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Report

Origin and progression of pregnancy-dependent mammary tumors induced by new mouse mammary tumor virus variants

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Summary

In order to study mechanisms of progression of mouse mammary tumor virus (MMTV)-induced pregnancy-dependent mammary lesions, we removed and serially transplanted 17 small tumors detected in MMTV-infected pregnant females. This gave rise to the same number of '*in vivo*' tumor lines. Hormone-dependency of the passages was determined by comparing tumor development in multiparous versus virgin hosts. We found that the first passages of most of these lesions (11/17) required pregnancy to grow. However, all these tumor lines lost their hormone-dependence through successive passages. The original pregnancy-dependent lesions were mostly multiclonal and showed high levels of estrogen and progesterone receptors. Alternatively, pregnancy-independent tumors arose as clonal dominant populations exhibiting a lower hormone receptor content. Our data show that the progression of hormone-dependent MMTV-induced mammary tumors is an irreversible process associated with the appearance of additional MMTV insertional events as well as alterations in the composition of the tumor cell population.

Introduction

Mouse mammary tumor virus or MMTV is a type B retrovirus causing tumors in susceptible mice by acting as an insertional mutagen in somatic cellular genes. MMTV is transmitted either horizontally as milk-borne exogenous variants or vertically as germ-line integrated provirus (Mtv). Mammary tumors induced by exogenous MMTV frequently arise from preneoplastic lesions termed hyperplastic alveolar nodules (HANs). HANs can be serially transplanted in female mouse hosts. Upon transplantation these lesions originate from preneoplastic clonal dominant cell populations from which, eventually, clonal-dominant mammary carcinomas and even metastases arise [1]. In consequence, it has been proposed that the new MMTV insertion in cell genome followed by clonal selection is the main mechanism by which this tumor progression sequence proceeds.

Alternatively, some MMTV strains are able to induce premalignant ductal lesions named plaques, which arise during pregnancy and regress after parturition [2]. Plaques have also been serially transplanted and it was found that upon this procedure, these lesions retain the ability to produce hormone-dependent

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(HD) tumors in pregnant females while they grow into normal ducts in non-pregnant hosts [3]. It has been shown that these HD tumor lines consist of mainly clonal dominant populations [4]. However, other authors have reported that the early passages of HD tumors from GR mice have different mutated loci than later passages showing hormone-independent (HI) behavior [5]. This suggested the possibility that the HD tumors were composed of polyclonal populations.

It has been previously shown that the identification of MMTV insertions by Southern blot analysis in a mammary cell population indicates their clonal or nearly clonal origin [6]. Unique virus–host restriction fragments constitute a specific and reproducible pattern of bands only if they are present in most of the cells in a population. In case, this was derived from the expansion of many different progenitor cells, then specific MMTV–host restriction fragments would be hard to detect, as retroviral DNA insertions occur randomly at multiple sites in cellular DNA [7]. In fact, it has been shown that the intact lactating MMTV-infected mammary glands which represent polyclonal populations – because secretory lobules develop from multiple progenitors at multiple sites – do not show a pattern of specific MMTV–host restriction fragments [6].

Three new exogenous MMTV variants (BALB2, BALB14, and LA) were described in our mouse colony [8, 9]. The three variants appearing together were designated MMTV(LA) in recent publications [8, 10]. BALB/c infected with MMTV(LA) showed a 90% incidence of mammary tumors. These tumors were initially pregnancy-dependent. They regressed completely or partially between pregnancies and they reappeared at the same site in subsequent pregnancies. Eventually, they progressed to become autonomous and grew independently of the female hormonal status [9].

Our aim herein was to investigate tumor cellular origin and progression mechanisms by analyzing the insertional events of these new exogenous MMTV variants. We found that the HD and hormone-responsive (HR) passages may arise as polyclonal populations. Alternatively, pregnancy-independent or HI tumors are mostly clonal-dominant populations. In addition, HI tumor passages arising from HD tumors usually showed additional bands, suggesting that, in this model, the progression towards HI is produced by the selection of specific cell subpopulations and not by epigenetic modifications in the tumor cell population as a whole.

Materials and methods

Mice

Female BALB/c mice from our mouse colony, 8–12 weeks in age and 20–25 g in weight, were used throughout. They were housed four per cage in air conditioned rooms at $20 \pm 2°C$, kept under an automatic 12 h light/12 h darkness schedule, and given pellets and tap water *ad libitum*. Animal care was in accordance with institutional guidelines.

In vivo *tumor lines*

MMTV (LA) infected BALB/c pregnant females were exhaustively examined in order to detect pregnancy-dependent mammary tumors. Then, a total of 17 small tumors (tumor area: 50 mm^2, approximately) growing under these conditions were removed. They were minced in sterile PBS and randomized fragments of 1–2 mm^3 were transplanted subcutaneously by trocar in both flanks of syngeneic females that were either maintained virgin or crossbred. Three to eight consecutive tumor passages were made, the HD tumor lines being subjected to more transplant generations in order to investigate their eventual progression to hormone-independence. Whenever a tumor line was undoubtedly behaving as HI, tumor cells were frozen in liquid nitrogen. In each transplant generation tumor fragments were implanted in eight (earlier passages) to four (later passages) mice. Tumor size was evaluated twice a week with a Vernier Caliper and tumor size was expressed as the product of two orthogonal diameters. We have not found significant differences when tumor size was expressed by the formula: tumor volume $= (ab^2)$, a and b being the larger and smaller diameters, respectively (data not shown). In each transplant generation, tumor hormone-dependency was determined by comparing passages in virgin versus multiparous females. Then, tumor behavior was classified by the following criteria: A tumor transplant was considered as HD when none of the tumor implants showed any development in virgin hosts for at least 5 weeks, while all their parallel passages were able to develop during pregnancy; HR when all tumor transplants were able to develop in virgin hosts, but latencies were significantly longer when compared with parous hosts ($p < 0.05$); and HI when there were no significant differences in tumor latency when implants in virgin hosts were compared with those in parous hosts.

DNA extraction and Southern blot analysis (SBA)

For each passage, fragments (30–40 mg) were excised from tumors and preserved at −70°C until nucleic acid extraction was performed. High molecular weight genomic DNA was prepared using the Wizard Genomic DNA Purification kit (Promega, Madison) following manufacturer's instructions. The nucleic acid concentration was determined by optical density reading at 260 nm. Fifteen micrograms of DNA were digested and subjected to electrophoresis on 1.2% agarose gels in 1% TAE buffer. Afterwards, gels were treated with 0.2 N HCl for 15 min, denaturing solution (1.5 M NaCl–0.5 M NaOH) for 40 min, neutralizing solution (0.5 M Tris–1.5 M NaCl–pH7.6) for 1 h, and blotted onto nitrocellulose filters according to the method of Southern [11]. After transferring, filters were pre-hybridized for 2 h at 42°C with a pre-hybridization solution (50% formaldehyde, 5 × SSPE, Denhardt's solutions, 1% SDS, 10% dextran sulfate and 50 μg/ml of denatured salmon sperm DNA), and then hybridized overnight at 42°C with ^{32}P-labeled probes generated by random priming. A fragment of MMTV-LTR(C3H) of 700 bp was used as mold [6]. Filters were washed once with 2 × SSC–1% SDS for 15 min at 42°C, once with 0.1 × SSC–0.1% SDS for 15 min at 42°C, twice with 0.1 × SSC–0.1% SDS for 15 min at 55°C and dried, before autoradiography.

Morphological and immunohistochemical studies

Tumors were fixed in 10% buffered formalin and embedded in paraffin for histological studies. The morphological features were evaluated in hematoxylin–eosin (H&E) stained slides. Immunohistochemical detection of estrogen- and progestin-receptor (ER and PR) was performed on formalin-fixed, paraffin-embedded tissues. Briefly, paraffin sections were dewaxed in xylene and rehydrated through graded ethanols. Endogenous peroxidase activity was inhibited using 3% H_2O_2 in distilled water. Sections were blocked in 5% normal goat serum and then incubated overnight with polyclonal anti-ER (clone MC-20) and anti-PR (clone C-20) (both antibodies were from Santa Cruz Biotechnology, Santa Cruz, CA) at 1:50 dilution. This was then followed by indirect immunoperoxidase antisera detection by Elite kit (Vector Laboratories, Inc.) following manufacturer's instructions. Negative controls were performed by replacing the primary antibody with normal rabbit serum. ER and PR content were evaluated by counting at least 1000 cells per analyzed tumor.

Western blot analysis

Equal amounts of proteins (100 μg/lane) were separated on discontinuous 7.5% (for PR) or 12% (for ER) polyacrylamide gels. Proteins were dissolved in sample buffer [6 mM Tris (pH 6.8), 2% SDS, 0.002% bromophenol blue, 20% glycerol, and 5% mercaptoethanol] and boiled for 4 min. After electrophoresis, proteins were blotted to a nitrocellulose membrane. The membranes were blocked overnight with 5% dry skimmed milk dissolved in 0.1% PBST (0.8% NaCl, 0.02% KCl, 0.144% Na_2PO_4, 0.024% KH_2PO_4, pH 7.4, and 0.1% Tween 20), washed several times with PBST, and probed with PR Ab-7 2 μg/ml (NeoMarker, Fretmont, CA) or ER MC-20 1 μg/ml (Santa Cruz Biotechnology, Santa Cruz, CA) in PBST at room temperature for 2 h. The blots were washed three times, 10 min each, and probed with peroxidase-conjugated sheep antimouse immunoglobulin (for PR) or peroxidase-conjugated donkey-antirabbit immunoglobulin (for ER; Amersham Life Science, Buckinghamshire, United Kingdom). The results were visualized with the ECL western blotting detection reagent kit (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) and exposed to a CUPRIX RP 1 (Medical X-ray Film, Agfa) for 1–5 min. Uteri obtained from mice primed with 10 μg/kg E_2 and muscle tissue were used as positive and negative control, respectively.

Results

First transplant generation behavior

Seventeen primary tumors from pregnant MMTV(LA)-infected females were transplanted in inbred uninfected BALB/c female mice. We found that most of them (11/17) were fully able to develop in female hosts when impregnated, but did not show any growth for more than 5 weeks when transplanted into virgin hosts. Tumor lines that showed this behavior were called pregnancy-dependent or HD tumors. Figure 1 shows that some of these HD lines ($n = 6$) indicate regression after each parturition (Figure 1(A), tumor D-2), while others do not ($n = 5$) (Figure 1(B), tumor 2236).

Alternatively, some of the primary tumors were able to grow in virgin hosts. However, some of these lines ($n = 4$) were still able to respond to pregnancy since tumor transplants grew earlier and faster in multiparous females than in virgin hosts (Figure 1(C), tumor 2216). We refer to them as pregnancy or HR

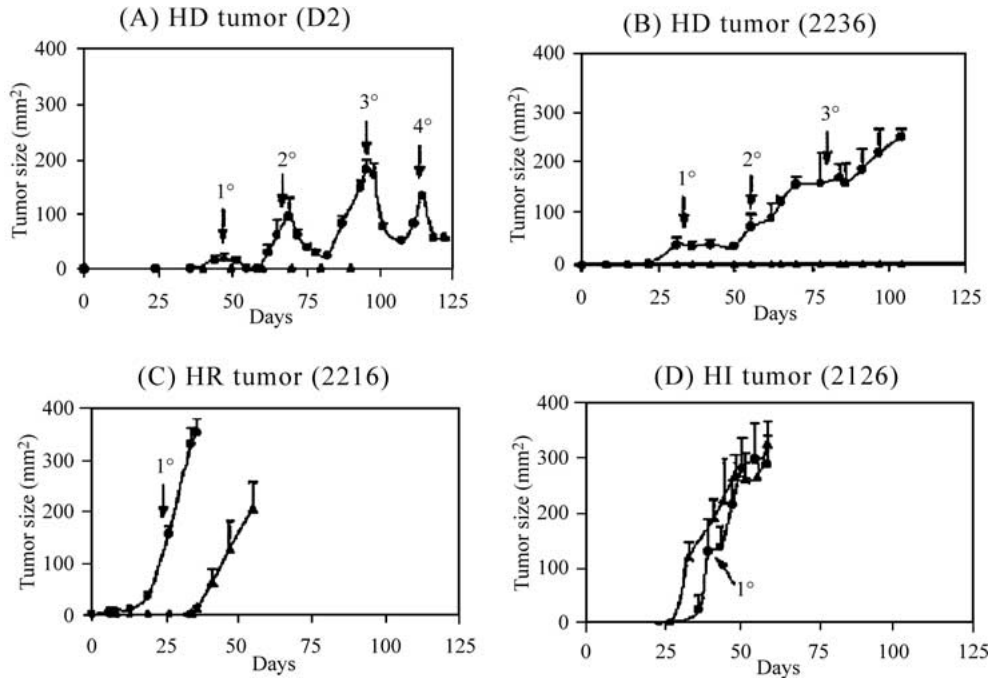

Figure 1. Examples of the four different tumor growth patterns at first transplant generation: (A) hormone-dependent (HD) tumor showing regression after each pregnancy, (B) HD tumor which does not regress after each pregnancy, (C) hormone-responsive (HR) tumor, (D) hormone-independent (HI) tumor. Each point represents the mean surface of 3–8 mammary tumor passages ± SD in multiparous (•) and (▲) virgin females. The arrows indicate the end of each pregnancy.

tumor lines. Only two of the primary tumor transplants grew in virgin hosts and their development was not stimulated by pregnancy (Figure 1(D), tumor 2126); these are referred to as pregnancy-independent or HI tumor lines.

Figure 2 shows Southern blot analysis of multiple first passages from six primary tumors (4 HD, 1 HR, and 1 HI). Only in a few cases, bands associated with MMTV insertions were clearly observed in all first transplant generation implants from an HD tumor. Figure 2(B) shows one of these cases in which all the passages show clear additional bands corresponding to exogenous MMTV insertion events occurring in most of the tumor cells. On the other hand, Figure 2(C) shows that most of the implants from HD tumors do not show a stable pattern of bands. In tumor 2144, bands corresponding to exogenous MMTV are observed in only one implant, while no exogenous insertions are detectable in the other six parallel transplants. Tumor 2280 shows two bands in one of the transplants, but only one of these bands is present in a second implant and possibly in the third one, but with lower intensity. In the case of tumor 2236, a single band corresponding to exogenous MMTV insertion was found in only two of the three assayed DNA samples. Figure 2(D) shows the band patterns corresponding to 2216 primary tumor and its successive passages. This tumor line showed an HR behavior. Once again, a stable pattern of bands was not found in the first transplant generation implants. Only one of these three implants showed an identical pattern to the one found in the primary tumor. Other implant showed an extra band, while the last one showed one band less. The fact that one of the tumor passages showed fewer bands than the primary tumor strongly suggests that the latter was composed of several cell populations. Eventually, one of these populations became predominant and gave rise to a clonal or quasi-clonal population as seen in passage 2 and 3.

When no exogenous MMTV associated bands were detected in *Eco* RI digested tumor DNA, it was possible that they were masked by bands corresponding to similar size fragments generated from endogenous MMTV-LTR sequences. Therefore, in those cases, other restriction enzymes such as *Hin*d III and *Bgl* II were used in an attempt to unmask putative hidden bands. However, analysis of HD first generation transplants, which did not show new insertion bands after *Eco* RI digestion, also failed to provide evidence of a homogeneously mutated tumor cell

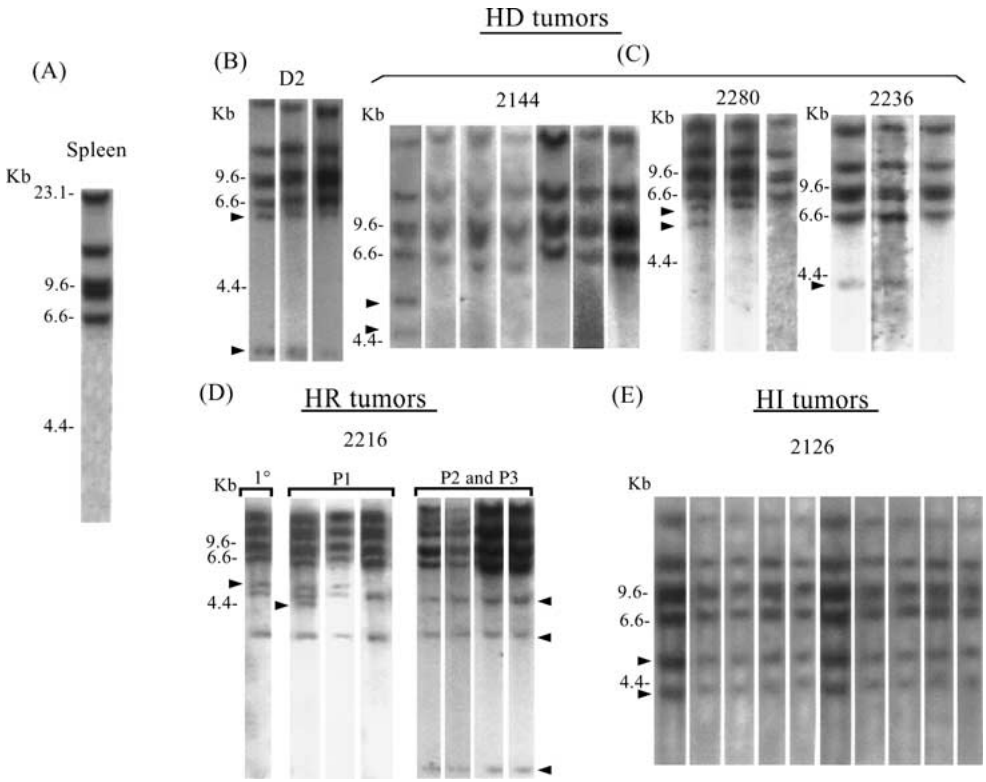

Figure 2. Southern blot analysis of *Eco* RI digested DNA from tumor transplants with different growth patterns: (A) spleen DNA digestion pattern from a BALB/c mouse is displayed in order to despict *Eco* RI restriction pattern of endogenous MMTV sequences. (B) and (C) Four HD tumors at first transplant generation; (D) HR, primary tumor (1°), first (P1), second and third (P2 and P3) transplant generation; (E) HI tumor, first transplant generation. DNA from each tumor was digested with *Eco* RI, and Southern blot analysis was carried out. In every case the blots were hybridized with a specific probe for MMTV-LTR sequences. The arrowheads indicate the location of host–viral restriction fragments indicative of the presence of exogenous MMTV proviral insertion within the somatic DNA. The five upper bands correspond to endogenous MMTV sequences present in BALB/c mouse genome.

population when treated with other enzymes (data not shown).

Passages from the HI tumor lines showed a very strong and stable pattern of bands corresponding to exogenous MMTV cDNA insertions, indicating that these tumors were mostly clonal-derived cell populations. An example of this, in which the 10 parallel transplants from HI tumor 2126 displayed the same two extra bands, is shown in Figure 2(E). Therefore, these results suggest that while MMTV(LA) tumors showing an HI pattern of growth are mostly clonal-derived cell populations, pregnancy-dependent lesions can be composed of several different cell populations, each of them with different MMTV(LA) insertion sites.

Pregnancy-dependent tumor progression

After consecutive passages, HD and HR tumors progressed to an HI behavior. We found that the selection of HI cells by a hormone-deprived environment was not necessary for the progression of these tumor variants. This was observed in at least four different HD tumor lines. For example, Figure 3 shows how D2 HI tumor variants appeared very early after implantation. In the first transplant generation, they appeared before and, in the second one, during the first pregnancy. SBA showed that in both of these HI lines, there were extra bands not present in the HD passages of this tumor line. These bands were observed in all parallel and subsequent passages of these HI variants. Interestingly, although they appeared independently, both D2 HI variants showed the same pattern of bands suggesting that they originated from the same cell subpopulation already present at a low percentage in the HD tumor.

Another pattern of progression was observed in which HD tumor passages after more than 4 months of dormancy grew during the next pregnancy or during hormone-stimulation, but behaved as HI tumors in the

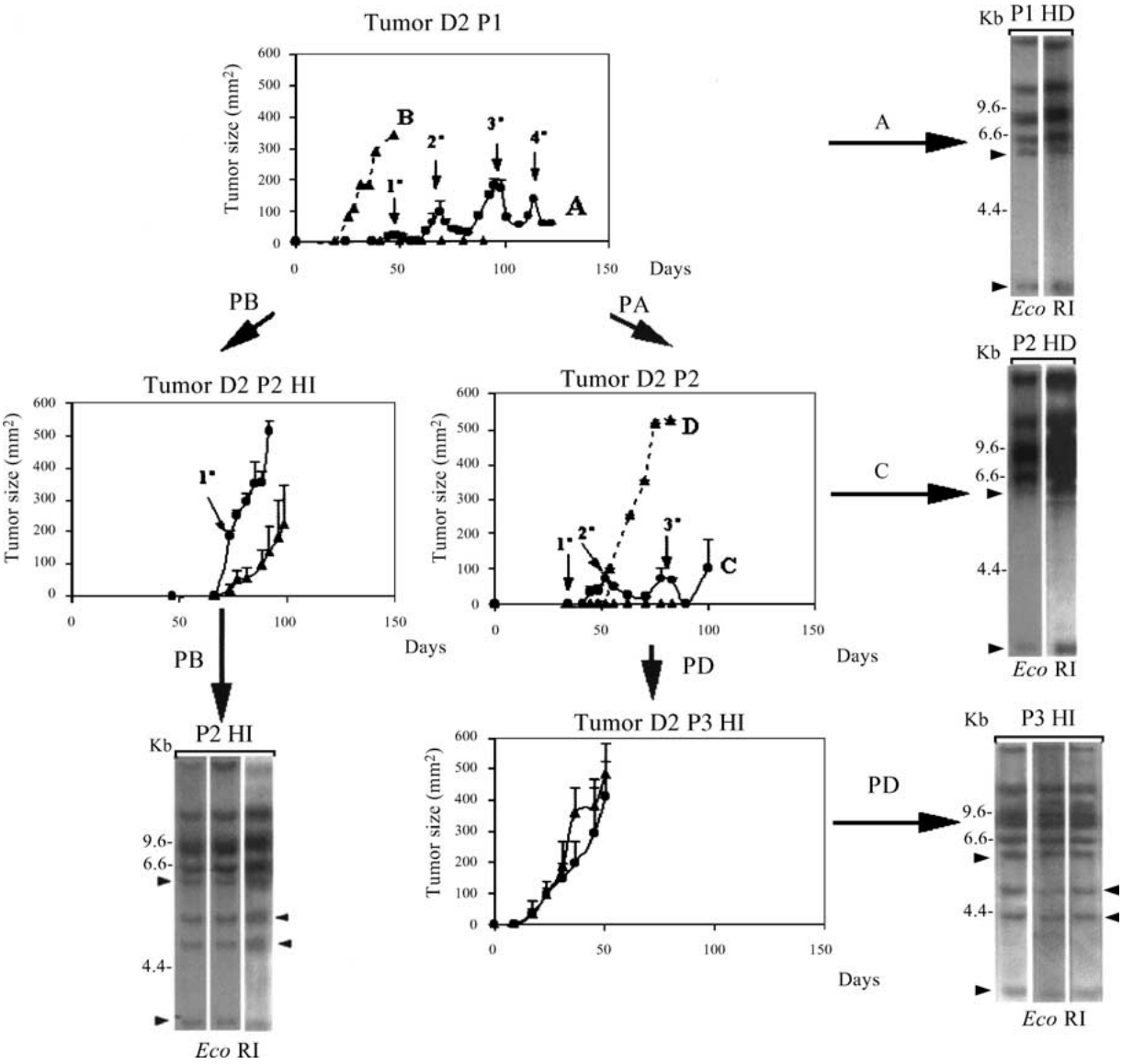

Figure 3. Tumor D2 progression to hormone-independence. P1: First transplant generation, P2: second transplant generation, P3: third transplant generation. A: HD first transplant generation ($n = 6$), B: HI first transplant generation ($n = 1$), C: HD second transplant generation ($n = 5$), D: HI second transplant generation ($n = 1$). PA: passages of tumor A, PB: passages of tumor B, PD: passages of tumor D. In tumor growth curves (when $n > 1$) each point represent mean ± SD of 4–6 tumor transplants in multiparous (●) and (▲) virgin females. Small arrows indicate tumor passages. Large arrows indicate Southern blot analysis of A, C, PB, and PD tumors. Tumor DNA was digested with *Eco* RI and hybridized with a probe specific for MMTV-LTR sequences. Arrowheads show exogenous MMTV proviral insertion sites.

following generation. This pattern of progression was observed in six different HD tumor lines (Figure 4(A) shows one of them).

We have found a single case in which HI clones were selected in a pregnancy hormone-deprived environment. First generation transplants of tumor 2144 did not grow in virgin females for at least 6 weeks. However, after that, tumor growth was observed in three out of six transplants carried out in virgin hosts. These passages showed an HI behavior upon transplantation (Figure 4(B)). SBA of the HI variants that appeared in pregnant as well as virgin hosts showed extra insertions present in all the parallel HI tumor passages, suggesting once

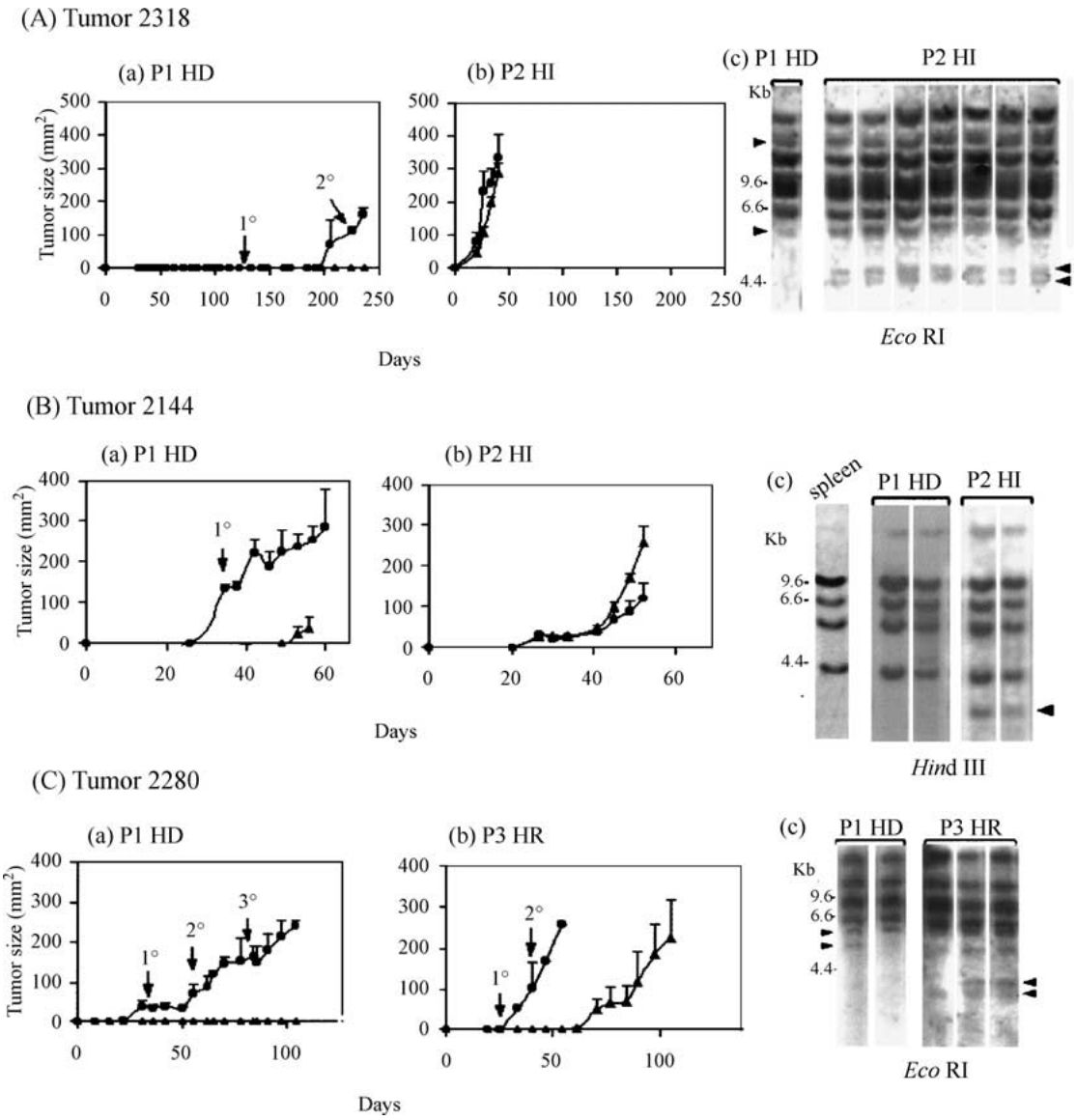

Figure 4. Progression to hormone-independence. (A) Tumor 2318: example of tumors that progressed following a dormant state. (B) Tumor 2144: tumor passages grew in virgin females after a longer latency. (C) Tumor 2280: an example of HD tumors that progressed through an HR state. (a) Growth at first transplant generation, (b) growth at a second transplant generation. Each point represents the mean. The arrows indicate the end of each pregnancy. (c) Southern blot analysis shows restriction pattern of MMTV proviral insertions. Arrowheads show exogenous MMTV viral insertions. P1 HD: hormone-dependent first transplant generation; P2 HI: hormone-independent second transplant generation; P3 HR: hormone-responsive third transplant generation. In panel showing tumor 2144 Southern blot analysis (B, c), a lane showing spleen DNA digestion pattern from a BALB/c mouse is displayed in order to depict *Hind* III restriction pattern of endogenous MMTV sequences.

again that HI variants had arisen as clonal-derived population.

Finally, in several cases, HD tumors progressed slowly to an HI phenotype, through several HD and/or HR passages. Tumor 2280 is an example of this. In this case, bands by SBA showing the predominance of a clonal subpopulation were already observed at passage 3, which showed an HR behavior (Figure 4(C)).

Tumor progression, histological, and ER and PR content analysis

At first transplant generation, all the HD and HR analyzed tumors were well-differentiated mammary adenocarcinomas with an either papillary–cystic or ductulo-acinar morphological pattern. Alternatively, HI tumors showed a variety of histological patterns,

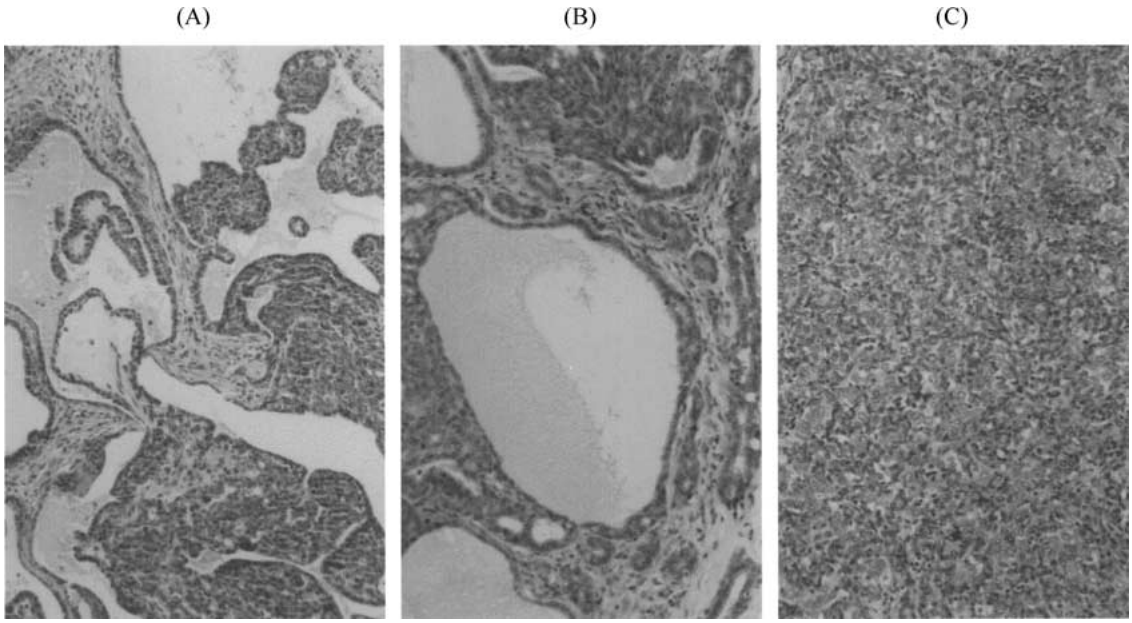

Figure 5. Histological features of MMTV-induced mammary tumors progressing from HD to HI phenotype. (A) Well-differentiated adenocarcinomas with papillary formations into lumen of cystic ducts and stromal invasion that were mostly found in HD tumors. (B) Adenocarcinomas with cystic and solid-cribiform pattern that were seen most commonly in HR tumors. (C) Poorly differentiated adenocarcinomas with predominant solid pattern were found in several HI tumors (H&E, 125×).

Table 1. Immunostaining for estrogen and progesterone receptors in HD and HI tumor transplants

	ER (% positive nuclei ± SD)	PR (% positive nuclei ± SD)
HD passages ($n = 7$)	55.40* ± 16.7	48.45* ± 9.99
HI passages ($n = 5$)	10.30 ± 2.1	11.53 ± 4.7

* HD tumor passages showed a significant higher percentage of positive nuclei for ER and PR by Student's two-tailed *t*-test ($p < 10^{-3}$).

while one of the HI tumor lines resembled the pattern found in HD carcinomas, the other one showed a very poorly differentiated architecture. Figure 5 shows examples of three different patterns found in our mammary tumor passages. First, a ductal–cystical papillary pattern commonly found in HD transplants is displayed. The second panel (Figure 5(B)) shows a well-differentiated glandular–cystic morphology found in an HR passage. Finally, a poorly differentiated adenocarcinoma is shown in Figure 5(C), corresponding to an HI tumor line. Noteworthy, scattered areas with the latter morphology comprised of 5–40% in HR tumors and up to 90% in HI ones.

A very good correlation was found between hormone-responsiveness and the expression of ER and PR. Immunohistochemical analysis showed high number of cells expressing these receptors in all the analyzed HD and HR tumor transplants (Table 1; Figures 6(A) and (C)). However, HI tumors – even the well-differentiated ones – always presented low ER and PR levels (Table 1, Figures 6(B) and (D)). Therefore, it can be concluded that progression to autonomous growth was associated with a significant loss of ER and PR content. Similar results were obtained when ER and PR content was analyzed by western blot analysis: HD tumors showed higher ER and PR expression levels when compared with HI passages. Representative western blots are shown in Figure 6(F).

Discussion

In the mouse mammary model, detection and analysis of exogenous MMTV integration in cellular genomic DNA has been widely used to deal with the question whether or not MMTV-induced premalignant and malignant lesions have a mono or polyclonal origin [4, 5, 12–14]. This debate started a long time ago and there does not seem to be a final answer. The results reported herein indicate that the pregnancy-dependent premalignant MMTV(LA)-induced

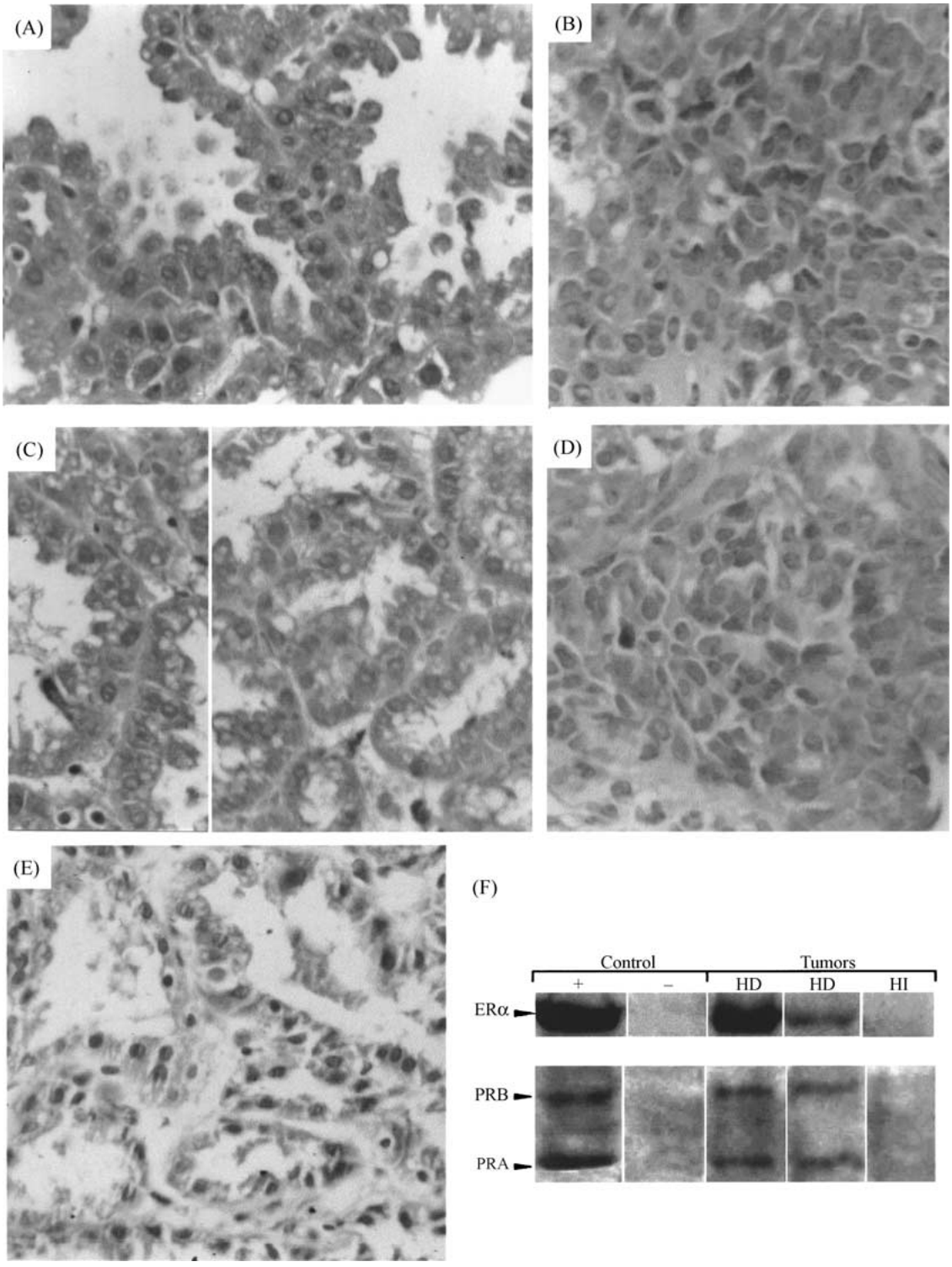

Figure 6. Immunostaining for estrogen (ER) and progesterone receptor (PR). In HD tumors (e.g., tumor 2228 HD P1), numerous nuclei were strongly stained for ER (A) and PR (C shows two regions in the same sample). In HI tumors (e.g., tumor D2 HI P2), few nuclei were weakly labeled for ER (B) and almost none was positive for PR (D) (DAB, 600×). (E) Negative control: HD tumor immunostanding (tumor 2228 HD P1) in which polyclonal rabbit antibodies that recognize ER or PR were replaced by normal rabbit serum (DAB, 400×). (F) Western blot of PR isoforms (PR A, M_r 83,000; PR B, M_r 115,000) and ERα in two HD tumors (tumor D2 HD, P2, and tumor 2314 HD P1) and an HI tumor (tumor 2216 HI P5). Similar bands were obtained in five different tested HD tumors; no bands were detected in four different tested HI tumors. Uteri obtained from mice with 10 μg/kg E_2 were used as positive control (control +) and mouse muscle tissue as negative control (control −).

mammary lesions can arise from polyclonal cell populations. The strongest proof resides in the lack of detectable MMTV-associated bands in several HD primary tumors or their passages. By serial dilution of mouse genomic DNA, we found that the MMTV insertion bands become undetectable only if they are present in less than 30% of the total cell population (data not shown). Therefore, it can be roughly estimated that each of these tumors originated from at least four different MMTV-infected cells.

In a few cases, we found that HD lesions can also originate from a single clone. Hormone-dependent tumor D2 showed a very stable pattern of bands that neither changed nor disappeared through multiple passages, suggesting a monoclonal origin. However, we have showed that the subclones with extra bands appeared rather early, most probably in the primary tumor. These data indicate that clonal dominant as well as polyclonal HD tumors are composed of unstable cell populations within which selection of specific clones or subclones is prone to happen.

A variety of experimental cancer models of progression towards hormone-independence indicate that it can be due to phenotypic or epigenetic changes in an HD tumor cell population [15–17]. It has been shown that *in vitro* loss of response to hormones follows a very stable pattern that fits better with an epigenetic program than with the random appearance of mutations. In addition, it has been postulated that in MMTV(BR6)-induced pregnancy-dependent lesions, the acquisition of further proviral elements during progression of the tumor towards hormone-independence probably reflects fortuitous superinfection and does not contribute to the phenotypic change [4]. Nevertheless, our study suggests that additional mutations may play a relevant role in tumor progression towards hormone-independence.

Interestingly, in experimental models in which mammary tumor cells gain the capacity to proliferate in a hormone-deprived environment retaining the ability to express relatively high ER and PR levels, progression to hormone-independence seems to be associated to epigenetic events [16–19]. Alternatively, in those HD mammary tumor experimental models, in which the ability to grow in a hormone-deprived environment is associated to the loss of ER and PR, genetic changes in the tumor cell population are involved [12, 20].

The model of tumor progression presented herein closely resembles other pregnancy-dependent mouse mammary tumor models described in other mouse strains such as GR [5, 20], RIII [2], DD [21], and BR6 [4]. In particular, similarly to what was previously observed in GR mice, in our model, HD tumors are mostly composed of several cell populations, and HI variants arise as clonal subpopulations expressing low levels of ER and/or PR that are able to proliferate in a hormone-deprived environment [20]. However, our results show that HI variants do not necessarily arise as a consequence of selective pressure against survival of HD cells. Even though in a few cases HI variants appeared after a long latency in virgin females (see tumor 2144 in Figure 4(B)), in most cases they arose in either multiparous females or very early after transplantation from multiparous hosts, while no tumor development was observed in the parallel passages made in virgin mice (see tumor D2 in Figure 3). These results suggest that the proliferating activity of HD cells that takes place in a rich hormonal milieu could provide factors that facilitate the appearance of HI clones instead of inhibiting it. It has been already suggested that in the GR mouse mammary tumor model, autonomous cells multiply at a faster rate than HD tumor cells do [20]. Therefore, HI clones could have selective growth advantage over HD cells even in non-hormone-deprived environments, similar to what has been reported for metastatic cells arising from primary solid tumors [22]. Then, the loss of hormone-dependence during tumor progression could be just a 'side-feature' of the better-adapted or just faster growing clones.

It has been suggested that the ER^+ cells do not in general proliferate in the normal human breast, but ER^+ mammary tumors have a high proportion of dividing ER^+ cells [23]. Interestingly, it was later found that in the normal breast, the number of proliferating ER^+ cells correlates positively with the level of risk of developing cancer [24]. In our model, as HD ER^+ tumors may be heterogeneous populations, it would be possible that the ER^+ cells still behave 'normally' and do not proliferate, but provide to the ER^- cells growth factors that allow them to cycle. Another possibility is that these ER^+ cells could be able to cycle, since in neoplastic tissue ER^+ cells can acquire the capacity to proliferate [23]. Taking into account these two possibilities, progression to HI could happen either because ER^- do no longer need growth factors provided by ER^+ cells, or because ER^- cells can proliferate more efficiently than their ER^+ counterparts do.

It has been reported that in the normal mammary gland there is a stepwise decrease in PR that occurs in two stages. The first decrease is completed by day 12

of pregnancy and maintained until day 19. Then, PR becomes undetectable only after parturition (day 2 of lactation). The first down-regulation during pregnancy would be caused by the negative effect of progesterone on estrogen-mediated increase in PR. However, the lack of PR during lactation is not related to the hormonal milieu of lactation, but is directly related to the secretory state of the mammary gland [25]. In our model, we found that the pregnancy-dependent tumor samples that have been taken during the second half of pregnancy express PR. More experiments need to be done in order to address the question whether or not regulation of PR expression in HD tumors during pregnancy and lactation is under the hormonal and local regulation observed in the normal mammary gland.

Results reported herein (Figure 4(A)) show a very special pattern of tumor progression. We have found that the hormone-stimulation after long periods of hormone-deprivation played a fundamental but intriguing role in tumor progression. HD tumor cells that were implanted in virgin females and remained dormant for more than 4 months grew under hormone stimulation but behaved as HI upon transplantation. We are now investigating the mechanisms underlying this pattern of progression.

In conclusion, our data indicate that: (i) all the HI tumor variants showed clear MMTV(LA) new insertion sites that were mostly absent in the HD cells from which they had arisen; (ii) in no case was progression to HI reversible; (iii) the appearance of HI variants mainly happened randomly, discarding a fixed pattern of epigenetic changes; and (iv) additional insertions in the MMTV band pattern were associated with HD tumor progression. We are now in the process of isolating and sequencing the MMTV insertion sites found in the HI tumors. This will allow us to determine – by PCR, for example – whether or not these mutations were present, even in small subpopulations, in the HD tumors from which they have progressed. Then, we will be able to determine whether the HI tumors arise from clonal outgrowths of HI subpopulations containing early MMTV insertions only present in small subpopulations, or whether they appear as a consequence of newly acquired mutations in the HD population that result in a rapid switch in tumor growth behavior.

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