Quantitative Studies of Ductal Versus Alveolar Differentiation from Rat Mammary Clonogens (44335)

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Abstract. **In a program aimed at defining and characterizing the cellular origins of radiogenic mammary cancer, we developed and have used a quantitative rat mammary cell transplantation model to investigate hormonal control of differentiation, growth, and response to ionizing radiation. In this model, in response to appropriate hormonal stimulation, a subpopulation of transplanted mammary epithelial cells gives rise to either alveolar colonies (AU) or ductal colonies (DU). The cumulative data support the conclusion that both types of colonies are derived from single clonogenic mammary cells. In the current experiments the glucocorticoid, cortisol, which does not induce mammary proliferation or differentiation alone, promoted AU formation with milk secretion from grafted clonogens when present with estrogen and/or pituitary MtH (primarily prolactin and growth hormone) from co-grafted MtT. Progesterone synergized with estrogen in a dose-dependent fashion in induction of DU formation in mammary cell grafts in ovariectomized MtT–co-grafted rats and antagonized glucocorticoid-dependent AU development in such grafts in adrenalectomized-ovariectomized MtT–co-grafted rats as well. We conclude that hormonal regulation of growth and differentiation of rat mammary glands *in situ* is mediated to a significant extent through effects on the mammary clonogens and their immediate progeny. The mammary clonogen transplantation model permits quantitative investigation of such hormone actions on a mammary clonogen basis *in vivo*.** [P.S.E.B.M. 1998, Vol 219]

Rat mammary glands contain subpopulations of epithelial cells that are capable of forming multicellular glandular structures when transplanted to gland-free fat pads of hormonally manipulated syngeneic recipient rats. The current studies of the formation of these glandular structures indicate that hormonal control of mammary growth and function is largely mediated through the effects on the clonogenic cells from which such structures arise in mammary cell grafts. In otherwise untreated recipient rats co-grafted with MtT (transplantable mammotropic pituitary tumors), the predominant structures that arise in mammary cell grafts resemble alveoli and hence are termed AU (alveolar units; Ref. 1). More recently different mammary structures termed DU (ductal units) were noted in small numbers in mammary cell grafts (2). The cumulative evidence supports the conclusion that the AU, and probably the DU, that develop in hormonally stimulated grafts of small mammary cell numbers are monoclonal in origin; the cells from which they arise are thus referred to as clonogens (3, 4).

The AU that develop by 3–4 weeks after mammary cell transplantation in MtT–co-grafted recipients are ductless spherical structures ~0.3–0.7 mm in diameter. They are composed of a single layer of highly secretory mammary epithelial cells surrounding a central lumen and in turn are surrounded by myoepithelial cells (1). The polarized epithelial cells have apical microvilli and contain secretory vacuoles with lipids and electron-dense protein particles at their luminal ends. The secretion in the distended lumina contains similar vacuoles and dense bodies and is reactive to

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anticasein antibodies. The surrounding myoepithelial cells are characterized by myofibrils.

A limiting dilution transplantation assay was developed for estimating the concentrations of clonogens in monodispersed suspensions of mammary cells by the formation of AU at the sites of mammary cell grafts stimulated by mammotropic pituitary hormones secreted by MtT (MtH) (1, 3). In such assays, serial dilutions containing known numbers of enzymatically monodispersed mammary cells are transplanted by injection into gland-free subcutaneous fat pads of MtT–co-grafted recipient rats. Three or four weeks later, whole mounts of the graft sites are examined for the presence or absence of one or more AU, and an alveolar dose 50% (AD_{50}) value is calculated. AD_{50} values are the numbers of mammary cells per graft site required to produce one or more AU in 50% of the sites, and are inversely related to the clonogenic fractions in the transplant inocula. It is to be emphasized that estimation of the concentration of clonogenic cells by this assay procedure depends on hormonal stimulation of formation of scoreable multicellular structures by as great a fraction of the clonogens present in the grafts as possible (3). Baseline estimates of clonogen concentrations are thus derived from observations of AU in the mammary cell graft sites in recipient rats that are endocrinologically manipulated to maximize AU formation from clonogens in the grafts.

These transplantation procedures have also been used to characterize and quantify the AU-forming clonogen subpopulation in a variety of investigations (4). Among these are histological studies of graft sites at intervals during AU formation (1), estimations of loss of tritiated thymidine-labeled cells from the graft sites following injection of transplantation inocula (1), observation of the quantitative decrease in AU-forming capacity with increases in the dose of ionizing radiation to the donor mammary glands before removal for transplantation (3–6), and the kinetics of post-irradiation recovery of AU-forming capacity in mammary cells irradiated *in situ* (7, 8) or in mammary organoids in culture (9). Other investigations include demonstration of the consistency of the results of assays of AU-forming capacities of mammary cell suspensions prepared from glands of untreated young adult female rats by the same or different individuals and/or from rats of different strains (9–11), concentration of the AU-forming cells by differential filtration of partial mammary gland digests and short-term mammary organoid culture (9) and fluorescence-activated cell sorting (10), investigation of the age-dependent development of subpopulations of AU-forming cells in the fat pad gland sites of prepubertal rats, and the lack of post-irradiation repair capacity by the AU-forming cells of prepubertal rats and its acquisition coincident with sexual maturation (11). The data from all of these studies are most consistent with the conclusion that the AU that develop in sites of transplantation of small mammary cell numbers are monoclonal in origin (4). Indeed, many of these data are inconsistent with the alternative that AU formation requires more than one cell.

Limiting dilution transplantation assays indicate that DU are also monoclonal in origin. They predominate in mammary cell grafts in MtT–co-grafted recipient rats that are also glucocorticoid deficient (2). DU are composed of multiple branched ducts with central lumina lined with two to four layers of epithelium surrounded by myoepithelium. The ductal lumina contain little or no secretion. The ducts terminate in multiple cell-layered end buds in which mitosis is common (2).

MtT strain W10 (2) was used as an indwelling source of mammotropic pituitary hormones in the current studies. Autonomous rat MtT have been derived by transplantation of pituitary adenomas, either spontaneous or induced by chronic estrogen treatment (12, 13). Estrogens, including diethylstilbestrol, directly stimulate the lactotropes of the anterior pituitary to both secrete and divide (14–16). During subtransplantation, autonomous MtT variants can be selected that secrete high levels of the primary pituitary mammotropin, prolactin, with lesser amounts of growth hormone (17). Some MtT substrains have acquired the capacity to secrete adrenocorticotropin as well (12). However, the lack of thymic atrophy in intact rats co-grafted with MtT W10 in the current study indicates that it had little or no adrenocorticotropic effect. The mammotropic secretory products of MtT W10 are referred to as MtH in this report.

The aim of the current studies was to examine further the hormonal control of formation and differentiation of AU and DU from transplanted mammary clonogens, and to test the usefulness of the clonogen transplantation model for quantitative study of the control of growth and differentiation at the mammary cell level *in vivo,* and particularly of those hormones and hormone combinations known to influence mammary cancer promotion. The experiments were thus designed to gain quantitative information on the effects of estrogen, progesterone, and cortisol alone and in combination with one another and with and without high levels of pituitary MtH on AU and DU formation and differentiation (2). The current results suggest that the effects of these hormones on the mammary glands *in situ* are mediated to a significant extent through effects on the subpopulation of clonogenic epithelial cells.

Materials and Methods

Animals. Virgin female WF (Wistar-Furth) rats (Harlan Sprague-Dawley, Madison, WI) 50–55 days old at the time of mammary cell transplantation were used throughout for mammary cell donors as well as recipients. Ovx (ovariectomized) and surgically untreated rats were given acidified tap water *ad libitum.* Adx (adrenalectomized) and Adx/Ovx rats were given their choice of acidified tap water or physiological saline as drinking water. Mammary cell transplants were made by inoculation of mammary cell suspensions into gland-free i.s. white fat pads under visual control through small incisions. Surgical procedures were performed with ether anesthesia, and incisions were closed with wound clips. The rats were fed commercial laboratory chow and were housed in a temperature (23 ± 2°C) and humidity (50%) controlled room with a 12:12-hr light:dark cycle. Rats were sacrificed by ether overdose. The rats were maintained throughout in accord with the NIH *Guide for the Care and Use of Laboratory Animals,* and experimental procedures were performed in accord with research protocols approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee.

Preparation of Monodispersed Mammary Cell Suspensions. The method is slightly modified from that described previously (1, 3). Mammary cell donor rats were sacrificed with ether, and their inguinal mammary fat pads were dissected free of lymph nodes, removed, and scissors-minced in chilled culture medium (α-MEM, Flow Laboratories Inc., McLean, VA). The minces were then incubated in collagenase solution (Type III, 2 mg/ml, Cooper Biomedical, Freehold, NJ) with shaking for 3 hr at 37°C; DNase solution was added to a final concentration of 0.002% 10 min before termination of the incubation.

The suspensions were then passed onto 40-μm pore size nylon mesh filters. Single epithelial and stromal cells and cell clumps passed through the filters and were discarded. The predominantly epithelial mammary organoids (duct fragments and end buds) that were trapped on the filter surfaces were collected by back-flushing with medium containing 10% fetal bovine serum (FBS, Sterile Systems, Inc., Logan, UT). The organoid suspensions were centrifuged at ~350*g* for 2 min, the supernates were discarded, and the washings were repeated twice. The organoids were then dissociated by incubation in 0.05% trypsin plus 0.02% EDTA with shaking for 10 min at 37°C.

After trypsinization, the primarily epithelial and monodispersed cell suspensions were passed twice through 40-μm nylon mesh filters to remove remaining cell clumps. The concentrations of morphologically intact cells in the preparations were determined at 400x with a phase microscope and hemocytometer. After appropriate dilutions were prepared, equal volumes of 50% v/v homogenates of syngeneic rat brain were added to each suspension. Addition of brain homogenate significantly increases retention of inoculated cells at the graft sites (1).

Hormone Preparation and Treatments. Progesterone (Prg), cortisol (C), and estradiol (E2) (Sigma Chemical Co., St. Louis, MO) were prepared for injection by dissolving the crystalline hormones in small volumes of ethanol and stirring in peanut oil. To dissolve the Prg in oil, the mixture was gently heated. A final concentration of 3%–5% ethanol was added to maintain the Prg in solution. Doses of 0.04, 0.4, 4.0, and 20 mg Prg, 1.0 mg C, and/or 0.01, 0.1, 1.0, and 10 μg E2 were injected s.c. daily where indicated.

Design of Mammary Cell Transplantation and Clonogen Assay Experiments. Two types of experiments were performed. First, in high cell dose studies, single 60-μl aliquots containing 2×10^5 morphologically intact mammary cells were injected into each of three sites in the interscapular white fat pad (i.s.) in each of a minimum of five recipient rats, for a total of at least 15 graft sites per experimental group. Second, in limiting dilution assays, serial dilutions of mammary cell suspensions were prepared, and 60-μl aliquots of each concentration were injected into a minimum of 15 i.s. pad transplant sites as described above. When indicated, recipients had been Ovx or Ovx-Adx 3 weeks before mammary cell grafting. MtT grafts were by intramuscular injection of cytosieve suspensions of MtT W10 in a hind leg 2 weeks before mammary cell grafting where indicated. Steroid hormone treatments were begun on the day of mammary cell transplantation in the indicated groups.

The recipient rats in both types of experiments were sacrificed and autopsied 3 weeks after mammary cell grafting. The i.s. pads containing the mammary graft sites were removed, fixed, stained with hematoxylin, and cleared as described previously (1, 3). These i.s. pad whole mounts were then examined under a dissecting microscope for the presence or absence of mammary AU and/or DU (2, 3).

Data Analysis. Data from the high cell dose studies (2×10^5 mammary cells per graft site) are presented as percentages of sites with AU or DU in rats subjected to various hormonal conditions. The data from limiting dilution assays were analyzed according to a modification (1, 3) of the transplantation model of Porter *et al.* (18). Briefly, the relationship between the fractions of graft sites with at least one AU or one DU and the numbers of mammary cells inoculated per site in each group of recipient rats were computer fit to the Porter *et al.* equation:

$$P = 1 - e^{-M}$$

In the current experiments, P is the probability that a given graft site will contain at least one AU or one DU, and M is the mean number of clonogenic cells that are capable of proliferation and differentiation to form an AU or a DU under the specific hormonal conditions in the recipient rat. M is derived according to the following equation:

$$\log M = \log K + S \log Z$$

where Z is the mean number of morphologically intact cells per inoculation site, and K—either K_A (AU-forming) or K_D (DU-forming)—is the clonogenic fraction (i.e., the fraction of inoculated cells capable of forming either an AU or a DU, respectively). S is the slope of the relationship. S and K are estimated with a maximum likelihood iterative procedure (1, 3).

It is worthy of note that in all the assay experiments reported herein, and in virtually all of the numerous mammary clonogen assays previously performed in these laboratories, S has not varied significantly from 1.0. This is of importance in that according to the Porter model, if the multicellular endpoint scored is monoclonal in origin, S will ideally equal 1.0. If two cells are required to form such a structure, S will be ~0.5; if more cells are required, S will be less than 0.5 (3, 18).

From the parameters derived as above, AD_{50}[4] and DD_{50}[4] values were calculated; that is, the mean numbers of cells per graft site required to produce at least one AU or one DU, respectively, in 50% of the graft sites. AD_{50} and DD_{50} values are inversely proportional to the clonogenic fractions, K_A[4] and K_D[4], respectively (2–4).

Mammary Gland DNA. As indications of changes in total cellularity in mammary glands *in situ,* DNA concentrations were determined in the partially defatted mammary glands from groups of MtT-grafted rats that were otherwise untreated or had been treated daily for 3 weeks with 0.1 μg E2 and/or 4.0 mg Prg. The rats were sacrificed, and their inguinal fat pads were removed as for preparation of cell suspensions. As much as possible of the nonglandular fat was dissected from the glands, and they were minced and homogenized in cold 0.5 *M* perchloric acid (19). The precipitates were washed in perchloric acid, and the RNA was hydrolyzed from the acid-insoluble fractions in 0.3 *M* KOH. After acid washings, the DNA samples were hydrolyzed from the insoluble material in hot 0.5 *M* perchloric acid. The concentrations of DNA were then determined spectrophotometrically by optical density at 265 and 290 nm (19). In view of the incomplete removal of fat and interstitial cells, results of these measurements are useful primarily as indicators of major changes in glandular cellularity.

Results

DU were first observed occasionally in mammary cell graft sites in intact MtT–co-grafted WF females (i.e., in rats with high levels of MtH). When stained and cleared fat pad whole mounts are examined with a dissecting microscope, DU are readily distinguishable from AU by their gross and histological morphology (2). DU are composed of branching ducts with terminal end buds, whereas AU are single spherical or ovoid structures (Fig. 1A). In mammary cell grafts in female WF recipient rats co-grafted with MtT W10 and otherwise untreated, AU formation was about 22 times more efficient than DU formation. The AD_{50} was a mean of 740 monodispersed mammary cells as compared to the DD_{50} of 16,051 monodispersed cells per graft site (Figs. 2A and 2B). These figures indicate that one of ~935 mammary cells in the graft inoculum was capable of forming AU whereas one of about ~20,288 such cells was capable of DU formation in this hormonal milieu.

In the current studies, in Ovx MtT–co-grafted recipients treated with 1 μg of E2 daily, the AD_{50} value was significantly greater (i.e., AU formation was less efficient) than in intact MtT–co-grafted recipients (Fig. 2A). In such E2-treated rats that had been grafted with mammary cell numbers in excess of the AD_{50}, clusters of AU were common (Fig. 1B). The addition of 4.0 or 0.4 mg Prg to 1.0 or 0.1 μg E2 daily in MtT–co-grafted Ovx rats did not significantly alter the AD_{50} values from that seen after E2 alone (Figs. 2A, 3A, and 3B).

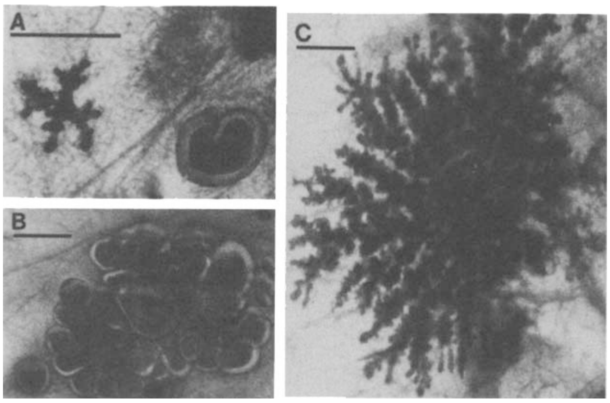

Figure 1. Mammary structures in hematoxylin-stained whole mounts of i.s. fat pads of W/F rats 3 weeks after transplantation of monodispersed mammary cells. Bars indicate 1 mm in each panel. (A) DU (left) and AU (right) in a graft site inoculated with 2 × 10^5 mammary cells in an MtT–co-grafted, otherwise untreated recipient rat. (B) Clusters of AU in an i.s. graft site in an Ovx MtT–co-grafted recipient treated with 1 μg E2 daily. The transplant inoculum was 9.8 × 10^3 mammary cells/site. (C) Cluster of DU in an i.s. graft site of an Ovx MtT–co-grafted rat treated with 1 μg E2 plus 4 mg of Prg daily. The transplant inoculum contained 9.8 × 10^3 mammary cells.

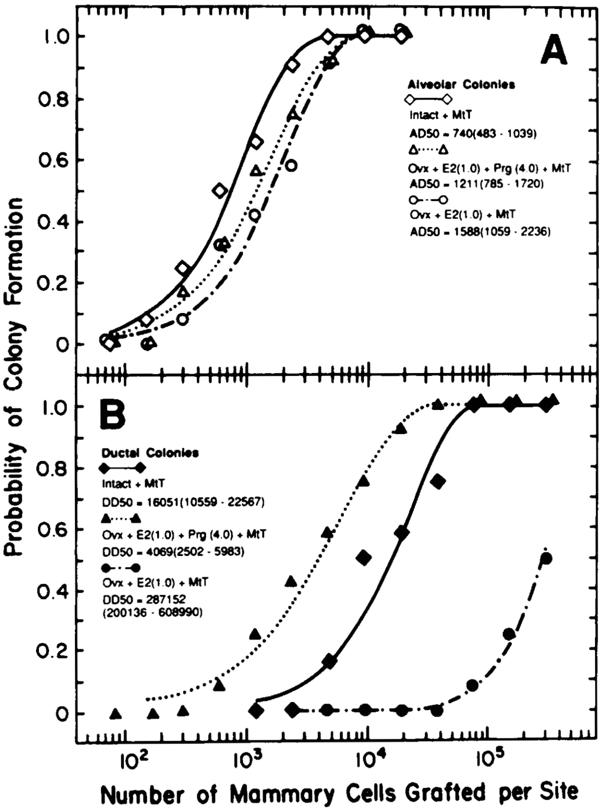

Figure 2. (A, upper) Dependence of fractions of graft sites with AU and (B, lower) with DU on the numbers of mammary cells transplanted in otherwise untreated, MtT–co-grafted recipients and in Ovx MtT–co-grafted recipients treated daily with 1 μg E2 alone or in combination with 4 mg Prg daily. AD_{50} and DD_{50} values are given with 95% confidence limits in parentheses.

In contrast, Prg markedly stimulated DU formation when given in combination with E2 to Ovx MtT–co-grafted recipient rats. The DD_{50} value in such recipients that re-

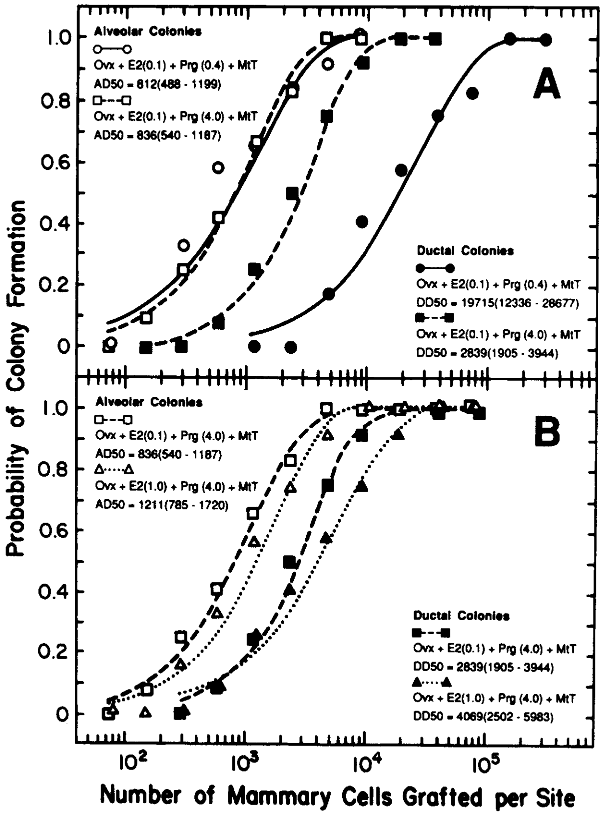

Figure 3. Dependence of the fractions of mammary cell graft sites with AU and with DU on the numbers of mammary cells grafted per site in Ovx MtT–co-grafted rats treated with different doses of E2 and Prg. (A) 0.4 and 4 mg of Prg combined with 0.1 μg E2 daily. (B) 0.1 and 1 μg E2 combined with 4 mg Prg daily. AD_{50} and DD_{50} values given with 95% confidence limits.

ceived 1.0 μg E2 alone daily was about 18-fold greater than that in MtT–co-grafted intact rats (Fig. 2B). In contrast, in such rats given both 1.0 μg E2 plus 4.0 mg Prg daily, the DD_{50} was about 4-fold less than in intact MtT–co-grafted recipients and about 70-fold less than in the Ovx MtT–co-grafted rats given E2 only (Fig. 2B). In Ovx MtT–co-grafted rats, the smaller 0.1-μg E2 dose in combination with 4.0 mg Prg was nearly 7-fold more efficient at stimulation of DU formation than 0.1 μg E2 with the smaller 0.4-mg Prg dose (Fig. 3). In E2 plus Prg-treated Ovx rats that had received mammary cell grafts greater than the DD_{50}, DU were frequently observed in large clusters (Fig. 1C).

Neither AU nor DU developed in grafts of 2 × 10^5 cells each in groups of Adx-Ovx recipients with neither co-grafted MtT nor E2 treatment (Table I). Only DU were observed in all such rats that received E2 alone, E2 plus Prg, or E2 plus Prg plus C. In those rats that received E2 plus C without Prg, all sites had AU of which 20% also had DU (Table I).

Both AU and/or DU developed in the graft sites transplanted with 2 × 10^5 mammary cells in all of the Adx-Ovx rats co-grafted with MtT in this experiment (Table I). The majority of colonies in MtT–co-grafted Adx-Ovx rats without exogenous steroid hormone treatment were DU; only 13% of the sites in such recipients also had AU. In accord with previous observations on the requirement of glucocorticoids for lactogenesis in mammary glands *in situ* (20–22), treatment of such rats with C alone or in combination with E2 or Prg or both caused a shift toward formation of AU (Table I). Both types of colonies were observed in rats given Prg alone, but twice as many sites had DU than AU. E2 alone or in combination with Prg promoted only DU formation in all of the sites in Adx/Ovx MtT–co-grafted recipients (Table I). Finally, in such rats treated with all three steroid hormones, both AU and DU developed in 100% of the graft sites.

Table I. Effect of E2, Prg, and C Alone and in Combination on AU and DU Formation in Mammary Cell Graft Sites in Adx-Ovx rats with or without MtT Grafts

	Percentage of graft sites with AU or DU[a]			
Hormones Injected[b]	No MtT graft		With MtT graft	
	AU	DU	AU	DU
None	0	0	13	100
C	0	0	100	0
Prg	0	0	38	76
Prg + C	0	0	100	7
E2	0	100	0	100
E2 + C	100	20	100	0
E2 + Prg	0	100	0	100
E2 + Prg + C	0	100	100	100

[a] Each i.s. graft site received 2 × 10^5 morphologically intact mammary cells. There were 21 graft sites in the group of MtT grafted rats that received Prg alone, and 15 graft sites in all other groups.

[b] Daily s.c. hormone doses were: C (cortisol), 1 mg; Prg (progesterone), 4 mg; E2 (estradiol), 1 μg.

To further quantify the interplay between E2 and Prg in DU formation in particular, groups of Ovx MtT–co-grafted rats were transplanted with 2 × 10^5 mammary cells/site and given 1.0 μg E2/day alone or with graded doses of 0.04 to 20.0 mg Prg daily. Conversely, other groups of Ovx MtT–co-grafted rats were given 4.0 mg Prg/day alone or with graded doses of 0.01–10.0 μg E2 daily. An additional group of mammary graft recipients were MtT–co-grafted and otherwise untreated. Both AU and DU developed in all 21 graft sites in this latter group of MtT–co-grafted, ovary-intact recipients (data not shown). In all but three of the Ovx MtT–co-grafted groups (see Fig. 4 and heading), AU developed in 100% of the graft sites regardless of E2 and/or Prg treatment. DU formation, however, was dependent on the E2 and/or Prg treatments of Ovx MtT–co-grafted rats, and there was evidence of E2/Prg synergism. In groups of rats given 1 μg E2 daily, addition of 0.04 mg Prg increased the fraction of graft sites with DU from 10% to 60%; addition of 0.4 or more mg of Prg caused DU formation in all the graft sites (Fig. 4). Similarly, in rats given 4.0 mg Prg daily alone or with 0.01 μg E2, the fractions of sites with DU were 40% and 47%, respectively; addition of 0.1 μg or more E2 daily with 4.0 mg Prg increased the frequency of sites with DU to 100%. In the absence of Prg, the frequen-

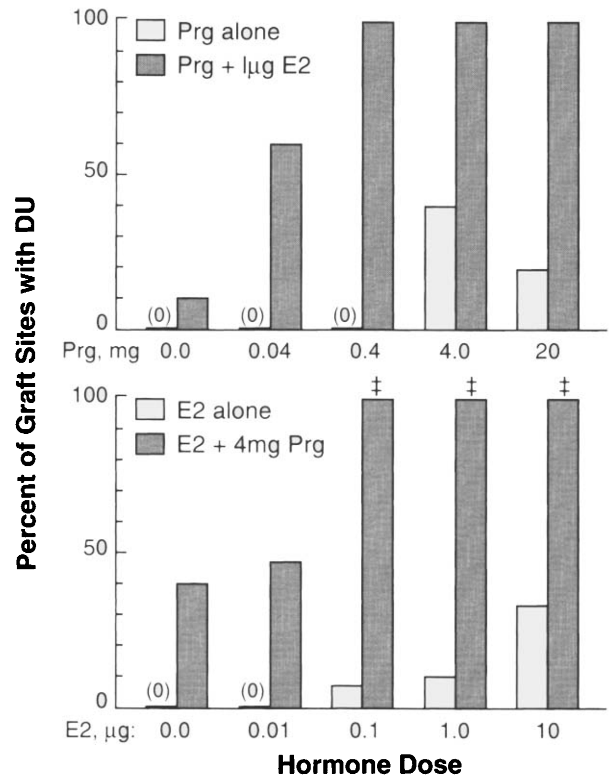

Figure 4. Interactions of E2 and Prg alone or in combination on the percentage of graft sites with DU after transplantation of 2×10^5 mammary cells per site in Ovx MtT-grafted recipient rats. (Upper Panel) Effects of 0–20 mg Prg daily with or without 1 μg E2. (Lower Panel) Effects of 0–10 μg E2 daily with or without 4 mg Prg. ‡AU were found in 85%, 90%, and 97% of the graft sites in the groups that received 4.0 mg Prg with 0.1, 1.0, or 10 μg E2, respectively. In all other groups, 100% of the graft sites contained AU regardless of whether and how much E2 and/or Prg they received (data not shown).

cies of graft sites with DU progressively increased from 0% at doses of 0 and 0.01 μg E2 per day to 33% at 10 μg E2 per day. Only the highest doses of 4.0 and 20.0 mg Prg alone per day induced DU formation in 40% and 20% of the graft sites, respectively (Fig. 4).

Synergism between the two ovarian hormones and MtH was further reflected in analyses of the total DNA in the caudal three mammary glands and adherent fat *in situ*. A dose of 4.0 mg of Prg alone daily to Ovx MtT–co-grafted rats did not significantly affect the mammary DNA concentrations; 1.0 μg E2 alone daily increased the total DNA content by ~40%. Prg combined with E2 increased the total DNA to ~400% of that in MtT–co-grafted Ovx rats given no ovarian hormones (Table II).

In summary, E2 in the presence or absence of elevated MtH from co-grafted MtT generally stimulated DU formation in mammary cell grafts in Adx/Ovx recipients, and favored AU formation in recipients with intact adrenals or co-treatment with C. Prg synergized with E2 particularly in induction of ductal growth in the presence or absence of co-grafted MtT and in Adx/Ovx and Ovx rats. Neither Prg nor C alone nor in combination induced DU or AU formation in Adx/Ovx rats in the absence of co-grafted MtT or E2 treatment. In the presence of elevated MtH from co-grafted MtT and/or E2 treatment, C stimulated AU formation with secretion whereas Prg favored DU formation with little or no secretion.

Table II. Effect of E2 and Prg Alone and in Combination on Total DNA in Inguinal Fat Pads Containing Three Caudal Mammary Glands in Ovx MtT-Grafted Rats

Treatment	Number	mg DNA + s.d. (% control)
Control	7	0.90 ± 0.07 (100%)
4 mg Prg	7	0.95 ± 0.10 (106%)
1 μg E2	7	1.26 ± 0.10 (140%)
Prg + E2	8	3.57 ± 0.42 (397%)

Discussion

The necessity of pituitary MtH (prolactin and/or growth hormone) for mammary gland growth and secretion (23–26), the dual role of estrogens in stimulating prolactin secretion (15, 16, 23) and in synergy with MtH to stimulate mammary growth and differentiation (22, 27, 28), and the requirement of glucocorticoids for differentiation for milk secretion (22–22) are characteristic of the actions of these hormones on rat mammary glands *in situ*. In addition to its recognized antilactogenic effect (29–31), in the presence of E2 and MtH, Prg has been shown to stimulate cell proliferation and growth of mammary ducts (32–34). In the current study, these effects have been observed during growth and differentiation of grafted mammary clonogens and their new progeny into AU and DU. We conclude that the effects of these hormones on mammary glands *in situ* is in significant measure mediated through stimulation of growth and differentiation of the clonogenic cell subpopulation and their immediate progeny. The mammary cell transplantation model readily lends itself to quantitative as well as qualitative study of such hormonal effects at the mammary clonogen level *in vivo*.

In the course of our studies with the transplantation assay, the lowest AD_{50}s and highest K_A[4] values for grafts of mammary cell suspensions prepared as in the current experiments from the glands of untreated young adult female donors have consistently been seen in otherwise untreated, MtT–co-grafted young adult female recipients. We have thus used this treatment of recipient rats as the standard for estimating the baseline concentrations of clonogens competent to form AU. For example, from an AD_{50} of 740 mammary cells/graft site in intact recipients with co-grafted MtT (Fig. 2A) and the Porter transplantation equation (cf. Materials and Methods; Refs. 3, 18) we may calculate that one cell of ~935 monodispersed mammary cells in the transplant suspension was a clonogen (i.e., the AU-forming clonogenic fraction, K_A, was $\sim 1.07 \times 10^{-3}$). This is in good agreement with the AD_{50} values of 704 cells and 786 cells per site ($K_A = \sim 1.12 \times 10^{-3}$ and $\sim 1.01 \times 10^{-3}$, respectively) in previous AD_{50} assays of similarly prepared cell suspensions from untreated WF strain and F344 strain donors in intact syngeneic recipients co-grafted with MtT W10 and MtT F4, respectively (2). It is not certain, of course, that all grafted mammary clonogens in intact MtT–co-grafted recipients in any given assay in fact gave rise to scoreable AU. The clonogenic fractions calculated from these assays are therefore conservative (i.e., are more likely to be underestimates than overestimates). The K_A values calculated from AU-based assays in MtT–co-grafted, otherwise untreated young adult female recipients are thus taken as the best available baseline estimates of ''real'' AU-forming clonogen concentrations.

Baseline estimates of DU-forming clonogen concentrations have been similarly derived. In previous studies, assays of aliquots of the same mammary cell suspension were performed in recipient rats with differing hormonal manipulations (i.e., in otherwise untreated, MtT–co-grafted WF female recipients, the DD_{50} value was 11,377 grafted cells/site ($K_D = 6.95 \times 10^5$), whereas in Adx MtT–co-grafted recipients, the DD_{50} was nearly 10-fold lower, 1244 cells (one DU-forming clonogen per ~1572 grafted mammary cells, $K_D = 6.36 \times 10^{-4}$; Ref. 2). The latter K_D value is among the largest thus far observed; such large K_D values have been seen consistently in assays in recipients with combinations of elevated MtH and glucocorticoid deficiency consequent to Adx. K_D values calculated from DU-based assays in Adx MtT–co-grafted recipients are thus here taken as the best baseline estimates of the ''real'' DU-forming clonogenic fractions. Like the baseline K_A values above, such K_D values are conservative.

Smaller K_A and K_D values calculated from AU-based or DU-based assays of mammary cell suspensions prepared as herein but from donors of different ages or treatment backgrounds assayed by grafting in recipient rats with the same endocrine manipulations used for baseline K_A or K_D estimations, respectively, as described above are interpreted as indicating lower concentrations of AU- or DU-forming clonogens in the donor glands. Such assays have included determinations of the clonogenic fractions of the mammary glands of irradiated young adults (5, 6) and of prepubertal rats (11). Smaller K values in assays of cell suspensions prepared from young adult females but grafted in recipients with different endocrine manipulations than those used in baseline AU or DU assays, as in the current experiments, are interpreted to be the result of less effective stimulation of the grafted clonogens present to form AU or DU, respectively, rather than to differences in clonogen concentrations in the transplant inocula. In this regard, it is of interest to note that the high baseline K_D value seen in MtT–co-grafted Adx recipients in the above comparative assays was accompanied by a ~190-fold decrease from the baseline K_A value from 1.12×10^{-3} in MtT–co-grafted otherwise untreated recipients to $<5.27 \times 10^{-6}$ in the MtT–co-grafted Adx rats (2). We conclude that the glucocorticoid deficiency in the Adx recipients inhibited MtH-stimulated AU formation and differentiation for milk secretion in the grafts; similar effects have been widely observed in whole mammary glands *in situ*.

The baseline clonogenic fractions calculated above reflect a two-to-three-fold concentration of the clonogens during preparation of the mammary cell suspension for transplantation when compared to those of our early reports (e.g., earlier AD_{50} value of ~2282 cells and $K_A = 3.47 \times 10^{-4}$; Ref. 6). In the current and other more recent studies (2), the clonogens were partially concentrated by first isolating mammary organoids that are composed primarily of epithelial cells; the collagenase-dispersed stromal fibroblasts and other nonepithelial cells were separated from the organoids by differential filtration and discarded. The epithelial cells of the organoids were then monodispersed for transplantation.

In the current limiting dilution assays, E2 and Prg synergized with each other and with MtH to stimulate AU and DU formation. The hormonal combinations tested in order of the most to the least effective in stimulation of DU formation in MtT–co-grafted Ovx recipient rats were: 0.1 or 1.0 μg E2 plus 4.0 mg Prg daily; 0.1 μg E2 plus 0.4 mg Prg daily; and 1.0 μg E2 alone daily. It is of interest that both E2 doses combined with the higher Prg dose were significantly more effective at stimulating DU formation than was the hormonal milieu in otherwise untreated MtT–co-grafted recipient rats. In contrast, all three combinations of E2 plus Prg given daily to Ovx MtT–co-grafted recipients yielded AD_{50} values insignificantly greater than the AD_{50} value in otherwise untreated MtT–co-grafted recipient rats; as with stimulation of DU formation, the least effective hormonal milieu for stimulation of AU development tested in such recipients was 1.0 μg E2 alone.

Mammary clonogens have been concentrated further during preparation for transplantation by monolayer culture of the isolated organoids for 2–4 days, selection of the epithelial monolayer outgrowths from such cultures (9), and further selection of clonogen enriched subpopulations by fluorescence activated cell sorting (10). A combination of these procedures resulted in epithelial cell suspensions with a clonogenic fraction ~27-fold greater than in our early AU-based assays and nine-fold greater than in the baseline AU assays of the current report (10).

Recent evidence indicates that some actions of steroid hormones, particularly estrogens, on mammary epithelia involve mechanisms that are dependent on stromal cells (22, 35, 36). The method for preparation of mammary cells for transplantation used in the current study yields suspensions composed primarily of epithelial cells, and, as noted, highly reproducible results in AU-based assays. If the effects of steroid hormones on clonal proliferation and differentiation observed in the current experiments were mediated through interactions with stromal cells, it seems likely that the latter were primarily of graft recipient origin (i.e., were present in the i.s. fat pads of the recipient rats before transplantation).

The current experiments are part of a program devoted to study of the characteristics, physiologic control, and radiation response of the cells of origin of radiogenic mammary cancer in rats (4). MtH had been shown to be the prime hormonal promoter of radiation-initiated rat mammary cells to overt cancer *in vivo* (37). Following the observation that growth of the mammary glands without milk secretion occurred in Adx-castrated MtT–co-grafted male rats (20), adrenalectomy was shown to further increase mammary carcinoma formation in whole body–irradiated female rats with elevated MtH, and this effect was counteracted by glucocorticoid administration (38, 39). The increase in cancer incidence consequent to Adx was attributed to blockage of the loss of initiated cells from potential cancer progenitor status by differentiation for milk secretion. Finally, mammary cancer occurs in very high frequency per surviving clonogen in grafts of irradiated rat mammary cells in Adx recipient rats with elevated MtH, and these cancers arise within the mammary structures that develop from the transplanted clonogens (40, 41).

These findings and the current studies are supportive of stem cell hypotheses of mammary cancer origin proposed by Dulbecco (33, 43), Russo (42), Smith and Medina (44), and others. They further support the conclusions that both AU and DU arise from mammary epithelial stem cells, and that a significant fraction of such stem cells are multipotent, able to respond to specific hormonal stimulation with formation of either AU or DU. Finally, the current results further illustrate the potential usefulness of the rat mammary cell transplantation assay for further quantitative investigation and characterization *in vivo* of the clonogenic progenitor cells from which radiogenic mammary cancers are derived.

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