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MMTV infectious cycle and the contribution of virus-encoded proteins to transformation of mammary tissue

Susan R. Ross
Department of Microbiology/Abramson Family Cancer Center, University of Pennsylvania, 421 Curie Blvd., Philadelphia, PA 1914, Phone: 215-898-9764, FAX: 215-573-2028, e-mail: rosss@mail.med.upenn.edu

Abstract

Mouse mammary tumor virus has served as a major model for the study of breast cancer since its discovery 1920's as a milk-transmitted agent. Much is known about *in vivo* infection by this virus, which initially occurs in lymphocytes that then carry virus to mammary tissue. In addition to the virion proteins, MMTV encodes a number of accessory proteins that facilitate high level *in vivo* infection. High level infection of lymphoid and mammary epithelial cells ensures efficient passage of virus to the next generation. Since MMTV causes mammary tumors by insertional activation of oncogenes, which is thought to be a stochastic process, mammary epithelial cell transformation is a by-product of the infectious cycle. The envelope protein may also participate in transformation. Although there have been several reports of a similar virus in human breast cancer, the existence of a human MTV has not been definitely established.

Keywords

retrovirus; mammary tumor; mouse model

Introduction

The study of virus-induced cancers in animals has played a critical role in the understanding of oncogenesis in humans. This is especially the case for retroviruses, which were first recognized as transmissible agents that cause cancer shortly after the turn of the previous century. In the 1920's, J. J. Bittner showed that there was a milk-transmitted agent in mice that was responsible for breast cancer induction (1). Since that time, this transmitted agent, now known to be the betaretrovirus, mouse mammary tumor virus (MMTV), has been used as an *in vivo* model for the study of mammary carcinogenesis (2,3). Here, I review the biology of MMTV, its *in vivo* transmission pathway and how it interacts with its host's biology. I also review the current literature regarding a putative related human mammary tumor virus (HMTV).

MMTV genome structure and proteins

Retroviruses can be classified as simple or complex. The genomes of simple retroviruses, such as murine leukemia virus (MLV), encode only the virion proteins and enzymes required for viral replication. In contrast, complex retroviruses, human immunodeficiency virus (HIV)-1 or human T cell leukemia virus (HTLV) 1, encode in addition a variety of non-structural proteins that facilitate various steps of the replication pathway or counteract cellular and immunological anti-viral host responses. While MMTV was initially classified as a simple retrovirus, it is now clear that it probably lies somewhere in between viruses like MLV and HIV-1 in complexity.

The MMTV genome is approximately 9 kb in size. At least five transcripts are generated from the viral genome, four of which initiate in the 5′ long terminal repeat (LTR) and terminate in the 3′ LTR; the different transcripts are generated by alternative splicing (Fig. 1). The LTR also contains binding sites for transcription factors that determine hormone-responsive and tissue-specific transcription, both of which are necessary for *in vivo* infection and optimal virus production. Specifically, the LTRs encode sites that regulate both mammary epithelial and lymphoid cell-specific expression, as well as glucorticoid/progesterone response elements that cause increased virus transcription during pregnancy and lactation, when virions are shed into milk (4–8). Because the MMTV LTR encodes transcriptional regulatory elements that direct high level expression in mammary epithelial cells, it has been widely used to drive transgene expression in mouse mammary tissue (reviewed in XXX, this volume).

Like all retroviruses, the full-length, unspliced MMTV RNA serves two functions. First, two copies are packaged into virions and thus provide the viral genome. Second, the full-length transcript serves as the mRNA for the gene products encoded by the *gag*, *dut-pro* and *pol* genes (9). The *gag* translation product is a polyprotein precursor that is processed by the viral protease, PR or Pro, into the capsid (CA) and nucleocapsid (NC) proteins, as well as several other peptides of unknown function. Both the Dut-Pro and Pol polyproteins are translated from the same mRNA as Gag, but in different reading frames, by a process termed ribosomal frameshifting. The *pro* gene encodes the viral protease and *dut*, a dUTPase, whose role in virus infection is not known. However, for other retroviruses that encode a dUTPase, such as equine infectious anemia virus (EIAV), it is believed that this protein contributes to pathogenesis by maintaining adequate nucleotide pools and thereby facilitating productive viral replication in non-dividing cells (10). Since MMTV infects dendritic cells (DCs), which are non-dividing *in vivo*, the dUTPase could play a similar role. The *pol* gene codes for reverse transcriptase (RT), needed to generate the double-stranded DNA, and the integrase (IN), which is required for integration of this DNA into the host chromosome.

A singly spliced mRNA is translated from the envelope (*env*) gene, which is cleaved by host furin enzymes to yield two polypeptides, the surface (SU) and transmembrane (TM) domains of the Env protein, required for binding of the virions to cell surface receptor(s) that mediate cell entry (9). The SU domain carries the receptor binding site (RBS), while the TM domain mediates virion-cell membrane fusion required for entry. The MMTV entry receptor is transferrin receptor 1 (TfR1) (11). TfR1 belongs to a class of cell surface receptors that traffic to the acidic endosome upon ligand binding. Unlike MLV or HIV-1, which enter cells via the surface or a neutral compartment, MMTV entry occurs in a late endosomal compartment and probably requires co-trafficking of virions with receptor. The identification of TfR1 as the MMTV entry receptor explains in part the in vivo tissue-specific tropism of this virus, since activated cells of the immune system and dividing mammary epithelial cells express some of the highest levels of this protein *in vivo* (12–14). However, cell-type restriction *in vivo* is also probably due to post-entry events. For example, the enhancer elements in the LTR function predominantly in mammary epithelia and lymphoid cells and thus, MMTV is not transcribed in many tissues (15).

Retroviral Env proteins can have other activities in addition to mediating cellular entry and recent work has indicated that the MMTV Env protein may play additional roles in *in vivo* infection and MMTV-mediated tumorigenesis. In addition to interacting with TfR1 to mediate viral entry, the Env protein has been shown to activate antigen presenting cells, like DCs and B cells, via Toll-like receptor 4 (TLR4) (16,17). TLR4 is a member of a family of receptors that contribute to innate immune responses to pathogens (18). Env interaction with TLR4 may facilitate initial infection of cells of the immune system (see next section). The Env protein may also participate in the transformation of mammary epithelial cells (see below).

At least two other proteins required for efficient MMTV infection are encoded by additional alternatively spliced mRNAs (Fig. 1). These include a viral protein with HIV-1 rev-like activity required for efficient transport of unspliced MMTV mRNA from the nucleus, termed regulator of export of MMTV (Rem) (19,20) and the viral superantigen (Sag), encoded in the 3′ LTR, which is dispensable for *in vitro* infection but plays a critical role in virus dissemination *in vivo* (see next section). Several other alternatively spliced mRNAs originating from the MMTV genome have also been described, but their gene products and role in infection have not been well-studied (21,22).

In summary, since MMTV encodes a number of accessory proteins, as well as an Env that plays multiple roles, it is clearly not a simple retrovirus. Unlike HIV-1 and HTLV-1, viral gene products equivalent to tat and tax, respectively, which function as transcriptional activators of virus and host transcription have not been found in the MMTV genome. However, MMTV does encode proteins that allow it to alter the host immune system, as well as a protein that facilitates transport of unspliced viral RNA out of the nucleus. MMTV may encode these accessory proteins, particularly Sag, because its *in vivo* infection pathway requires trafficking through diverse cell types, as described in the next section.

MMTV *in vivo* infection pathway initiates in lymphoid cells

MMTV has two routes of acquisition *in vivo*. Susceptible strains acquire exogenous virus through milk and can be freed of MMTV by foster-nursing on uninfected mothers, while other strains inherit endogenous copies of the provirus (2). Virtually all laboratory strains have from 2 to 8 endogenous proviruses (termed *Mtv* loci and numbered in order of their discovery). In general, the endogenous proviruses do not encode functional viruses, although a few inbred strains, such as the GR strain which was selected for high mammary tumor incidence, have retained active endogenous proviruses (23). These active endogenous proviruses probably represent recent germ line integrations, since it has been estimated that endogenous MMTVs have been present in the mouse genome for 20 million years (24,25). Interestingly, while most of the endogenous MMTVs sustain mutations in the coding regions for the virion proteins, almost all retain intact Sag coding regions. This suggests that there is selection for the retention of endogenous *sag*s.

Although mammary epithelial cells are the ultimate targets for MMTV infection, cells of the immune system play multiple critical roles in *in vivo* infection (3). During milk-borne infection, MMTV first infects DCs in the small intestine and Peyer's patches (17) (Fig. 2). Retroviral infection is a multi-step process, initiating with the binding of the viral Env protein to one or more cell surface receptors and terminating with the migration of reverse-transcribed viral DNA into the nucleus and integration into the chromosomes. For most retroviruses, including MMTV, this latter step is dependent on the nuclear membrane breakdown that occurs during cell division, because the reverse-transcribed replication complex cannot cross the nuclear membrane. MMTV both infects and activates DCs (26,27). MMTV accomplishes the initial activation of DCs in part because the virion Env interacts with TLR4 (16,17,28). DC activation by MMTV also induces increased expression of the entry receptor TfR1, thereby potentially facilitating their infection (17). Virus interaction with TLR4 also induces their migration to the lymph node, by causing increased expression of CCR7, the receptor for the chemokine macrophage inflammatory protein 3β (26,28). Upon binding to ligand, members of the TLR family also activate signal transduction pathways that result in the production of anti-inflammatory cytokines and interferons, which in turn can influence the adaptive immune response (29). Indeed, there is evidence suggesting the MMTV's interaction with TLR4 shifts the adaptive immune response from a protective TH1 (cytolytic T cell) to a nonprotective TH2 (antibody) response (30).

MMTV amplification in the lymphoid compartment also depends on T cells. After milk-borne acquisition of virus, infected DCs present the MMTV Sag via major histocompatibility (MHC) class II proteins expressed on the surface of infected antigen presenting cells, to T cells bearing specific T cell receptor Vβ chains. Different MMTV strains interact with particular Vβ-bearing T cells because they encode Sag proteins with different C-terminal amino acid sequences (termed the hypervariable region); this region of the Sag protein contacts the TCR Vβ molecule. Sag-mediated lymphocyte activation is a requisite step in the infection pathway (3). The Sag-cognate T cells proliferate, provide B cell help and produce cytokines that stimulate and recruit additional DCs, B and T cells, resulting in the establishment of a reservoir of infection-competent and infected cells (31). Sag presentation causes the proliferation of specific Vβ-bearing T cells when it is recognized as foreign but deletion of such T cells when it is recognized as self. Thus, mice infected as neonates show initial activation of Sag-cognate T cells, followed by their gradual deletion (32). Since except for milk there is no cell-free MMTV *in vivo*, infected lymphocytes are also critical for virus spread to the mammary gland (2,33,34) (Fig. 2).

MMTV-induced tumorigenesis

Infected lymphocytes carry virus to the mammary gland (33,34). Mammary epithelial cells become infected with MMTV at a time when they are driven to divide, that is, during the hormonal stimulation that accompanies puberty and pregnancy. Once MMTV infects mammary cells, virus amplification within this tissue is required both to maximize virion production and to induce mammary tumors. MMTV is a non-acute transforming retrovirus and mammary tumorigenesis takes place after proviral DNA integration near cellular proto-oncogenes that activates their transcription (Fig. 3); indeed, the mammalian Wnt gene family and several other oncogenes were discovered because of their association with MMTV (35) (see the article by Callahan and ? in this volume). Because MMTV integration does not appear to be site-specific (36), the more virus produced, the more likely it is that proviral DNA will integrate near a proto-oncogene. Thus, latency and incidence of tumor formation are proportional to virus load (37). Moreover, since the mammary glands of virgin mice go through fewer cycles of cell division than those of multiparous mice, virgin mice have fewer MMTV-infected epithelial cells and thus a lower incidence and longer latency of tumor induction; estrogen-treated males also develop MMTV-induced mammary tumors (2).

The Env protein may also play a role in mammary tumorigenesis. Ectopic expression of the MMTV Env in normal mammary epithelial cells results in their phenotypic transformation and an activation motif termed the Immunoreceptor tyrosine-based activation motif (ITAM) in this protein is critical to this activity (38). Expression of the MMTV Env alone in transgenic mice causes increased lobuloalveoar budding in their mammary glands, but not mammary tumors, indicating that ITAM signaling is not sufficient for cellular transformation *in vivo* (39). However, Env signaling clearly participates in MMTV-mediated transformation, since mutation of the ITAM within the context of an infectious MMTV reduces virus-induced mammary tumorigenesis without affecting infection levels (39). ITAMs are commonly found in receptors expressed in hematopoietic cells and are negatively regulated by cell-type specific modulators. Uncontrolled signaling by the envelope protein in epithelial cells, which lack such negative modulators, may be an early step in the MMTV transformation process (Fig. 3). Interestingly, other oncogenic viruses, such as Epstein Barr Virus (EBV) and Kaposi's Sarcoma Herpes Virus (KSHV), encode viral proteins with ITAMs that play a role in the transformation of non-hematopoietic cells (40–42) and the Env protein of the Jaagsiekte sheep retrovirus (JSRV), which signals through the Akt pathway, is known to be required for transformation of lung epithelial cells (43–45).

While EBV and KSHV cause both lymphoid and non-lymphoid cancers, MMTV is primarily associated with mammary tumors. However, variant MMTVs have been isolated from T cell lymphomas with altered LTRs (46). These MMTV variants most likely cause lymphomas because the LTR alterations create novel enhancers that allow high level of virus expression in T cells (47,48). Interestingly, the lymphoma-causing MMTVs integrate near c-myc, Notch family and other oncogenes rather than Wnt1 and fibroblast growth factor (fgf) family members (49,50). Although, like EBV, MMTV also infects B cells, it has not been associated with any B cell malignancies.

Genetic resistance MMTV-induced mammary tumors

Interest in genetic predisposition to human breast cancer led to breeding experiments between mouse strains with high mammary tumor incidence and those with low tumor incidence, as a means of identifying breast cancer susceptibility genes (51,52). A number of mouse strains were identified that were resistant to MMTV-induced mammary tumors, but thus far, most have been shown to control susceptibility to virus infection rather than tumorigenesis itself. In some strains, resistance to virus infection is due to the inability of certain major MHC class II genes to present Sag, thereby aborting the *in vivo* infection process at an early step (53). For example, C57BL/6 mice (MHC haplotype H-2^b) that lack the I-E chain required for efficient presentation of most MMTV Sags are not readily infected by virus. This resistance phenotype can overcome by the introduction of the I-E molecule as a transgene, although only infection and not mammary tumorigenesis was examined in this study (54). Similarly, the retention of endogenous *sag* genes with the same Vβ-specificity as those encoded by infectious virus precludes infection because mice delete Sag-responsive T cells during the shaping of the immune repertoire and thus, lack a reservoir of infection-competent cells (3). Not surprisingly, both B cells and DCs are also required for efficient MMTV infection, most likely because they both present Sag and serve as virus reservoirs (28,55). Interestingly, while there is an absolute requirement for DCs, B and T cells, the requirement for lymphocyte activation, either via TLR4 or Sag, is not absolute. Mice with mutant *Tlr*4 genes still get infected with virus, although the kinetics is somewhat delayed (30)(Rassa and Ross, unpublished observations). Similarly, Sag activity seems to be required primarily for lymphocyte amplification but not initial infection in the gut (56). This may be because MMTV can enter lymphocytes or DCs that are activated by commensal organisms in the gut.

Other mechanisms of resistance to MMTV infection also occur. The I/LnJ strain shows wild-type infection of lymphocytes, yet little or no transfer of virus to mammary tissue because these mice develop high titer anti-MMTV antibodies as they age that block efficient mammary gland infection (57). B10.BR mice are resistant to MMTV infection because their T cells have an attenuated, MHC-independent signaling response to the viral Sag and thus, there is little amplification of lymphocyte infection (58). Similarly, MMTV can infect and by transmitted by YBR/Ei mice, but virus production is severely attenuated through an as-of-yet uncharacterized T cell-mediated restriction (59). It has also recently been shown that BALB/c congenic mice lacking endogenous *Mtv* loci are resistant to infection; the mechanism of this resistance is not yet known (60).

There are a few indications that there may also be genetic differences in tumorigenesis. Impaired recognition of tumor cells by the cellular immune system might be responsible for the increased susceptibility of BALB/c mice to MMTV-induced mammary tumors (61). Finally, there is evidence that MMTV integration into the commonly targeted integration sites (CIS) are different in tumors induced in various inbred mouse strains (35,39). This suggests that the biology of the mammary epithelial cells of different inbred strains dictates which particular MMTV insertionally-altered oncogenes will result in mammary tumors.

Is there a HMTV?

Shortly after MMTV was shown to be a retrovirus, the search for an equivalent human virus began. Early studies reported finding MMTV-like proteins in human breast cancer biopsies and antibodies against the mouse virus proteins in human breast cancer patients (62,63). However, a more recent study that tested sera from almost 100 patients with breast cancer, using Western blots as the readout for the presence of antibodies against MMTV proteins rather than indirect methods, indicated no immunological reactivity against MMTV proteins (64).

Several groups have reported MMTV-like sequences in up to 30% of human breast cancers (65–68). Unlike previous reports that showed a high degree of homology between the *pol* genes of human endogenous retroviruses such as HERV-K and MMTV, these investigators found MMTV-like *env* sequences by PCR amplification of DNA from human breast cancer tissue. Although MMTV-induced mammary tumors in mice are associated with high level infection of normal as well as transformed mammary tissue, which is necessary to achieve insertional activation of cellular oncogenes, the HMTV sequences have only been found in tumor and not normal tissue from the same patients (66). While HMTV sequences have been reported by several investigators, others have been unable to replicate these studies (69–71).

There have also been a number of reports that continuous passage of certain strains of MMTV on human breast cancer cell lines results in adapted viruses that infect human cells (72,73). The mechanism by which MMTV infection of human cells occurs is unclear. The human TfR1 does not function as an MMTV entry receptor (11,74). The HMTV sequences are highly related to MMTV, particularly the endogenous *Mtv* loci, and there are no consistent changes in the Env protein of the HMTVs that would predict a changed tropism for the human TfR1. Moreover, some of the human cell-adapted MMTV envelopes still show tropism for mouse and not human TfR1 and these viruses appear not to have spread in the human cultures (75). More recently, one group has shown that MMTV can infect cultured human mammary breast cancer cells by an undefined mechanism (76,77). Taken together, these data suggest that neither the HMTVs nor MMTV use TfR1 for entry into human cells.

The mode of transmission of a potential HMTV has also not been described. If HMTV was transmitted through milk, then breast feeding should be associated with increase cancer risk in daughters. However, a large epidemiological study showed that there is no increase in breast cancer incidence in the breast-fed daughters of mothers who developed breast cancer compared those who were not breast-fed (78). Moreover, as described above, pregnancy in MMTV-infected mice is associated with greatly increased tumor incidence, yet in humans, pregnancy appears to have a protective effect (79) and breast cancer rates have gone up at the same time that breast feeding rates have decreased (80). Based on an epidemiological analysis showing that high breast cancer incidence in humans geographically co-localizes with the prevalence of mus domesticus in the environment, it has been suggested that MMTV may spread to humans from feral mice (81). However, the only clearly established mode of transmission of infectious MMTV in mice is through nursing, most likely because with the exception of milk, all of the virus *in vivo* appears to be cell-associated; there is little evidence for virions in blood, saliva or seminal fluid (2,33). Additionally, mice infected as adults have life-long, high-titer antibodies against MMTV (82). Thus, if HMTV was a zoonotic transmission from mice, humans with breast cancer should have anti-MMTV antibodies, which at least in a recent study, have not been detected (64).

Concluding Remarks

Much has been learned about the biology of MMTV since its discovery early in the last century, largely through the use of classical genetics and the more modern use of genetically altered mice. Indeed, the MMTV *in vivo* infection pathway is probably one of the best-characterized

both with regard to the virus and the host. While MMTV has served as a valuable model, particularly through the use of its LTR to direct transgene expression to mammary tissue, it is currently unclear whether a similar transmissible agent exists in humans. Confirmation of such a virus awaits the cloning of HMTV insertion sites in human mammary tumors and the identification of the means by which this virus infects, both at the level of the cell and organism.

Abbreviations

MMTV
: mouse mammary tumor virus

HMTV
: human mammary tumor virus

Sag
: superantigen

Env
: envelope

MLV
: murine leukemia virus

HIV-1
: human immunodeficiency virus-1

HTLVI
: human T cell leukemia virus I

CA
: capsid

NC
: nucleocapsid

LTR
: long terminal repeat

EIAV
: equine infectious anemia virus

Env
: envelope

SU
: surface

TM
: transmembrane

TfR1
: transferrin receptor 1

Rem
: regulator of export of MMTV

TLR4
: Toll-like receptor 4

DCs
: dendritic cells

ITAM
: immunoreceptor tyrosine-based activation motif

EBV
: Epstein Barr Virus

KSHV
: Kaposi's Sarcoma Herpes Virus

CIS
: common integration site

fgf
: fibroblast growth factor

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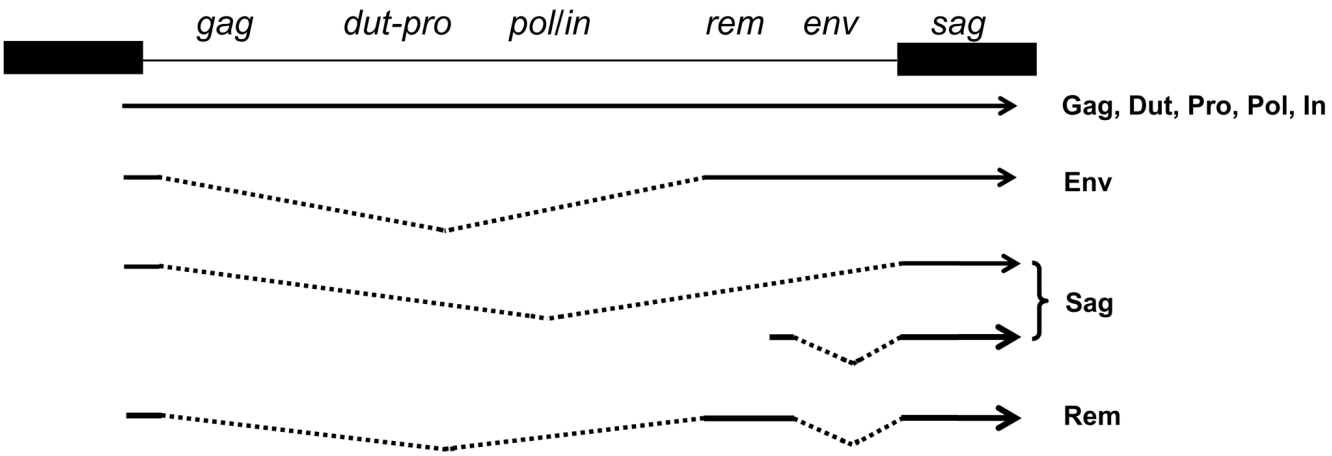

Fig. 1.
MMTV proviral genome and gene products.

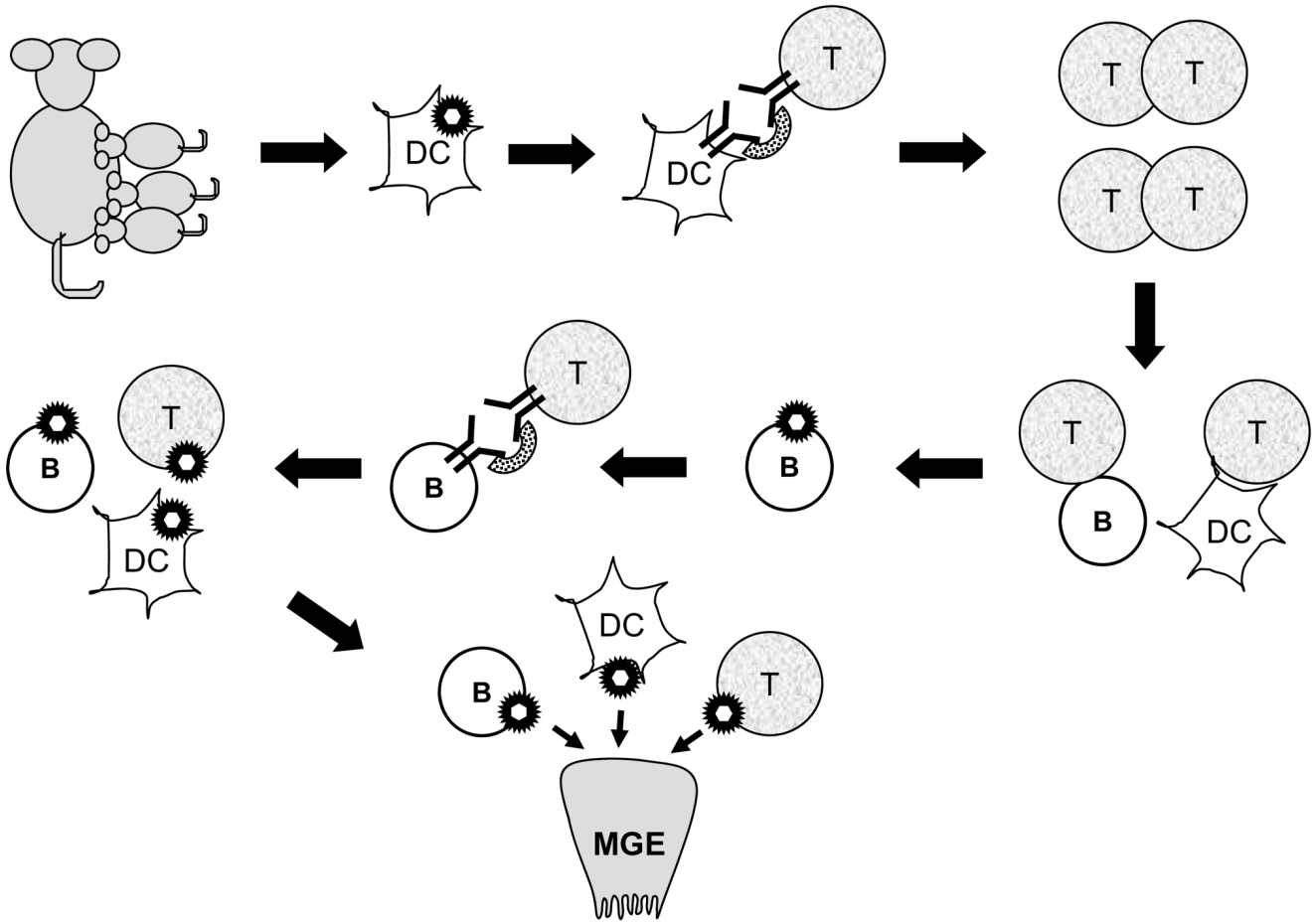

Fig. 2.

In vivo infection by MMTV. DCs in the gut get infected by MMTV, then traffic to the lymph node where they present Sag to cognate T cells. The T cells get activated, secrete cytokines and provide B and DC cell help, thereby creating a reservoir of infection-competent lymphocytes. Infected B cells then further amplify infection by presenting Sag to T cells. The infected lymphoid cells traffic to the mammary gland, where they transmit virus to mammary epithelial cells (MGE).

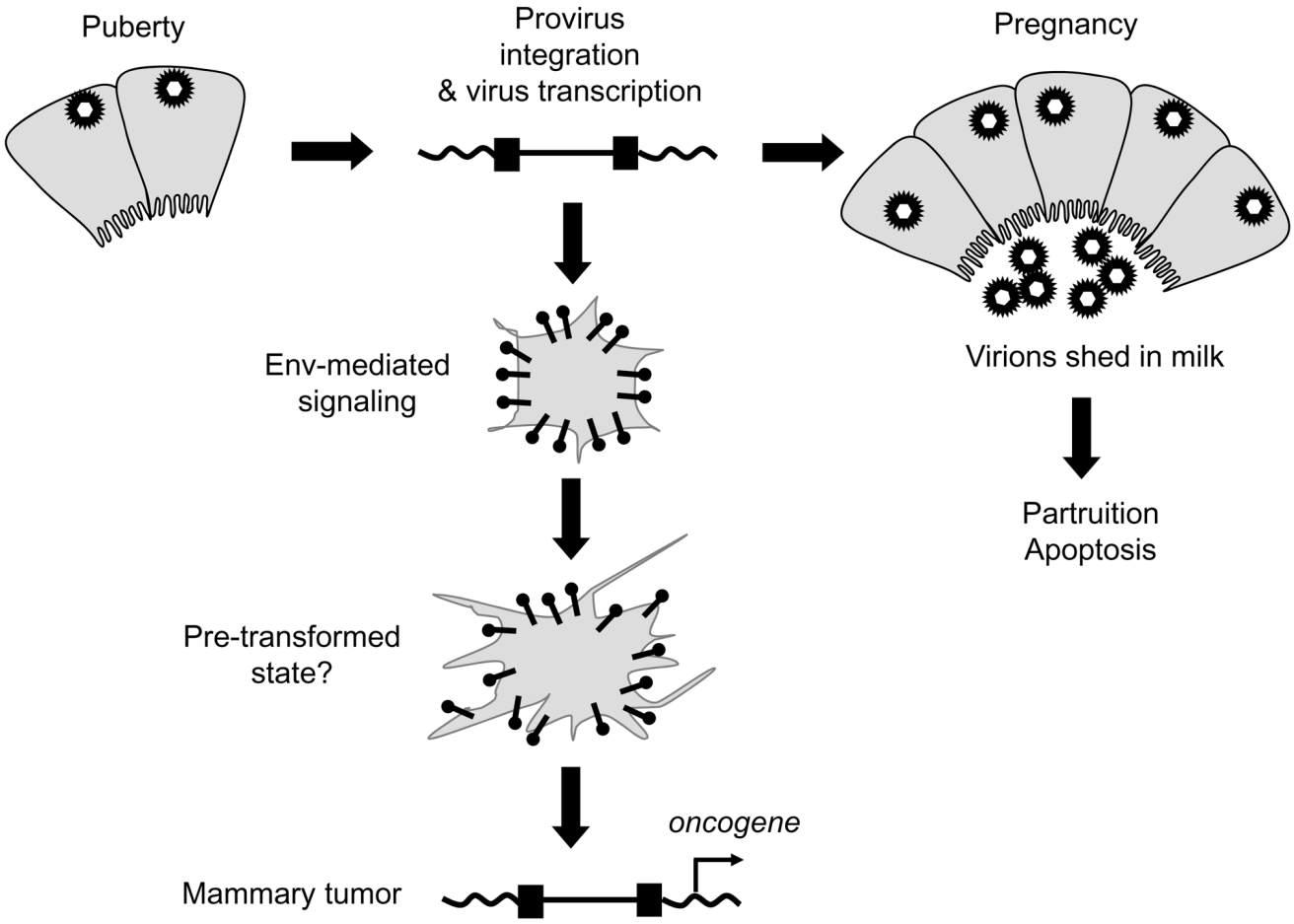

Fig. 3.

MMTV-induced mammary tumors. Virus infects dividing mammary epithelial cells during puberty and viral DNA integrates in the genome. Further proviral integration occurs in mammary epithelial cells stimulated to divide during pregnancy. During lactation, virus is shed into milk and transmitted to the next generation; the fully differentiated mammary epithelial cells undergo apoptosis during partruition. Infected mammary epithelial stem cells express the Env protein, which through ITAM signaling, may predispose mammary cells to more rapid transformation in conjunction with proviral integration near a cellular oncogene.