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# Tissue interaction in androgen response of embryonic mammary rudiment of mouse: Identification of target tissue for testosterone

(testicular feminization/sexual differentiation/epithelio-mesenchymal interaction)

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**ABSTRACT** **In the androgen response of the embryonic mammary rudiment of the mouse, both gland epithelium and surrounding mesenchyme are visibly involved. The question whether this is due to a direct action of testosterone on both tissues was investigated in experimental combinations of mammary epithelium and mammary mesenchyme, derived either from normal or from androgen-insensitive ($X^{Tfm}$/Y) embryos. A typical androgen response occurred in combinations of androgen-insensitive epithelium with normal mesenchyme, whereas all combinations of normal epithelium with androgen-insensitive mesenchyme failed to respond. It is therefore concluded that only the mesenchyme of the mammary rudiment is the target tissue for testosterone, and that all changes in the gland epithelium, including its necrosis, are secondarily caused by testosterone-activated mesenchymal cells.**

Organogenesis in vertebrate embryos depends on developmental interaction of the tissues involved (1). It seems therefore reasonable to suppose that an external influence affecting the development of such an organ can do so by interfering in the process of tissue interaction. Hormones are external stimuli capable of inducing or modifying morphogenetic processes in compound organs, as shown for instance by the many complex morphogenetic changes taking place during androgen-induced sexual differentiation. Although such a response typically involves all tissues of an organ affected, e.g., epithelium and mesenchyme of an accessory male sexual gland, the hormone could nevertheless initiate these changes by acting only on one tissue, which then in turn would influence its partner through interactive processes.

It has been speculated before that hormones exert their effect through tissue interaction—especially in epithelio-mesenchymal organs (2, 3)—but these studies so far have only demonstrated the need for tissue interaction in hormone-induced development. To our knowledge, no attempts have been made to establish whether a given hormone acts directly on both epithelium and mesenchyme, or whether, on the other hand, it has only one target tissue. An answer to this question can be expected from experiments combining epithelium and mesenchyme of the same organ, in the same and appropriate developmental stage, but differing in their ability to respond to the hormone tested.

A suitable material for such a type of experiment with androgenic hormones is available in the *Tfm* mutant of the mouse. This X-bound mutation, recovered by Lyon and Hawkes (4), renders hemizygous ($X^{Tfm}$/Y)* "males" insensitive to androgens, most probably by specifying a nonfunctional androgen receptor (5, 6). Consequently, *Tfm*-affected animals lack all secondary male sex characteristics.

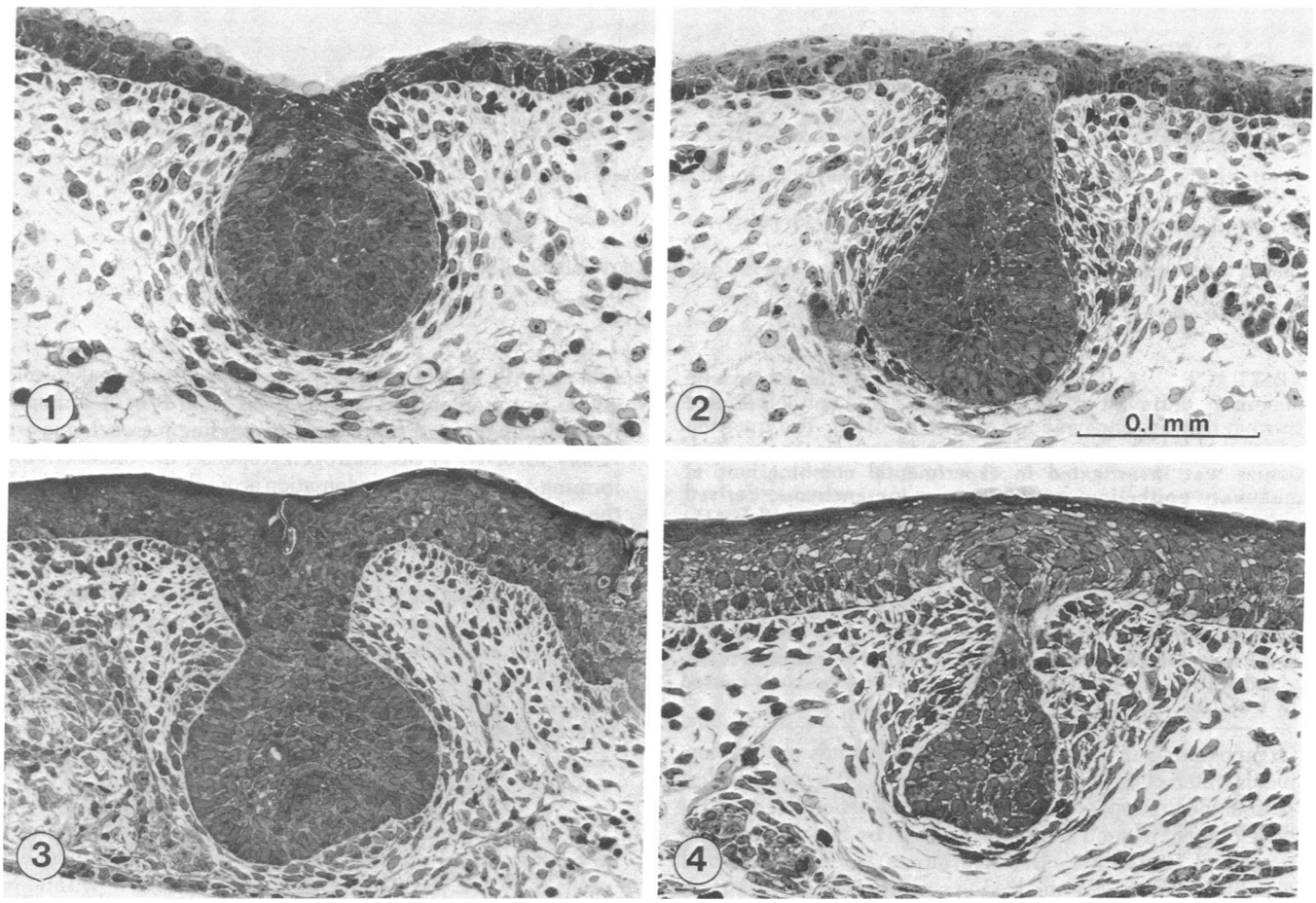
As test organ, we have selected the rudiment of the mouse mammary gland (see Fig. 1) because it can readily be kept in organ culture (7); it responds to testosterone *in vivo* (8) as well as *in vitro* (9); and, in contradistinction to accessory male sexual organs, is present in, and thus available from *Tfm*/Y-animals. Both mammary epithelium and surrounding mesenchyme are visibly involved in the androgen response, the mesenchyme forming a conspicuous condensation around the epithelial bud, the gland epithelium separating from the epidermis while undergoing extensive or even complete necrosis (ref. 10; and manuscript in preparation). In experimental combinations of mammary epithelium and mesenchyme of $X^+/X^+$ or $X^+$/Y ("wild type") on one hand, and of $X^{Tfm}$/Y genotype on the other, only one component will be capable of responding to testosterone. A hormone response occurring in one of the two types of combination would then reveal the target tissue of testosterone.

* *Tfm*/Y and $X^{Tfm}$/Y have the same meaning.

## MATERIALS AND METHODS

The X chromosome carrying the *Tfm* mutation is propagated in a mouse colony derived from three pairs kindly provided to us by Mary F. Lyon. The transmission of the $X^{Tfm}$ within the colony is followed by tagging one of the other two X chromosomes with the coat marker tabby (*Ta*) and the other with blotchy (*Blo*). *Tfm*/Y embryos were obtained by mating female carriers ($X^+/X^{Tfm}$) with C3Hf males. However, at the desired stage of 12 days, $X^{Tfm}$/Y embryos were not distinguishable from $X^+$/Y littermates. All male embryos (i.e., all embryos with testes) were therefore handled individually, the glands of one side were used for the combination experiments, the contralateral glands (at least three per embryo) were explanted and tested for androgen sensitivity. Their failure to respond to 0.1 μM testosterone was taken as evidence for an $X^{Tfm}$/Y genotype. In culture, mammary rudiments proved to be much more reliable indicators of androgen sensitivity than genital ducts: of about 1500 mammary explants observed so far in this laboratory for various investigations, not one has failed to show the typical response when exposed to testosterone in concentrations of 5 nM or higher.

"Wild-type" mouse embryos (with respect to the *Tfm* allele) were BALB/c × C3Hf hybrids. Combination experiments were done with tissues of 12-day embryos, the day of detection of a vaginal plug counting as day zero of pregnancy. The epidermis with the mammary buds could be separated cleanly from the underlying mesenchyme after 30 min incubation in an ice-cold trypsin (Difco 1:250)/pancreatin (Difco, National Formulary) solution (2.25% and 0.75%, respectively, in Ca-, Mg-free Tyrode's solution). Experimental combinations were cultured, epidermis upwards, at the medium–gas interface, supported by a Millipore filter (THWP, 25 μm thick) or—for better visibility—on an agar film spanning a 3 mm hole in a stainless steel grid. The medium was Eagle's minimal essential medium (Flow Laboratories, Irvine, Scotland), supplemented with 10% horse serum (Flow), 10% 9-day chick embryo extract, 2 mM gluta-




FIGS. 1–4. Mammary gland rudiments (×300). *Fig. 1.* Mammary gland rudiment of a 14-day female mouse embryo. *Fig. 2.* Mammary rudiment of a 14-day male embryo, in an early stage of its response to androgens. Note the beginning of a mesenchymal condensation around the epithelial bud. *Fig. 3.* Experimental combination of "wild-type" epithelium with *Tfm*/Y (i.e., androgen-insensitive) mesenchyme, fixed after 89 hr in culture, 46 hr in medium containing 0.1 μM testosterone. Note the absence of any visible androgen response. *Fig. 4.* Experimental combination of *Tfm*/Y epithelium with "wild-type" mesenchyme, 42 hr in culture, 28 hr with 0.1 μM testosterone. The response to the hormone is seen in the beginning condensation of mesenchymal cells around the gland epithelium, especially at the neck region.

mine, and 50 units each of penicillin and streptomycin. Testosterone concentration in the medium was 0.1 μM (or 29 ng/ml), i.e., about 100 times the minimal concentration effective in mammary explants (9).

**Criteria for the Androgen Response of Mammary Rudiments.** So far, the androgen response has only been described in morphological terms (ref. 10; and manuscript in preparation). First, densely packed mesenchymal cells surround the epithelial bud and especially its stalk; this stalk then stretches and finally ruptures, thereby separating the epithelial gland rudiment from the epidermis. This remaining gland epithelium frequently shows budding activity while many or all of its cells become necrotic and are eventually taken up by phagocytic mesenchymal cells. A reliable criterion at the electron microscopic level is the disappearance of the adepithelial basal lamina of the mammary bud. Mesenchymal condensation and separation of the gland epithelium from the epidermis are clearly seen in living explants of individual rudiments (9). In the large epithelio-mesenchymal combinations, an evaluation of living explants is somewhat more difficult. To avoid such uncertainty, the data of this paper are therefore based on the inspection of histological sections. The presence of the characteristic mesenchymal condensation (Fig. 2) and—at later stages—rupture of the stalk and necrosis of the gland epithelium were considered as indicators for an androgen response.

## RESULTS

Experiments were done with a total of 58 male embryos from 14 *Tfm*-carrier females. Explanted test glands of 31 of these 58 embryos were unresponsive to 0.1 μM testosterone. Such a result was to be expected from an $X^{Tfm}/Y$ genotype, as the *Tfm* mutation is known to affect all secondary male sex characteristics (with the exception of Müllerian duct regression) and adult $X^{Tfm}/Y$ animals do have nipples with mammary glands attached to the epidermis. All test glands of the remaining 27 male embryos responded to testosterone, thereby indicating an $X^+/Y$ genotype.

Thus, four types of experimental tissue combinations were tested for androgen responsiveness: (*i*) $X^{Tfm}/Y$ epithelium with wild-type (BALB/c × C3Hf) mesenchyme; (*ii*) wild-type epithelium with $X^{Tfm}/Y$ mesenchyme; (*iii*) $X^+/Y$ epithelium with wild-type mesenchyme; (*iv*) wild-type epithelium with $X^+/Y$ mesenchyme.

Although $X^+/Y$ and "wild-type" embryos were derived from different mouse colonies, their tissues can be expected to have the same properties as specified by the *Tfm* locus. Combinations *iii* and *iv* were made because the genotype of the embryos was not known at the time of explantation. They were useful, however, as controls to check whether possible strain differences not due to the *Tfm* locus would affect the outcome of the experiments. Differences in the extent of androgen-induced

mammary gland destruction between various strains of mice had been reported (11).

In 31 combinations of *Tfm*/Y epithelium with "wild-type" mesenchyme (type *i*) a total of 49 mammary gland rudiments were recovered after 3 days *in vitro*. Of these, 33 exhibited the typical features of the androgen response, already seen in the living explants and verified by histological examination in 27 cases (Figs. 4 and 5). In all six glands investigated with the electron microscope, the adepithelial basal lamina was missing. The occurrence of the response varied widely in time and in 12 glands (eight of them examined histologically) no definite sign of a reaction could be detected. Responding and nonresponding rudiments were found in the same explant (Fig. 5). The tissue combinations were fixed when the majority of the glands were in regression and it is therefore conceivable that the few failures might have responded after longer culture periods.

The 31 reciprocal combinations of "wild-type" epithelium with *Tfm*/Y mesenchyme (type *ii*) again yielded 49 glands; 42 of them were examined histologically. In contradistinction to the previous group, none of these glands showed any sign of a testosterone response. No mesenchymal condensation was seen, the epithelial rudiment of the mammary glands remained attached to the epidermis, and no necrosis was observed (Figs. 3 and 5).

In both types of combination involving tissues of $X^+/Y$ and "wild-type" embryos (type *iii* and *iv*) we found gland rudiments responding to testosterone (Fig. 5). Since the unresponsive experimental combination contained "wild-type" epithelium and *Tfm*/Y mesenchyme, the relevant control group was the one where "wild-type" epithelium was associated with mesenchyme of $X^+/Y$ embryos, the littermates of the *Tfm*/Y animals. From the appearance of living explants, 26 out of 30 such glands recovered were scored as responding. Twelve glands were investigated histologically; 10 of them clearly exhibited the hormone response (Fig. 5).

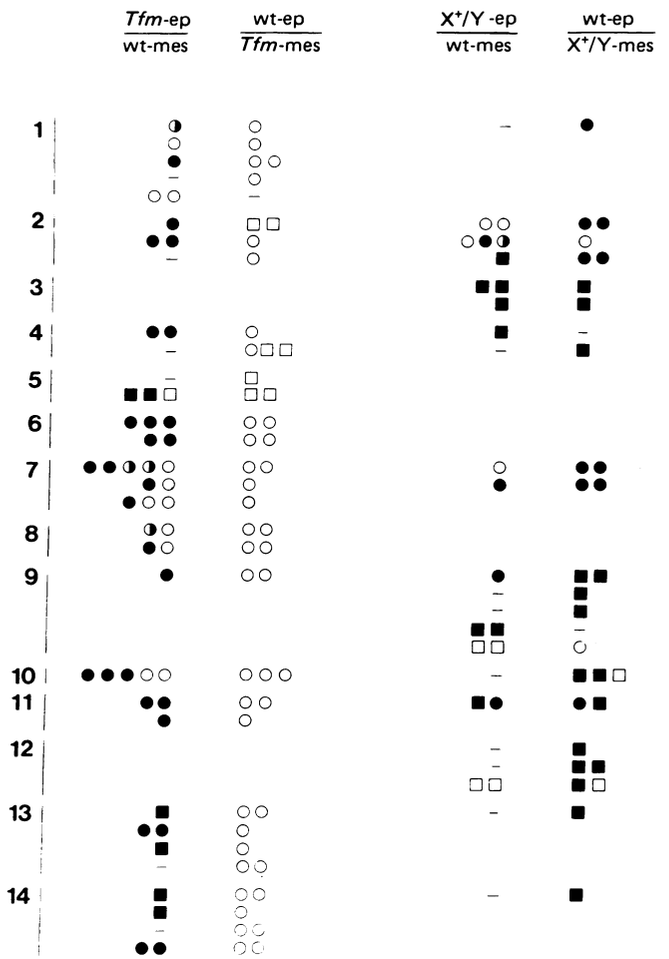



FIG. 5. Occurrence of an androgen response in four types of tissue combination. Columns from left to right: (*i*) $X^{Tfm}/Y$ epithelium combined with "wild-type" (wt) mesenchyme, (*ii*) wild-type epithelium with $X^{Tfm/Y}$ mesenchyme, (*iii*) $X^+/Y$ epithelium with wild-type mesenchyme, and (*iv*) wild-type epithelium with $X^+/Y$ mesenchyme. Individual mammary rudiments within such combinations are represented by circles when examined in histological sections, by squares when scored in living explants only. Glands responding to 0.1 $\mu$M testosterone are shown as filled symbols. Note that an androgen response occurred in all combinations except the one containing androgen-insensitive (*Tfm*) mesenchyme. (Numbers at left identify the 14 *Tfm*-carrier females from which 31 $X^{Tfm}/Y$ and 27 $X^+/Y$ embryos were obtained. Each embryo was used in two types of combination, one containing his epidermis with the mammary buds, up to five, the other his mesenchyme. Five $X^+/Y$ embryos were omitted from this graph since no mammary gland was recovered in either combination.)

## DISCUSSION

The combination experiments with "wild-type" and with *Tfm*/Y (i.e., androgen-insensitive) tissues have shown (*a*) that a testosterone response of the mammary rudiment takes place only when the mesenchyme is of wild-type character (with respect to the *Tfm* locus), and (*b*) that this response occurs independently of the genotype (with respect to *Tfm*) of the epithelium. This allows the conclusion that—at least in our experimental combinations—testosterone has only one target tissue, the mesenchyme. Since a typical response was observed in combinations containing androgen-insensitive (*Tfm*/Y) epithelium, it seems likely that testosterone does not act directly on mammary epithelium in the intact gland as well.

This result may appear somewhat surprising, since androgen receptors have been demonstrated in an (epithelial) mammary tumor cell line (12). Androgen receptors, however, were found to be present in various cell types that do not respond to testosterone and they may even be of ubiquitous occurrence (13). On the other hand, the histological appearance of the mammary rudiment during its androgen response has already provoked the speculation that the gland epithelium is destroyed by the surrounding mesenchymal cells (10). In a recent investigation of male $X^{Tfm,Blo}/X^{+,+}$ mice (sex reversed by the autosomal dominant *Sxr* gene) Ohno *et al.* (14) found concordance between the expression of the *Blo* phenotype and the presence of "female" mammary glands (due to *Tfm*). Since *Blo* expresses itself through mesodermal cells (15), Ohno suggested that the same type of cells may also be responsible for the mammary rudiment's responsiveness to testosterone.

The identification of the mesenchymal component as the only target tissue for testosterone in the mammary rudiment necessitates the assumption that all changes observed in the gland epithelium after testosterone exposure are caused indirectly through the action of testosterone-stimulated mesenchyme. The epithelial reaction, therefore, is a reliable indicator for the existence of a hormone-induced tissue interaction. From this it follows that the extensive epithelial necrosis during the androgen response of the mammary rudiment (in preparation) does not reflect hormone-induced cell death, but rather hormone-induced cell "killing." The mechanism by which mammary mesenchyme exerts its destructive influence on the gland epithelium, and the basis for the strict discrimination between mammary epithelium and adjacent epidermis (which is unaffected) remain unknown.

We think that hormone-induced tissue interactions of this kind offer some unique experimental advantages for the study of tissue interaction in general. One difficulty for their analysis lies in the fact that developmental tissue interactions are sequential and reciprocal processes (1), presumably starting before the first visible formation of an organ rudiment. Experimental combinations of tissues derived from different regions (in the skin—ref. 16), from different species or even classes (17, 18), or from normal animals and morphogenetic mutants (19) have yielded important information on the contribution of each tissue to the development of the compound organ. Nevertheless, in many cases it still seems difficult to determine which tissue takes the initial lead in the morphogenetic interaction. Hormone-induced tissue interactions, by contrast, have a precise starting point in the developmental history of an organ and, under experimental conditions, can be triggered at will. Moreover, such a development is initiated by an external signal and the tissue starting with the ensuing interactive process must be the one responding directly to that signal. For all morphogenetic processes induced by androgenic hormones, the *Tfm* mutation should allow identification of this "leading" tissue and thereby the first step in the interactive process. This could eventually enable us to reconstruct a complete series of interactive steps from its very beginning.

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