

An entire functional mammary gland may comprise the progeny from a single cell

Edith C. Kordon and Gilbert H. Smith*

Laboratory of Tumor Immunology and Biology, Building 10, Room 8B07, National Cancer Institute, 9000 Rockville Pike, Bethesda, MD 20892, USA

*Author for correspondence (e-mail: smithg@litiblp.nci.nih.gov)

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SUMMARY

Any epithelial portion of a normal mouse mammary gland can reproduce an entire functional gland when transplanted into an epithelium-free mammary fat pad. Mouse mammary hyperplasias and tumors are clonal dominant populations and probably represent the progeny of a single transformed cell. Our study provides evidence that single multipotent stem cells positioned throughout the mature fully developed mammary gland have the capacity to produce sufficient differentiated progeny to recapitulate an entire functional gland. Our evidence also demonstrates that these stem cells are self-renewing and are found with undiminished capacities in the newly regenerated gland. We have taken advantage of an experimental model where mouse mammary tumor virus infects mammary epithelial cells and inserts a deoxyribonucleic acid copy(ies) of its genome during replication. The insertions occur randomly within the somatic genome. CzechII mice have no endogenous nucleic acid sequence homology with mouse mammary tumor virus; therefore all viral insertions may be detected by Southern analysis provided a sufficient

number of cells contain a specific insertional event. Transplantation of random fragments of infected CzechII mammary gland produced clonal-dominant epithelial populations in epithelium-free mammary fat pads. Serial transplantation of pieces of the clonally derived outgrowths produced second generation glands possessing the same viral insertion sites providing evidence for self-renewal of the original stem cell. Limiting dilution studies with cell cultures derived from third generation clonal outgrowths demonstrated that three multipotent but distinct mammary epithelial progenitors were present in clonally derived mammary epithelial populations. Estimation of the potential number of multipotent epithelial cells that may be evolved from an individual mammary-specific stem cell by self-renewal is in the order of 10^{12} - 10^{13} . Therefore, one stem cell might easily account for the renewal of mammary epithelium over several transplant generations.

Key words: Stem cell, Mammary, Transplantation, Mouse

INTRODUCTION

A wealth of evidence supports the concept that mammary tumors and hyperplasias in mice are clonal dominant populations and probably represent the progeny of a single cell (Young et al., 1971; Cohen et al., 1979, 1980; Cardiff et al., 1983; Kordon et al., 1995b). Similar conclusions have been reached for human breast cancer, carcinoma of the colon, uterine, cervix and bladder, ovarian teratomas and many hematological neoplasms (Fearon et al., 1987; Wainscoat and Fey, 1990; Siddransky et al., 1992). This implies that mammary tumors and hyperplasias are developed from tissue-specific epithelial stem cells and therefore represent populations of mutated stem cells and their differentiating progeny. According to the concept of maturation arrest of stem cell differentiation, malignant stem cells arise from the normal tissue-determined stem cells required for tissue renewal and produce tumors that are caricatures of the normal renewal process because of the imperfect differentiation of their proliferation-competent progeny (Sell and Pierce, 1994).

The technique of tissue fragment transplantation into mammary fat pads cleared of mammary epithelium was originally devised by DeOme (DeOme et al., 1959). It was demonstrated that virtually any portion of the mammary gland could recapitulate the entire glandular structure upon transplantation into a cleared mammary fat pad. Similar results are obtained with isolated mammary epithelial cells, although larger numbers of cells are generally needed due to technical considerations (Smith, 1996). Age and hormonal status of the donor does not seem to alter the innate regenerative capacity present in the mammary epithelial population (Young et al., 1971). However, regenerative senescence is induced by repeated serial passage of portions of the outgrowth (Daniel et al., 1968, 1971; Daniel and Young, 1971; Young et al., 1971). The senescence of regenerative growth is strongly linked to the frequency of mitotic activity that has occurred in the transplant fragment that is selected. For example, outgrowths generated from fragments of the peripheral portions of a given outgrowth, which presumably represent cells that have undergone a greater

number of mitoses, show a senescent growth phenotype much earlier when selected exclusively for passage than outgrowths of fragments taken from the central portions of the same serially transplanted outgrowths (Daniel and Young, 1971). Utilizing this technique, DeOme and others demonstrated that MMTV-induced premalignant mammary epithelial lesions were present in the mouse mammary gland (DeOme et al., 1959, 1968; Medina, 1973; Smith et al., 1984). Premalignant outgrowths do not show regenerative senescence or postlactational involution and therefore represent epithelial populations that have attained or retained a proliferative 'immortality'. In all likelihood, this characteristic is shared among all premalignant epithelial populations, irrespective of species or tissue type (Sell and Pierce, 1994).

The mouse mammary tumor virus (MMTV) infects mammary epithelial cells and randomly inserts its proviral DNA into the somatic cell DNA during its replicative cycle (Ringold et al., 1979). Some of these random insertions have been shown to cause deregulation of cellular (INT) genes leading to premalignant transformation and subsequently to tumor progression (Callahan, 1996). We speculated that normal mammary epithelial stem cells in CzechII mice might become infected by MMTV and acquire mutations. All MMTV proviral insertions can be detected by Southern blot analysis in CzechII mice because of the absence of host sequences related to MMTV. We further speculated that normal mammary outgrowths from implanted fragments might be clonal populations from a single stem cell. To test this hypothesis, we transplanted mammary epithelial fragments, chosen randomly, from mature, parous MMTV-infected female mice into gland-free mammary fat pads of syngeneic mice (see Experimental Design in Fig. 1). Unique virus-host restriction fragments will be detectable and constitute a specific pattern of bands only if the outgrowths are clonal (or nearly clonal) because they will be present in all (or nearly all) the epithelial cells. If mammary outgrowths are derived from the expansion of many different mammary epithelial cells, then specific MMTV-host restriction fragments will not be detectable, since retroviral DNA insertions occur randomly at multiple sites in the cellular DNA (Withers-Ward et al., 1994). Intact, lactating MMTV-infected mammary glands represent polyclonal populations because secretory lobules develop from multiple progenitors at multiple sites along an existing mammary ductal tree. Therefore, only limited locally developed progeny will reflect a given MMTV insertional event(s). Since none of these patterns is predominant in the whole population, DNA from the host mammary epithelium is not expected to produce a pattern of specific MMTV-host restriction fragments upon Southern analysis. We found this to be the case.

MATERIALS AND METHODS

Mice

Czech II mice (Callahan et al., 1982) and FVB mice were used as donors and hosts of the mammary epithelial transplants. The mice were held in a closed colony, maintained on a 12 hour light/dark cycle under controlled temperature and humidity, and were given laboratory chow supplemented with bird seed and water ad libitum. Bedding was hardwood chip and was autoclaved prior to use.

Tissue transplantation

The surgical procedures for clearing the mammary epithelium from the #4 inguinal fat pads of 3-week-old female mice and the method

of implanting tissue fragments or cell suspensions have been described in detail in earlier publications (DeOme et al., 1959; Daniel et al., 1968; Medina, 1973; Smith et al., 1980, 1984, 1991). Generally, the surgical procedures required to remove the host epithelium from the fat pads were performed immediately prior to insertion of the transplant or inoculation of cultured cells. Fragments (~1.0 mm³) of mammary epithelium were taken randomly from virgin females or postlactation (involved) glands. The fragments were implanted as described above and the hosts were bred after 3 weeks. The implanted glands as well as host glands were taken 1 day postpartum. Roughly 80% of the implant was removed for analysis (Fig. 1). The remainder was left intact for later study. Subsequent to lactation, secondary transplants were made from selected primary outgrowths following complete involution to determine if clonally related populations could also be generated from random fragments of the primary outgrowth. 8-10 fragments were selected from each of these populations for transplantation as described above.

To determine the relative total amount of DNA in cleared and implanted fat pads in hosts 1 day after parturition, the #4 pads of 3-week-old CzechII and FVB females were cleared of epithelium by removing all of the gland from the nipple to the central lymph node and including the lymph node leaving approximately 70% of the fat pad intact and free of epithelium. The left #4 pad was implanted with a fragment taken from the autochthonous epithelium. The contralateral pad was left epithelium-free. The hosts were subsequently bred and the individual cleared and implanted fat pads were collected at delivery for isolation and determination of total DNA content.

DNA extraction and Southern blot analysis

DNA was extracted from individual glands by phenol-chloroform as previously described (Gallahan and Callahan, 1987) or using DNAzolTM (Invitrogen) following the manufacturer's instructions. 9-13 µg of DNA were digested with restriction enzymes as indicated and separated in a 0.8% agarose gel at 56 V overnight to be then analyzed by Southern blotting (Southern, 1975). Briefly, the gels were blotted onto a Genetran nitrocellulose membrane and hybridized with a randomly primed ³²P-labeled probe. The probes were either the whole MMTV-LTR (C3H) fragment (1.3 kb) generated by PCR or a *NOTCH4/INT3* cDNA probe (1.2 kb) which has been previously described (Gallahan and Callahan, 1997). The membranes were hybridized overnight at 42°C and then washed twice in 2× SSC and once in 0.2× SSC (0.15 M NaCl, 0.015 M sodium citrate) at 65°C, before autoradiography.

Individual #4 fat pads (epithelium-free or containing lactating outgrowths) were extracted using DNAzol (GibcoBRL) to estimate mammary total DNA content. The DNA concentration was obtained by reading the A₂₆₀ (conversion factor= 44.0). Total DNA content was calculated by multiplying the DNA concentration by the total volume of the mammary DNA isolate. RNA contamination was eliminated by the use of RNase and confirmed by electrophoresis of a portion of the isolate on agarose gels. The accuracy of the determination of total DNA content was also confirmed by using the DNA dipstick method (Invitrogen) on serial dilutions of selected isolates.

Tissue culture

Preparation of primary cell cultures of mouse mammary epithelium has been described elsewhere (Smith, 1996). Briefly, the implanted #4 fat pads were removed from the hosts 8-12 weeks after transplantation, minced into 1-2 mm³ pieces in the presence of 1.0 mg/ml Collagenase Type 1A (Sigma) in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum and incubated overnight at 37°C. The fragments were then triturated through a 10 ml pipette and washed by low-speed centrifugation through several changes of digestion medium without collagenase. The resulting epithelial organoids were plated in 60 mm Falcon culture dishes for 1 week in Dulbecco's minimal essential medium with 10% fetal calf

serum, 4 $\mu\text{g/ml}$ porcine insulin and 10 pg mouse epidermal growth factor. Fibroblasts were removed at 2-day intervals by treatment of the cultures with trypsin under conditions favoring the retention of the epithelial colonies.

RESULTS

Mammary outgrowths exhibit detectable MMTV insertions

CzechII mice lack endogenous MMTV genes but may be infected by a strain of MMTV that is transmitted congenitally through the milk (Callahan et al., 1982; Gallahan and Callahan, 1987). These mice can be foster nursed with other strains that do not carry any exogenous MMTV (like BALB/c) to produce CzechII mice that do not carry exogenous MMTV. In the following experiments, mammary epithelial fragments were taken from CzechII MMTV-positive females and subsequently transplanted in either CzechII MMTV-positive or CzechII MMTV-negative recipients depending upon availability (Fig. 1).

In all, 52 randomly chosen pieces (~1.0 mm in diameter) of MMTV-infected mammary glands from 7 different multiparous CzechII MMTV-positive mice were transplanted into 3-week-old CzechII virgin females, who were subsequently bred (Fig. 1). These transplantation experiments were conducted over a period of one year as 3-week-old CzechII recipients became available. At parturition, the fully developed individual mammary implants were taken and DNA was extracted from each of them. DNA from each individual gland was digested with *EcoRI* and Southern blot analysis was carried out. Czech mouse MMTV proviral DNA has three internal *EcoRI* restriction sites none of them in the LTR sequences (Fig. 2). Two specific viral-host DNA fragments containing LTR sequences will be generated during *EcoRI* digestion from each inserted proviral copy. The blots were hybridized with a probe specific for MMTV-LTR DNA. DNA was successfully isolated from 30 fully lactating implants. Of these, 20 of 30 DNAs showed a discernible and unique pattern of host-viral junction fragments following digestion with *EcoRI* and hybridization with an MMTV-LTR-specific probe. Some of these results are shown in Fig. 3. In Fig. 3A, lanes 2, 3, 5, 7, 10 and 11 possess MMTV-LTR-positive restriction fragments

(arrowheads) indicating the presence of host-viral junctions; acquired MMTV insertions were also present in B lanes 1-6 and in C lanes 1-3. DNA isolated from lactating MMTV-infected donor glands, which represent expanded randomly integrated populations of mammary epithelium derived from multiple stem cells, never produced detectable viral-host restriction fragments following *EcoRI* digestion. DNA from MMTV-infected host lactating mammary gland is shown in (Fig. 3C, lane 4).

Southern blots of individual outgrowths hybridized with an MMTV-LTR probe frequently showed two bands in common, one at approximately 3.0 kb and sometimes another around 5.5 kb (e.g., Fig. 3A, lanes 4, 8, 9, 12). During its replicative cycle in mammary epithelial cells, MMTV genomic RNA is reverse transcribed to form a double-stranded cDNA of approximately 9.9 kb containing two Long Terminal Repeat (LTR) sequences. Unintegrated proviral DNA may exist as a linear structure or as a closed circle which may be supercoiled (Fig. 2). Examination of the restriction map of proviral MMTV DNA shows that *EcoRI* digestion of unintegrated linear and supercoiled MMTV DNA would produce DNA fragments around 3.0 kb and 5.5 kb containing the viral LTR. We tested for the presence of unintegrated MMTV DNA by digesting DNA from MMTV-infected outgrowths and lactating donor mammary glands with *NotI*, a restriction enzyme that does not

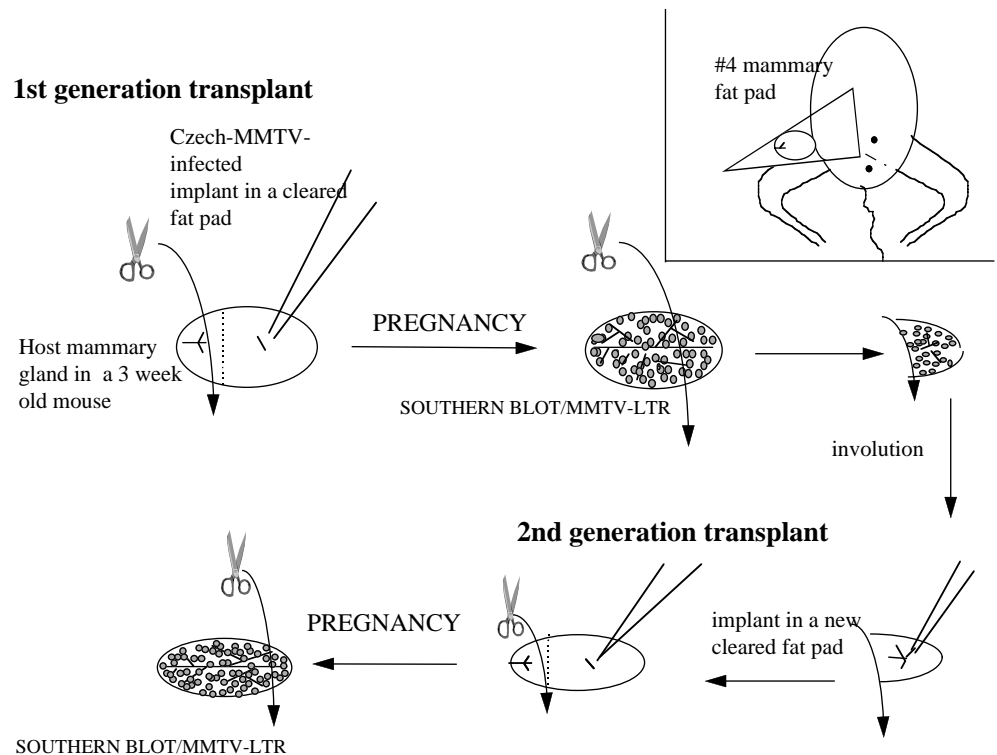


Fig. 1. Experimental design: Individual mammary fragments from MMTV-infected epithelium were transplanted into the cleared #4 fat pads (inset) of virgin CzechII mice. The mice were subsequently bred and roughly 80% of the lactating outgrowth was removed for Southern blot analysis in order to detect clonal growth. Fragments from the involuted portion of mammary outgrowths whose DNA had shown a clonal pattern upon Southern blot analysis were taken for a second cycle of transplantation into individual cleared fat pads. These host females were also bred and the resulting outgrowths were taken at lactation to perform a second round of Southern blot analysis. A third generation from clonally derived outgrowths was generated for producing primary epithelial cell cultures for dilution transplantation studies (not pictured).

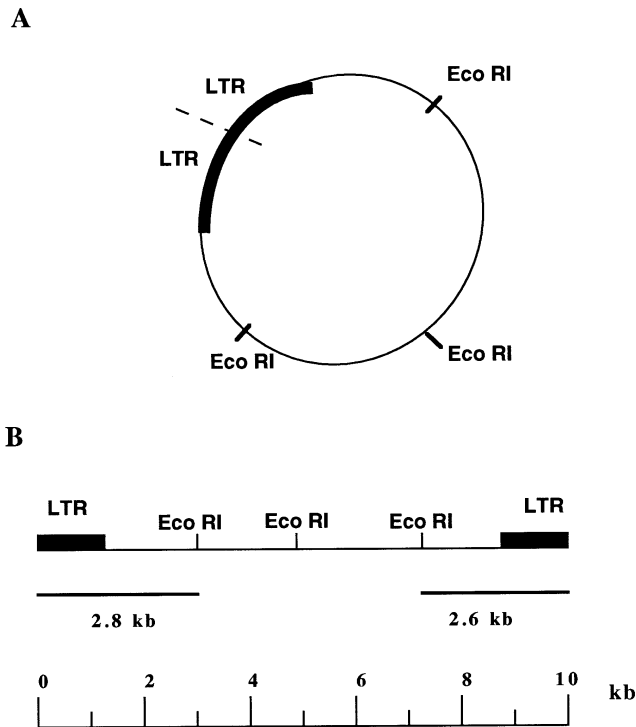


Fig. 2. *EcoRI* restriction map of proviral circular (A) and linearized (B) MMTV. During its replication cycle in mammary epithelial cells, MMTV forms a double-stranded circular DNA of approximately 9.9 kb containing two Long Terminal Repeat (LTR) sequences. Czech mouse MMTV proviral DNA has 3 internal *EcoRI* restriction sites, none of them in the LTR sequences. Therefore, each insertional event will generate two specific viral-host DNA fragments containing LTR sequences when cellular DNA is digested with *EcoRI*. If the DNA is not integrated, then *EcoRI* would produce two ~3.0 kb LTR-containing fragments from the linear MMTV genomic DNA and one ~5.5 kb LTR-positive fragment from the closed circular form.

cut within proviral MMTV DNA and then performed Southern blot analysis using the MMTV-LTR probe. The recognition site for *NotI* is 8 bp, therefore host restriction fragments which include the MMTV proviral DNA inserted in the cellular DNA should be on average larger than 75 kb because the average size fragment produced by complete digestion with *NotI* is 65.536 kb. Two bands were detected with the MMTV-LTR probe in DNA from either host or transplanted mammary glands with the apparent fragment size of approximately 10.0 and 7.0 kb (Fig. 4). The slower band corresponds to the size of an unintegrated linear provirus, while the faster one corresponds to the size predicted for an unintegrated supercoiled circular form (Fig. 4). This demonstrates that DNA isolated from MMTV-infected mammary glands may contain detectable levels of unintegrated proviral cDNA. The presence of unintegrated MMTV DNA most probably accounts for the common LTR-positive fragments at ~3.0 kb and ~5.5 kb observed in the Southern blots of *EcoRI*-digested MMTV-infected mammary gland DNA.

Stem cells with MMTV insertions are self-renewing

To distinguish whether the outgrowths bearing detectable host-viral restriction fragments represented populations derived

from multiple MMTV-infected progenitors or from a single stem cell, fragments from involuted mammary outgrowths that produced a clonal restriction pattern in the first generation were transplanted again. 8-10 fragments from each of these involuted outgrowths were implanted into individual cleared fat pads. As before, the host females were bred and the lactating implants were taken to perform a second round of Southern blot analysis (see Experimental Design in Fig. 1).

Fragments from seven different involuted primary implants, which had shown a specific pattern of MMTV-LTR bands, were transplanted into cleared mammary fat pads. Implants from two of these failed to produce lactating glands at parturition in the new hosts, either because of technical error or because the stem cells were senescent. Fragments from the other five involuted glands, produced fully functional glands in the #4 mammary fat pads of the pregnant hosts. A pattern of viral-host restriction fragments identical to those observed in the original outgrowth was reproduced in the second transplant generation. Fig. 5A-C shows the Southern blots from three different clonal outgrowths and their second generation transplants. Fig. 5B shows DNA from individual outgrowths originated from seven individual fragments from one involuted primary outgrowth; Fig. 5C compares the DNA from three second transplant outgrowths produced from another. In some cases, individual second generation transplants from the same primary outgrowth (Fig. 5B) show additional minor viral-host bands (see Fig. 6). This suggests that extra MMTV insertions may occur in the stem cell DNA during regeneration of a lactating outgrowth. The last lane of Fig. 5B contains DNA from intact, non-transplanted, MMTV-infected mammary host DNA. The single LTR-positive band in this lane is generated from *EcoRI* digested of unintegrated linear proviral DNA as described earlier.

MMTV insertions represent 'single copy' events (i.e., present in only one of a chromosome pair) and occur only in epithelial cells. Therefore, a maximal signal from a MMTV-inserted DNA fragment in a clonal outgrowth would be less than half the intensity of that of an equivalent DNA fragment from a normal cellular gene. One of the blots shown in Fig. 5B was hybridized with a probe of comparable complexity to the MMTV-LTR probe, but directed towards the sequence of a cellular gene (*NOTCH4/INT3*). The intensity of the *NOTCH4/INT3* band in each of the lanes represents a relative maximal signal for a homozygous gene sequence in proportion to the amount of DNA transferred. Examination and comparison of the intensity of these bands with those representing the integrated proviral fragments supports the interpretation that the epithelium in these outgrowths may be clonally derived from an individual mammary epithelial stem cell (Fig. 6). It is possible that the intensity of the lower band in the lanes 1-5 may be affected by the presence of unintegrated MMTV DNA in these preparations since *EcoRI* digestion of unintegrated linear viral DNA produces an LTR-positive fragment near 3.0 kb. Lane 6 represents DNA isolated from non-transplanted, MMTV-infected lactating mammary gland DNA and has a positive band near 3.0 kb. In all probability, the presence of unintegrated viral DNA contributes to the relative difference of hybridization between the upper and lower bands in lanes 1-5.

MMTV insertions are rare in prepubertal mammary gland

Transplants were performed using MMTV-infected,

prepubertal, 3-week-old females as donors, instead of multiparous mice as in the experiments described above. 18 ductal fragments from different, prepubertal, MMTV-infected females were implanted into individual #4 cleared fat pads and the glands were taken at parturition of the host to perform Southern blot analysis. DNA from only one out of fifteen successful transplants showed a host/viral junction fragment pattern indicative of clonal expansion of an infected stem cell. Subsequent transplants from this positive outgrowth did not exhibit a MMTV-LTR/host restriction fragment pattern (not shown). None of the other 14 outgrowths yielded DNA that showed any specific pattern of bands, although all 5 tested for the presence of unintegrated circular proviral DNA were positive indicative of MMTV infection and replication (not shown). Strong bands of approximately 5.5 kb were seen in each of these. Subsequent transplantation from these MMTV-positive outgrowths also indicated the absence of MMTV integration into individual mammary stem cells although these outgrowths remained positive for unintegrated MMTV DNA (not shown).

Determination of the number of epithelial cells in transplanted #4 fat pad

The following experiments were designed to estimate how many epithelial cells are produced to complete the entire epithelial cell repertoire of a lactating mammary outgrowth. One group was designed to determine the total DNA in an epithelium-filled #4 fat pad of a nulliparous female. In order to make these determinations comparable to outgrowth DNA values, the fat pads were surgically altered to mimic the size of a epithelium-divested #4 fat pad. The bilateral #4 fat pads of six, 8-week-old, FVB mice were surgically altered. They were not bred and 2 months later, the #4 glands were taken for quantification of total DNA. The total DNA in an epithelium-containing virgin 'sham-cleared' #4 fat pad in an adult female was 25.05 μg (s.d.=7.07, $n=11$). In a cleared, epithelium-free #4 fat pad, the DNA total was 11.15 μg (s.d.=2.64, $n=9$). Therefore epithelium accounted for an average of 13.9 μg of DNA per operated #4 fat pad. To estimate the number of epithelial cells that this DNA quantity might represent, we divided this number by 6 pg, the amount of DNA reported to be present in a single diploid mouse liver cell (Sober, 1970). From this calculation, it was estimated that a typical 'sham-cleared' #4 fat pad

contained $\sim 2.3 \times 10^6$ epithelial cells in a mature nulliparous FVB mouse.

In another experiment, both #4 mammary fat pads of FVB and Czech mice were cleared at the third week of age. A fragment from the excised mammary epithelium was reimplanted in the left #4 pad, the contralateral mammary fat pad was left empty. These mice were then bred and the #4 fat pads were taken at parturition for DNA quantification. Table 1 shows the quantity of DNA that was extracted from the cleared fat pads in FVB and Czech female lactating hosts compared to the contralateral pads that had received an implant. The total DNA content and the total number of epithelial cells is given for each of 12 individual hosts (9 FVBs and 3 Czechs). The epithelial contribution to the glands was estimated as explained above, but, in order to reflect the variability in the extent of lactational development among implanted glands, calculations were made individually for each mouse using the DNA numbers from the contralateral glands. Assuming for theoretical purposes that each of these populations was clonally derived, the number of doubling events required to reach the total number of cells in the lactating outgrowth from a single epithelial cell was calculated using the following formula:

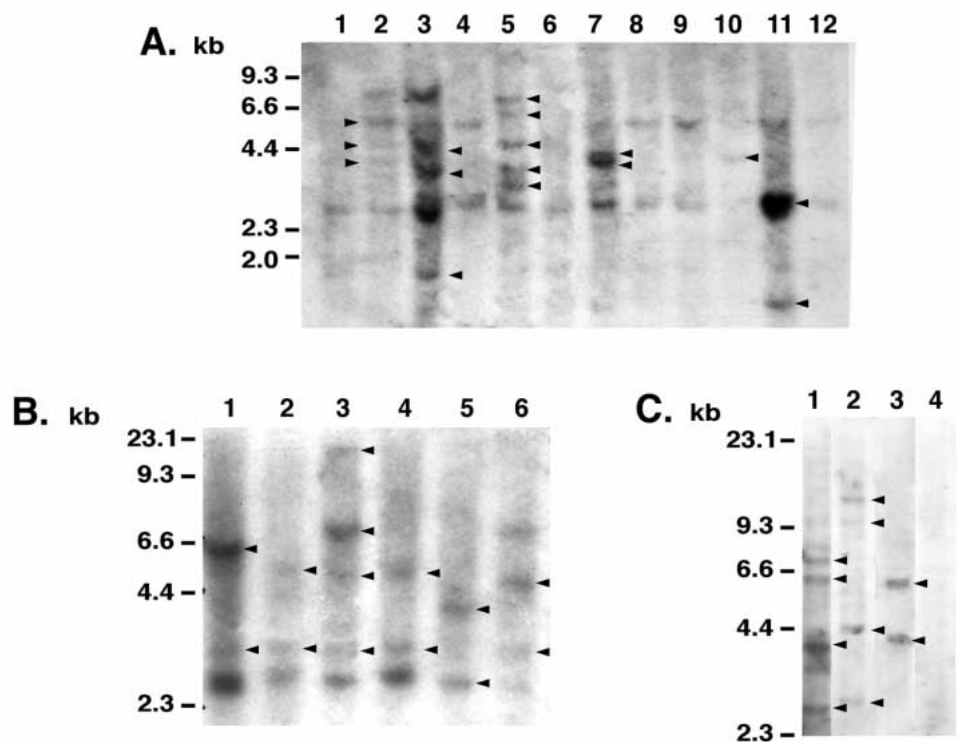


Fig. 3. Southern blot analysis of twenty one outgrowths started from single fragments. Fragments were implanted, individually, into Czech II cleared mammary fat pads. At lactation, the fully developed mammary glands were taken and DNA was extracted from each of them. DNA from each individual gland was digested with *EcoRI*, and Southern blot analysis was carried out. In every case, the blots were hybridized with a 1.3 kb dsDNA probe specific for MMTV-LTR sequence. (A-C) Southern blots of 21 independent lactating outgrowths. The arrowheads indicate the location of host viral restriction fragments indicative of the presence of specific MMTV proviral insertions within the somatic DNA (in A, lanes 2, 3, 5, 7, 10 and 11; in B, lanes 1-6; and in C lanes 1, 2 and 3). Lane 4 in C represents *EcoRI*-restricted intact mammary gland DNA from the MMTV-infected CzechII host. (A) Common MMTV-LTR-positive bands at ~ 5.5 kb can be seen in lanes 4, 8, 9, 10, 11 and 12 and nearly all the lanes show an LTR-positive band at ~ 3.0 kb. Subsequent analysis demonstrated that these bands are generated from unintegrated MMTV linear and circular DNA by *EcoRI* digestion (see Fig. 4).

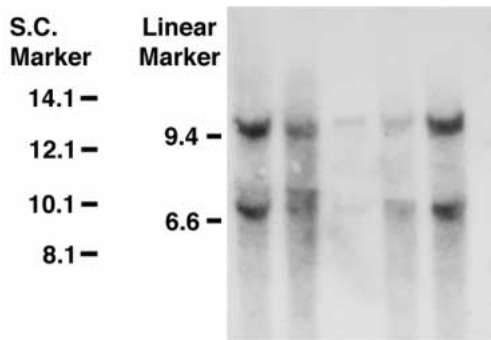


Fig. 4. Southern blot analysis using an MMTV-LTR probe of *NorI*-digested DNA isolated from MMTV-infected transplant outgrowths (lanes 1-3, counting from the left) and from the corresponding MMTV-positive lactating host mammary tissue (lanes 4-5). Supercoiled and linear molecular weight markers are shown on the left. Two uncut MMTV-LTR-positive bands are present in *NorI*-digested MMTV-infected cellular DNA preparations. These represent supercoiled (lower band) and linear (upper band) unintegrated MMTV proviral DNA.

$[\log_2(\text{no. epithelial cells}) + 1]$. The number of mitotic events, assuming a single progenitor, required to repopulate any given fat pad at parturition was relatively uniform varying between 25 and 27 doublings.

Epithelial stem cell repertoire in clonally derived outgrowths

Previous transplantation studies carried out in our laboratory indicated that three distinct mammary epithelial cell progenitors were present in primary cell cultures derived from

mouse mammary epithelium (Smith, 1996; Chepko and Smith, 1997). The three were distinguished by limiting dilution transplantation in pregnant hosts. Two were limited in their developmental capacity; one capable of producing only lobular structures without branching ductal morphogenesis; another produced only branching ducts without lobular development and a third produced a complete (both lobules and ducts) lactating gland which completely filled the fat pad. It was not clear from these experiments whether these progenitor types were maintained independently from one another or whether one ‘master stem cell’ was responsible for the generation of all. To distinguish among these possibilities, primary epithelial cultures from tertiary (third generation) MMTV-infected CzechII outgrowths, which appeared to be generated by a single stem cell were initiated and the resulting cells were inoculated into cleared Czech fat pads at a limiting dilution (1.4×10^4 cells). Three types of outgrowths were found in the small number of outgrowths resulting from these inocula. Under these conditions, only 37.5% of the inoculated fat pads had epithelial growth. The positive implants produced outgrowths (one each) which showed complete filling of the fat pad with ductal branching and full lobular development (Fig. 7B), partial filling comprised of secretory lobules alone (Fig. 7C,E), or limited growth defined by branching ducts alone with no alveolar development (Fig. 7D,F). Similar results were obtained when random fragments from a fully lactating, clonally derived outgrowth were transplanted, subsequent to the unexpected demise of the transplant-bearing host. In this instance, the fragments produced varied outgrowths (not shown), in approximately equal numbers (3:3:4), which were characterized by limited growth with lobulogenesis only,

Fig. 5. MMTV-LTR Southern blot analysis of secondary transplants. Three examples (A-C) of second generation transplants from clonally derived outgrowth. DNA from these first and second generation transplants were analyzed using a MMTV-LTR probe. (A,C) Comparison of the results from analysis of the first generation transplant with multiple second generation transplants established from the involuted primary outgrowth. (B) Southern blot analysis and comparison of the DNA from the original transplant generation with DNA from a single second generation transplant. DNA from all the second generation transplants show the same pattern of virus-host restriction bands found in the first generation. To aid in the comparison of virus-host restriction fragments between the first and second generations, we have used arrowheads. The only lane showing host lactating mammary DNA is lane 9 in 5A. The LTR-positive band in this lane is generated by *EcoRI* digestion of unintegrated linear proviral DNA present in the MMTV-infected mammary gland. Inspection of lanes 4 and 5 in A indicate the presence of new host-viral fragments. Because the secondary outgrowths shown in lane 2-8 were generated from nearly adjacent individual fragments dissected from the scanty portion of involuted original implant remaining in the non-lactating host (Fig. 1), it is probable that these new insertions were acquired by individual stem cells either in situ during the growth and development of the original implant or very early in the generation of the secondary outgrowth.

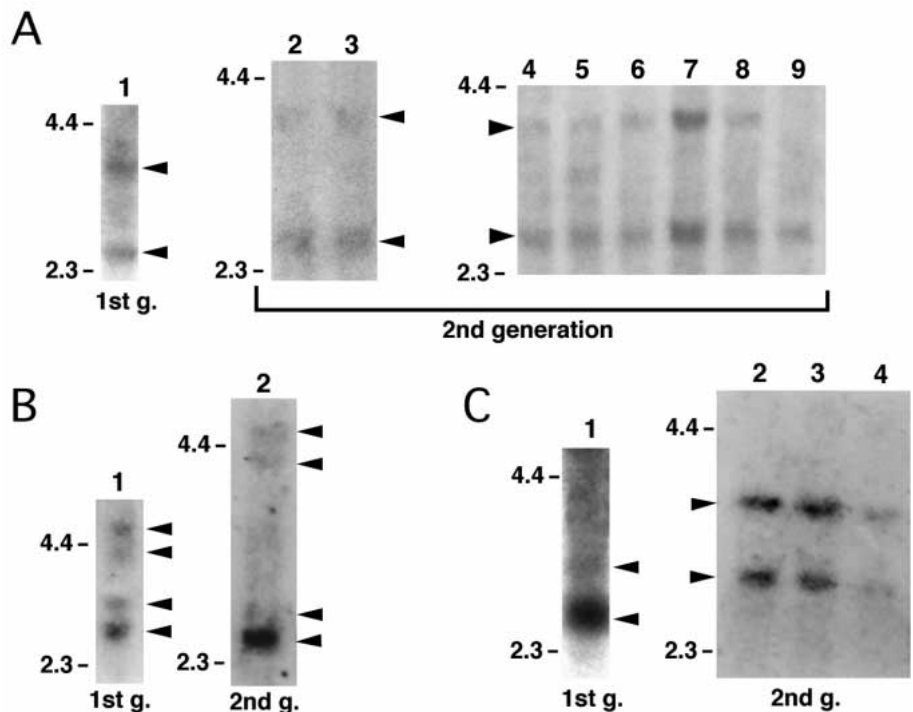


Table 1. Total DNA and epithelial content of operated #4 inguinal mammary fat pads

FVB mice	Lactating gland DNA (μg)*	Cleared pad DNA (μg)†	Epithelial cell content ($\times 10^6$)‡	Estimated doubling events§,¶
Mouse 1	225	07.8	36.2	26.1
Mouse 2	230	10.8	36.5	26.1
Mouse 3	470	10.2	76.6	27.2
Mouse 4	148	07.2	23.4	25.5
Mouse 5	149	10.8	22.8	25.4
Mouse 6	258	10.2	41.3	26.3
Mouse 7	412	34.8	62.9	26.9
Mouse 8	345	09.6	56.0	26.7
Mouse 9	177	15.6	27.0	25.7
Czech mice				
Mouse 1	245	06.0	39.8	26.2
Mouse 2	106	03.0	17.2	25.0
Mouse 3	150	04.2	24.3	25.5

*Total DNA content of #4 inguinal mammary fat pad with lactating outgrowth.

†DNA content of contralateral cleared fat pad

‡Epithelial cell content determined for individual outgrowths by subtracting the total DNA from the contralateral cleared pad from total DNA of implanted pad and dividing by DNA content of single mouse liver cell (6 pg) (see text).

§Doublings required to reach the total No of epithelial cells from a single cell was calculated as $[\log_2(\text{no of epithelial cells}) + 1]$

¶The average number of doublings required was 26.05 ± 0.54 ($n=12$).

limited ductal branching with minimal lobular formation and completely filled fat pads with fully developed lactating glands.

DISCUSSION

A minimalist's definition of tissue-specific stem cells might be "Stem cells have the capacity both to self-renew and to generate all of the differentiated progeny represented in the tissue". Our data demonstrate that an entire functional mammary gland may comprise the progeny from a single epithelial stem cell and that this process may be repeated in serial transplant generations (see Fig. 1). The capacity for self-renewal is demonstrated in the second generation transplants, which possessed the same MMTV insertion(s) as the first. The original epithelial stem cell not only produced additional stem cells scattered throughout the reconstituted gland and all the differentiated epithelial types, but also gave rise to two distinct lineage-limited epithelial cell progenitors. The latter were identified in primary cell cultures from these clonal populations by transplantation to subsequently impregnated hosts. These lineage-limited mammary epithelial progenitors, which we have described before (Smith, 1996), gave rise to epithelial outgrowths restricted to either lobular development alone or branching ducts without secretory lobules. The extent to which these outgrowths were able to fill the cleared fat pad was also limited suggesting that the lineage-limited lobular and ductal progenitors possessed a finite capacity for growth. In contrast, the outgrowths that contained all epithelial components, both lobular and ductal, completely filled the pad. Limited outgrowths i.e., lobular only or duct only, were also obtained when individual, randomly selected, fragments from fully

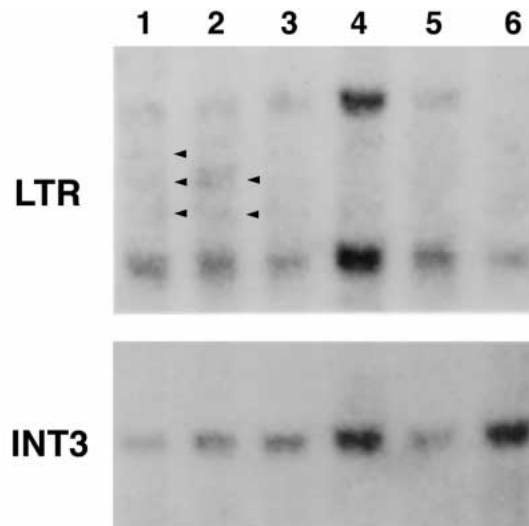


Fig. 6. Relative intensities of MMTV-LTR, a single copy gene present only in one sister chromatid and only in epithelial cells versus *INT3/NOTCH4*, a normal homozygous gene present in all cells. The upper panel shows Southern blot analysis of DNA from five independent second generation outgrowths (originated from the same primary transplant) hybridized with a MMTV-LTR probe (same as shown in Fig. 5A, lanes 4-9); the lower panel shows the same blot hybridized with a probe of similar complexity specific for *INT3/NOTCH4*, a normal cellular gene which is present in both sister chromatids (i.e., two copies) and present in all cell types. The relative intensities of the upper band in the upper panel (the density of the lower band may be affected by the presence of unintegrated proviral DNA) with respect to the *INT3/NOTCH4* bands in the lower panel, is consistent with the interpretation that these transplants are clonally derived and that most of the epithelium in the outgrowths contain the inserted proviral DNA. The arrowheads point out new MMTV-LTR/host restriction fragments in lanes 1 and 2 that are described in the legend for Fig. 5.

lactating clonal outgrowths were transplanted into cleared fat pads and the hosts subsequently impregnated (not shown). It appears, therefore, that three distinct, proliferation-competent and multipotent epithelial cells reside within mammary epithelial populations ostensibly generated from a single stem cell. We consider each of these progenitors multipotent because each is capable of giving rise to multiple epithelial cell types, e.g. myoepithelial and luminal epithelial cells. All types of mammary epithelial progenitor appear to be present in the fully differentiated, 'clonally derived' outgrowth and also in cell cultures produced from 'clonally derived' outgrowths after involution. Significantly, the developmental properties exhibited by the lineage-limited multipotent epithelial cell types appear to be 'cell intrinsic', since each is capable of producing only lobular or ductal growth in a hormonal environment that allows both processes to occur simultaneously. On the other hand, the tissue-specific epithelial stem cell responsible for regeneration of the entire gland seems to give both lobular and branching ductal expansion as the hormonal environment dictates. In that case, cleared fat pads in virgin hosts are completely filled with a branching ductal system without significant lobular development, whereas in the impregnated host ductal branching morphogenesis and lobular development occur simultaneously in the transplant.

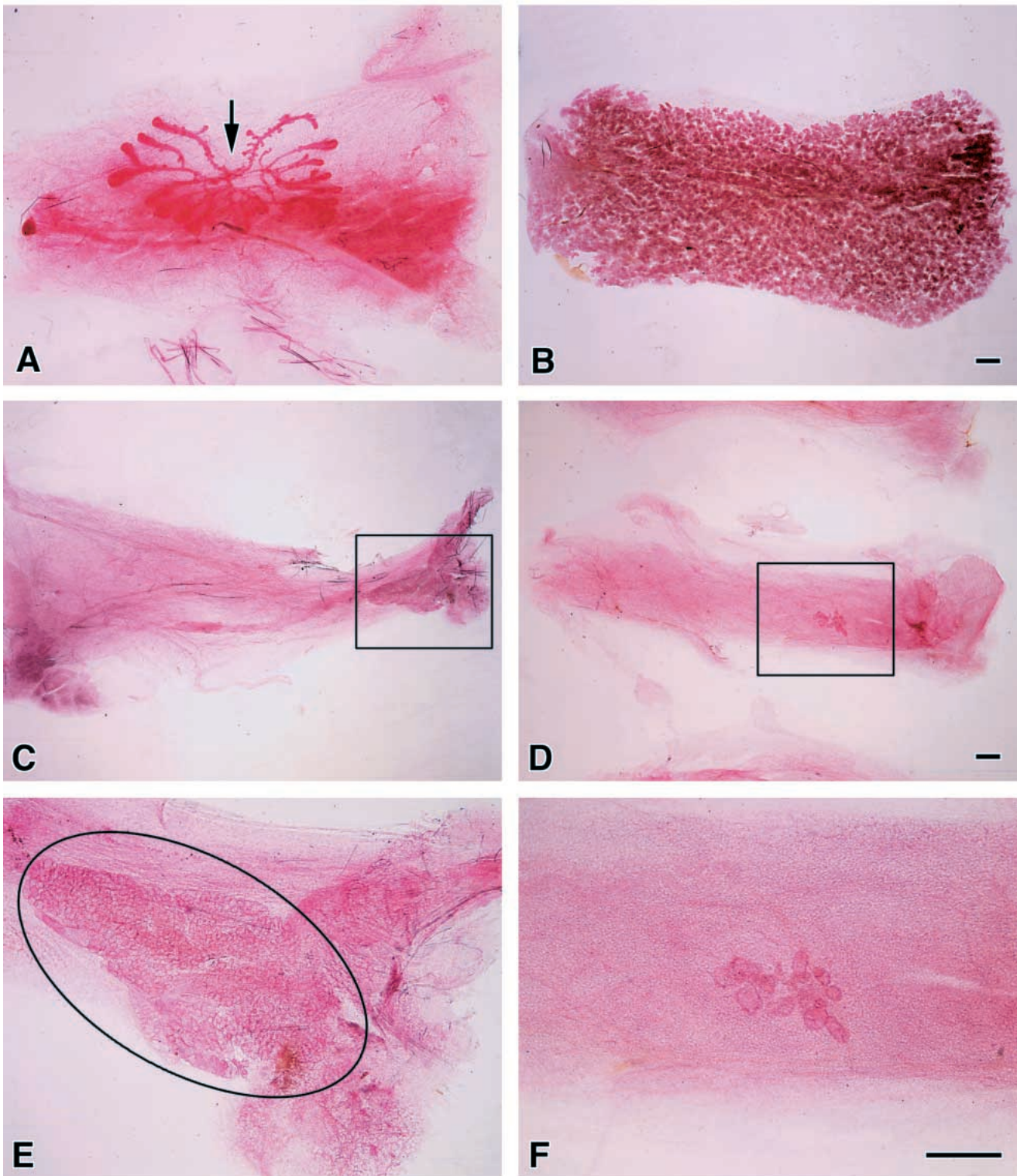


Fig. 7. Whole mounts. (A-D) The major portion of a cleared inguinal mammary fat pad and the epithelial growth from implants. (A) The mammary development from a typical fragment of epithelium 11 days after being implanted and on the 4th day of pregnancy; the arrow indicates an apparent single starting site for ductal growth. (B-F) The three different types of mammary outgrowths found in 1 day lactational hosts after inoculation with a limiting dilution of 14,000 epithelial cells. The epithelial cells were from a single primary culture derived from a third generation clonal outgrowth. (B) The inoculum produced an entire functional lactating mammary gland and the fat pad is filled with secretory epithelium. In C,E and D,F, the inoculated cells developed a structure consisting of secretory lobules alone (C,E) or a sparse epithelial growth composed of branching ducts (D,F). (C,E) The same lobular-limited outgrowth at different magnifications. (D,F) The same ductal-limited outgrowth at different magnifications. This was done so that the viewer could ascertain the actual extent of epithelial growth of each of these lineage-limited structures attained relative to the complete outgrowth pictured in B. Magnification: A-D, $\times 8$; E,F, $\times 30$. Bar, 500 μm

The potential replicative power of mammary stem cells

Nicoll and Tucker (1965) reported that mammary epithelium contains approximately 14% of the total DNA content of a #4 mammary fat pad in a virgin C3H strain mouse and about 88% of the total DNA of the same fat pad at the end of the gestational period. They used the method of surgically clearing the #4 fat pad to obtain their data. From their DNA measurements, we determined that the average number of epithelial cells in a filled surgically altered #4 fat pad in C3H virgin mouse was 2.5×10^6 . The average number of epithelial cells that we find in surgically altered, epithelium-filled virgin FVB #4 mammary glands is very close to the number that we computed from the values reported by those authors in C3H mice (2.3×10^6 versus 2.5×10^6). However, the lactating implants (Table 1) show that the absolute number of epithelial cells may vary considerably when comparing individual outgrowths from one mouse to another mouse. Some of this variation may be due in part to variations in the amount of fat pad that remains after surgery. Our theoretical estimation of the number of doublings (mitoses) to produce these lactating population if they were composed of the progeny from one cell varied between 25 and 27 (Table 1) with an average of 26 ± 0.5 . The difference among the populations in the lactating outgrowths therefore vary by only 1 population doubling on average, a remarkably uniform result. Similar calculations, again assuming a single progenitor, using the figures presented by Nicoll and Tucker (1965) for total average epithelial cell DNA in lactating surgically altered #4 C3H fat pads ($n=10$) gave a theoretical doubling of 27 ± 0.1 . These authors did not implant these fat pads so the increase in epithelium at lactation was from the intact epithelial ductal system and therefore not clonally derived. Nevertheless, the total number of epithelial cells present in the surgically altered #4 fat pads of lactating C3H females in their study closely agrees with our calculations from FVB and CzechII mice.

It seems unlikely to us that a multipotent stem cell responsible for regeneration of the entire functional glandular structure would be able to divide 25 to 27 times during this process and maintain its integrity as a tissue-specific stem cell. It seems more probable that much of this cell division is carried out regionally by the developmentally committed lobular and ductal progenitors described above, which have a limited capacity of proliferation. Unfortunately, because of their limited growth potential, we have not been able to demonstrate directly that these limited outgrowths possess the same MMTV insertions as the original clonal outgrowth. Nevertheless, these limited outgrowths have arisen from limiting dilutions, which contain less than one clonogenic epithelial unit per bolus as indicated by the small number of successful outgrowths (3 out of 8). By Poisson distribution, one clonogenic component per inoculum would provide approximately 3 positive takes out of 5 implants. Although we hypothesize that these lobular and ductal committed progenitor cells may be mostly responsible for cell divisions needed to fabricate the ductal and lobular compartment of the lactating mammary gland, there is at present no evidence that they are normally capable of self-renewal. In addition, in our hands, their proliferative capacity is quite low.

Mammary tissue-specific epithelial stem cells do not have

unlimited renewal potential (as shown by Daniel et al., 1968), although they are able to renew for the several transplant generations. Generally, growth senescence is reached by the fourth transplant generation (Daniel et al., 1968). Our earlier estimate of the number of stem cells within the mammary epithelial population capable of producing complete mammary development upon transplantation is around one in 2500 cells (Smith, 1996). This is in good agreement to the reported frequency (1 in 1100) of clonogenic epithelial cells in rat mammary gland cells transplanted into scapular fat pads (Tai et al., 1997). Using our calculated frequency of stem cells and dividing it into the total number of epithelial cells in a virgin #4 fat pad as determined from our own data or from Nicoll and Tucker's figures (1965), there are between 900 and 1000 stem cells in a typical repopulated #4 mammary fat pad (i.e. $2.3\text{--}2.5 \times 10^6$ divided by 2500). If this number is similar in glands regenerated from a single stem cell then, the tissue-specific mammary epithelial stem cell would need to divide symmetrically (self-renew) roughly 11 times or, in other words, undergo 11 doublings (to reach ~ 1000). In practice, serial transplants of normal mammary epithelium approach senescence at the fourth transplant (Daniel and Young, 1971). After 4 serial transplants, assuming clonal expansion, the original, clone-generating stem cell would have doubled between 40 and 50 times. This number of doublings fits very well with the number predicted by Hayflick and Moorhead (1961) to represent the maximum number of doublings that a eukaryotic cell may undergo before reaching proliferative senescence.

Stem cell biology and mammary cancer

As shown in Fig. 7 and in previously published data (Daniel et al., 1971; Kordon et al., 1995a; Smith et al., 1996) lobular-limited or ductal-limited outgrowths look senescent and are not able to fill a fat pad even during pregnancy. Therefore, in order to have a relevant impact on the mammary epithelial population, mutations in these limited progenitors must provide a net increase in proliferative capacity or provide the potential for self-renewal. However, any mutation in the mammary epithelial stem cell population will be highly relevant for the entire epithelial population because it will be irrevocably conserved and repeatedly inherited by all the progeny of the stem cell. We have demonstrated in this paper that mammary epithelium stem cells may be mutated during organogenesis and that these mutations are conserved in all their subsequent progeny and during self-renewal. Therefore, the implication for tumorigenesis that arises from these observations is that these self-renewing, organ-specific stem cells may represent the major potential risk among the mammary epithelium for carcinogenesis due to their vast proliferative capacity and their potential for self-renewal.

It appears that a single mammary epithelial stem cell may have the capacity to produce approximately $10^{12}\text{--}10^{13}$ multipotent, proliferation-competent offspring (we calculate approximately 11 symmetric doublings per transplant generation \times 4 transplants to reach senescence, i.e. $(1+2^{43})$ divisions or 8.7×10^{12} stem cells), before reaching proliferative senescence. Mammary epithelial fragments from females virtually of any age, multiparous or nulliparous, have the capacity to recreate complete mammary glandular structures for several transplant generations in mammary fat pads (Young

et al., 1971). Therefore, these prodigiously proliferative and self-renewing cells persist throughout the lifetime of the animal and represent an epithelial subpopulation that is continually in peril of oncogenic transformation. The observation that mutated, albeit normally functioning, tissue-specific stem cells persist among the population of aging epithelium in the mammary gland of the mouse argues that these cells may be responsible for the early manifestations of malignancy and begs for an exhaustive study of their ontogeny and biology in the Human.

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