration may be difficult to distinguish from lobuloalveolar hyperplasia induced by oestrogenic compounds.

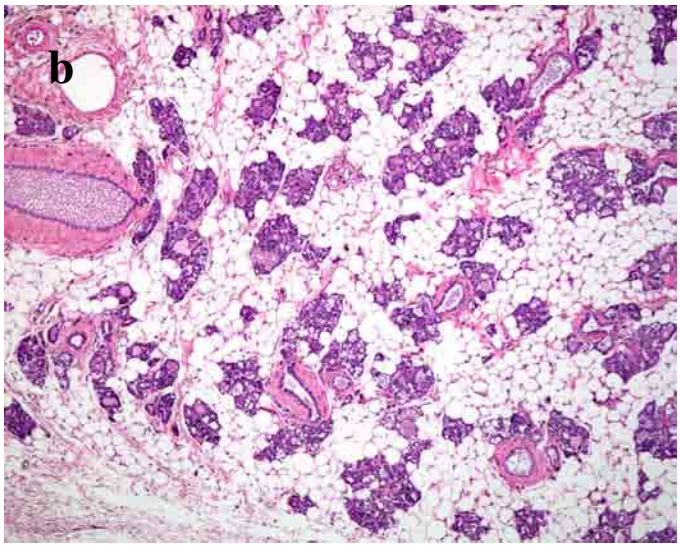


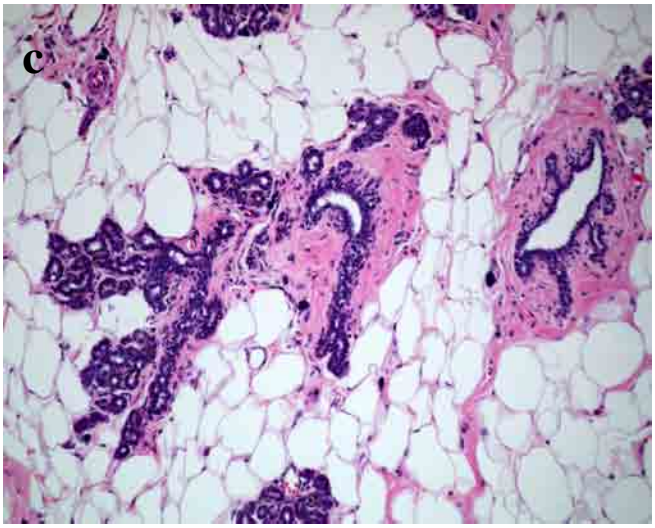
Normal female tubuloalveolar differentiation (left) may be changed to lobuloalveolar differentiation (right) following exposure to androgens e.g. methyl testosterone. Loss of discrete lumina and development of foamy vacuolation

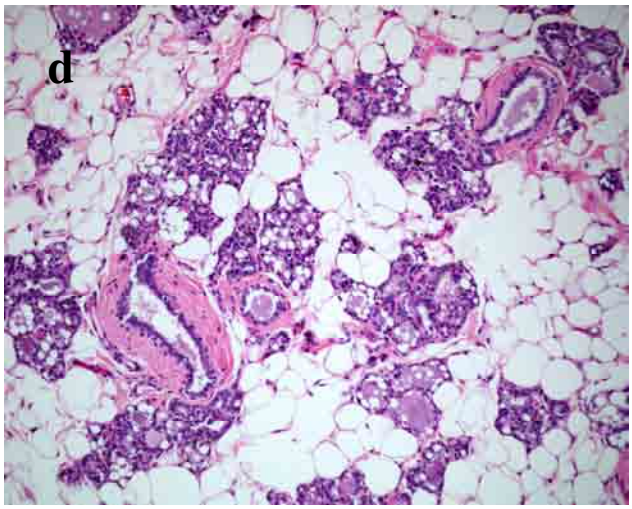
Female alveolar hyperplasia, ductal dilation, enhanced secretory activity

Hyperplasia of the female mammary gland occurs with administration of oestrogen and prolactin or oestrogenic and prolactinemic xenobiotics (Biegel *et al.*, 1998; Okazaki *et al.*, 2001; Harvey *et al.*, 2006). The change is characterized by multifocal or diffuse hyperplasia of the alveoli with or without ductal dilation and increased secretory material in the ducts and/or alveoli. The main difference between the response to oestrogens versus prolactin appears to be the additional stimulation of secretory activity.





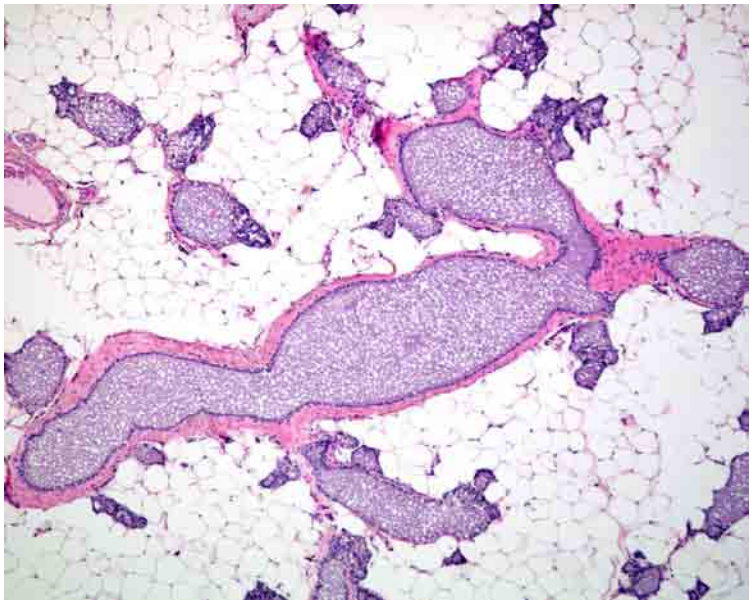


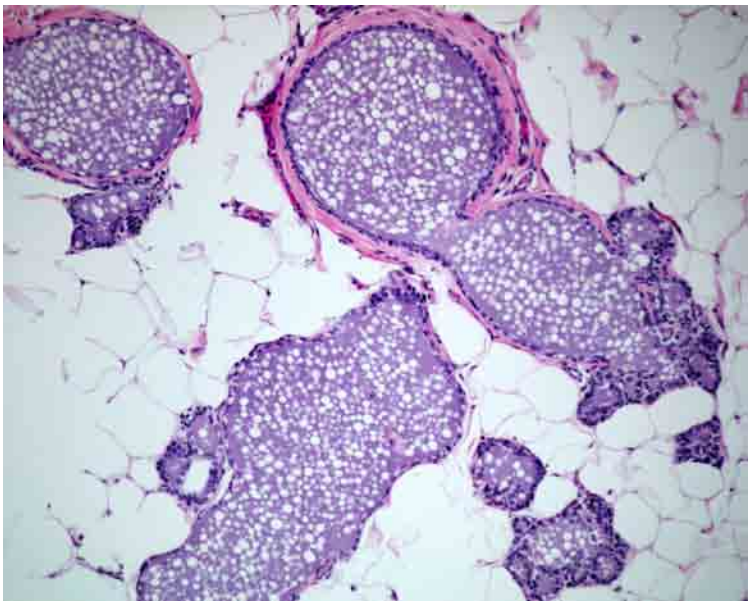


Normal female gland (a,c). Hyperplasia of the alveoli with increased secretory activity (b, d) may be seen with estrogenic and prolactogenic compounds. Ducts do not generally show hyperplasia but may become dilated and filled with secretion

Cystic dilation of ducts and enhanced secretory material has been observed with administration of oestrogenic or progestogenic components of combination steroid contraceptives and may be an indicator of disturbance of the hypothalamic-pituitary-gonad axis. Ducts and alveoli may also become dilated and cystic and filled with proteinaceous secretory fluid (galactoceles), in response to hyperprolactinemia

Although hyperplasia of the ductal epithelium is a common response to hormonal disturbance in man and non-human primates, it is not a common change in rats except following chronic exposures or as an age related change.





Ducts and alveoli may become dilated and cystic and filled with proteinaceous secretory fluid (galactoceles), in response to hyperprolactinemia

Recommended Terminology and Severity Grading for Histopathological Findings

Male Mammary Gland

Feminization: Replacement of normal "male" lobuloalveolar pattern by "female" tubuloalveolar form. This may be complete replacement or only partial, leaving a mixture of the two patterns

Atrophy: Reduced size and number of alveolar cells and reduced volume of alveolar lobules while maintaining foamy eosinophilic characteristics.

Female Mammary Gland

Masculinization: Replacement of normal "female" tubuloalveolar pattern by "male" lobuloalveolar form. This may be complete or only partial replacement.

Atrophy: Decreased size and number of alveolar and/or ductal cells and reduced volume of groups of alveoli.

Alveolar hyperplasia: Increased numbers of alveoli with hypertrophic and hyperplastic epithelium forming small lobules of glandular tissue surrounding ducts. This finding is distinguished from lobuloalveolar (male) differentiation by the presence of discrete lumina in the hyperplastic alveoli and easily identifiable ductular structures. This change is also generally accompanied by increased secretory activity within ducts and alveoli.

Increased secretory material: Increased proteinaceous secretory fluid in the alveoli and/or ducts

Ductal ectasia: Dilation of ducts often containing secretion and sometimes forming galactoceles

Severity Gradings:

Severity gradings ranging between minimal: "smallest degree of change that can be consistently distinguished from normal background variation" and severe: "greatest degree of change that is likely to occur" are subjective. The following gradations are generally used to define a 5 grade severity system.

Minimal = very few/very small
Slight = few/small
Moderate = moderate number/moderate size
Marked = many/large size
Severe = very many/very large size

Critical aspects of histopathological evaluation

Ensure that an adequate section of mammary gland has been sampled for examination. A potential change in the male gland in response to antiandrogens is “lobular atrophy”. Since the amount of glandular tissue can vary with the location or the plane of sectioning, it is particularly important to ensure that the gland has been sampled consistently.

Terminal branches of glandular development in the male will begin as tubuloalveolar structures before differentiating into lobuloalveolar form. This is a normal feature. Weak oestrogens or low doses of strong oestrogens can produce a mixture of male and female differentiation, but the altered structures should be spread throughout the gland.

Ensure you have a good knowledge of the normal structure of the male and female gland and the normal range of glandular volume.

It may be difficult to distinguish alveolar hyperplasia of the normal female tubuloalveolar glandular elements in response to oestrogen and prolactin from the “masculinization” of the female gland in response to androgenic molecules. Possible critical features are the presence of discrete alveolar and ductal lumina, increased secretion and ductal ectasia in the hyperplastic female alveoli. This contrasts with the large foamy epithelial cells of the masculinized alveoli, which lack a discrete lumen and the paucity of recognizable ductal structures.

Similarly, it may be difficult to distinguish “atrophy” from “feminization” of the male mammary gland. The main distinguishing feature of the atrophic male mammary gland is the presence of reduced volume of foamy eosinophilic cells and the lack of obvious ductular structures in the atrophic gland contrasted with the low cuboidal basophilic epithelium associated with recognizable ducts with lumens in the feminized gland. Consistency of sampling is critical to be able to make this distinction.

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