Androgen-independent proliferation of LNCaP prostate cancer cells infected by xenotropic murine leukemia virus-related virus

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Abstract

Xenotropic murine leukemia virus-related virus (XMRV) is a novel gammaretrovirus that was originally isolated from human prostate cancer. It is now believed that XMRV is not the etiologic agent of prostate cancer An analysis of murine leukemia virus (MLV) infection in various human cell lines revealed that prostate cancer cell lines are preferentially infected by XMRV, and this suggested that XMRV infection may confer some sort of growth advantage to prostate cancer cell lines. To examine this hypothesis, androgen-dependent LNCaP cells were infected with XMRV and tested for changes in certain cell growth properties. We found that XMRV-infected LNCaP cells can proliferate in the absence of the androgen dihydrotestosterone. Moreover, androgen receptor expression is significantly reduced in XMRV-infected LNCaP cells. Such alterations were not observed in uninfected and amphotropic MLV-infected LNCaP cells. This finding explains why prostate cancer cell lines are preferentially infected with XMRV.

1. Introduction

Xenotropic murine leukemia virus-related retrovirus (XMRV) is a novel human gammaretrovirus that was originally isolated from human prostate cancer tissues [1]. Although it is widely believed at present that XMRV is not the etiologic agent of prostate cancer, human prostate cell lines are frequently infected with XMRV [2].

It is known that some retroviruses play a critical role in leukemogenesis in various mammalian species including human [3,4]. The xenotropic MLV infection receptor (XPR1), which is also recognized by XMRV [5,6], varies among wild mice species as a mechanism of resistance to xenotropic virus infection [7,8]. The latter observation suggests that xenotropic viruses may be pathogenic in some species and implies that XMRV may affect growth of certain cell lineages.

Prostate cancer cell lines exhibit a propensity for infection by XMRV when compared to other types of human cancer cell lines [2,9]. It has been reported that amyloidogenic fragments originating from prostatic acid phosphatase greatly increase XMRV infections of primary prostatic epithelial and stromal cells [10]. In vivo infection of macaques with XMRV has confirmed that prostate tissue has a high affinity for XMRV, and the prostate tissues remain continuously infected even after 5 months, when XMRV was undetectable in blood [11]. Dihydrotestosterone (DHT) stimulates XMRV expression in cells expressing a functional androgen receptor (AR) [12,13]. These results suggest that XMRV infection specifically confers an advantage to prostate cancer cells.

In this study, we aimed to determine whether XMRV infection affects androgen-dependent growth of the LNCaP human prostate cancer cell line. Our results indicate that XMRV infection may

provide an androgen-independent growth advantage to prostate cancer cells.

2. Materials and Methods

2.1. Cells

PC-3 and LNCaP cells were obtained from ATCC. PC-3 cells were cultured in RPMI 1640 medium (Wako) supplemented with 8% (v/v) fetal bovine serum (FBS) (Biofuies), L-glutamine and penicillin-streptomycin (both from Sigma-Aldrich). LNCaP cells [14] were maintained in the same medium but additionally supplemented with 10 nM dihydrotestosterone (DHT) (Sigma-Aldrich). Rat F10, human HeLa, and human 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 8% FBS and penicillin-streptomycin. All cell lines were grown in a tissue culture incubator at 37°C with a 5% CO_2 atmosphere.

2.2. Retrovirus infection

The XMRV plasmid DNA was obtained from Dr. R. H. Silverman and Dr. B. Dong [1] through the AIDS Research and Reference Reagent Program (NIAID, NIH, USA) and was used for transfection of rat F10 cells. Culture supernatants of transfected F10 cells were used to inoculate target cells in presence of polybrene (4 μg/ml) (Sigma). Inocula containing MLV were from culture supernatants of amphotropic MLV-producing cells, obtained from Dr. Y. Iwatani. Infected LNCaP cells were maintained in presence of DHT. In tests of androgen responses, target cells were cultured in various combinations of DHT (10 nM) and bicalutamide (10 μM).

2.3. Cell counts and viability

The cells to be counted were collected and stained with trypan blue. Numbers of unstained (viable) cells were counted using a counting chamber under a microscope to estimate cell viability.

2.4. Western blot analysis

Cell lysates were subjected to electrophoretic separation in SDS-containing polyacrylamide gels (BioRad), after which proteins were transferred onto a PVDF membrane. The membrane was first treated with the primary antibodies: mouse anti-β-actin (Santa Cruz Biotechnology), goat anti-dynamin (Santa Cruz Biotechnology), rabbit anti-human AR (Santa Cruz Biotechnology), goat anti-MLV p30 gag (ViroMed), or goat anti-MLV SU (ViroMed) antibody. Following these procedures, the membrane was treated with secondary horse radish peroxidase (HRP)-conjugated anti-mouse IgG antibody, or HRP-conjugated protein G (Bio-Rad). Secondary antibody- or protein G-bound polypeptides were detected by ECL Western Blotting Detection Reagents (GE healthcare).

2.5. Semi-quantitative RT-PCR

Total RNA and genomic DNA samples were isolated by standard protocols. Fist-strand cDNA was synthesized using reverse transcriptase (TaKaRa) from the total RNA (500 ng). Semiquantitative PCR was performed to detect XMRV env, AR, and GAPDH sequences. Nucleotide sequences of the PCR primers for the XMRV *env* sequences were 5'-GACTTGTGTGATTTAGTTGGAGAC-3' and 5'-CCCCGGTGTGGCACC-3'; for AR, 5'-AGCCCCACTGAGACAACC-3' and 5'-ATCAGGGGCGAAGTAGAGCAT-3'; and for GAPDH,

5'-AGGTXGGAGTXAAXGGATTTGGT-3' and 5'-GTGGGCCATGAGGATCCACCAC-3'. These primers were synthesized by Genenet Inc.

2.6. Statistical analysis

Differences between two sets of data were determined by Student's t-test, and these differences were considered significant when $P<0.05$.

3. **Results**

3.1. XMRV infection converts LNCaP cells to an androgen-independent phenotype

To analyze the effect of XMRV infection on androgen-dependent growth of LNCaP cells, the proliferation of XMRV-infected and -uninfected LNCaP cells was compared. As it has been reported that XMRV can replicate in rat cells but not in human 293T cells [12,13,], virus was first rescued by transfection of an XMRV expression plasmid [1] in rat F10 cells. Undiluted culture supernatant from these cells was then added to LNCaP cells with polybrene and cultured for 24 h. The XMRV-infected and -uninfected cells were maintained in the presence of 10 nM DHT for more than 3 months. Uninfected LNCaP cells did not grow in the absence of DHT (Fig. 1A) but did in the presence of DHT (Fig. 1B), indicating a strong androgen-dependent growth requirement, as reported [14]. As a control for specific AR effects, DHT-induced growth of uninfected LNCaP cells was shown to be abrogated by the antagonist bicalutamide, an androgen blocker (Fig. 1C). Bicalutamide (10 μM) alone had no effect on the growth of either infected or uninfected LNCaP cells (Fig. 1D). LNCaP cells chronically infected with XMRV grew even in the absence of DHT (Fig. 1A), and bicalutamide did not suppress growth of XMRV-infected LNCaP cells (Figs. 1C and D). In the presence of DHT, the number of XMRV-infected LNCaP cells was greater than control uninfected cells after 3 days in culture (Fig. 1B). Three independent XMRV-infected LNCaP cell pools were constructed, and all of them could grow in the absence of DHT. When uninfected LNCaP cells were maintained in the presence of DHT, the cells did not gain androgen-independent growth property during this study. These results indicate that XMRV infection converts LNCaP cell growth from androgen dependence to independence.

On the other hand, LNCaP cells chronically infected with amphotropic MLV did not efficiently proliferate even in the presence of DHT (Figs. 1A, B, C, and D), suggesting that the amphotropic MLV infection is cytotoxic for LNCaP cells.

To determine the time course of the conversion of XMRV-infected LNCaP cells to androgen independence, growth kinetics were analyzed after XMRV infection (from 1–2 months, 2–3 months, and >3 months). Cultures initially contained 5×10^3 cells and were counted again after 6 days, because differences between the uninfected and XMRV-infected LNCaP cells in androgen dependence were apparent 6 days after the culture was started (Figs. 1A, B, C, and D). DHT dependence of LNCaP cell growth was reduced by XMRV infection, but DHT still activated cell proliferation 1–2 months after XMRV infection (Fig. 1E). Cell numbers of the infected LNCaP cells in the absence of DHT were comparable to those in its presence 2–3 months after infection. These results suggest that the complete conversion of LNCaP cells to androgen independence takes more than 2 months. The XMRV infection did not increase cell numbers in the absence of DHT 1–3 months after the XMRV inoculation, but cell increases were observed longer than 3 months after inoculation, showing that the activation of LNCaP cell growth by the XMRV infection requires at least 3 months.

Uninfected PC-3 cells, whose growth is androgen-independent [15], grew as efficiently as XMRV-infected PC-3 cells in the absence or presence of DHT (data not shown). These results indicate that XMRV infection did not affect growth of androgen-independent PC-3 cells.

3.2. XMRV infection inhibits androgen receptor expression in LNCaP cells

Because androgen agonistic (DHT) and antagonistic (bicalutamide) effects are mediated through androgen receptor (AR), we analyzed the effects of XMRV infection on its expression in LNCaP cells. As demonstrated by western blot analysis, the expression of AR protein gradually decreased after XMRV infection of LNCaP cells (Fig. 2A). Expression was significantly decreased but still detectable 2–3 months after infection, but by >3 months no expression was observed. Therefore, the reduction of AR expression occurred in parallel with the conversion to androgen-independent proliferation. In LNCaP cells chronically infected with amphotropic MLV, AR expression was not affected (last lane of Fig. 2A).

To determine whether the reduction of AR protein expression by XMRV infection was associated with decreased AR transcript levels, we examined mRNA expression by semiquantitative RT-PCR. As the result, we found that the AR mRNA level in LNCaP cells is counteracted by the XMRV infection (Fig. 2B). These findings demonstrated that the XMRV infection induces the androgen-independent growth and attenuates the *AR* gene transcription in LNCaP cells.

LNCaP cells have been shown to exhibit androgen-dependent expression of the prostate-specific antigen (PSA) [14]. We therefore analyzed PSA expression in LNCaP cells converted to androgen independence by XMRV. PSA expression was not detected in HeLa, 293T, or androgen-independent PC-3 prostate cancer cells (Fig. 2C). Uninfected LNCaP cells expressed PSA, but chronically XMRV-infected LNCaP cells did not, even though the cells were cultured in the presence of DHT.

3.3. The expression of XMRV proteins depends on androgen stimulation

Because it has been reported that XMRV expression is dependent on androgen and AR [12,13], we analyzed XMRV Gag and Env protein expression in infected LNCaP cells. Our results show that expression of XMRV Gag gradually decreased after infection, correlating with the time course of conversion to androgen-independent growth (Fig. 3A). Three months after XMRV infection, both the Gag precursor and mature protein levels were much lower than after 1–2 month. The XMRV Env protein was expressed for as long as 3 months after infection, but at periods longer than 3 months expression was not detected (Fig. 3B).

Amount of XMRV sequence integrated into genomes of chronically infected LNCaP cells were comparable to that at shorter than 1 month (Fig. 4A), indicating that XMRV-infected cells were maintained during the culture. XMRV RNA level at periods longer than 3 months after XMRV infection was lower than that at shorter than 1 month (Fig. 4B). These results indicate that XMRV expression is reduced during LNCaP cell phenotypic conversion and support the conclusion that XMRV expression is dependent on androgen [12,13].

The XMRV Gag proteins were not detected by western analysis of XMRV-infected PC-3 cells (Fig. 3A), but the XMRV env sequence-containing RNA was detected by RT-PCR (Fig. 4C), showing that XMRV genome was integrated and transcribed at low level in PC-3 cells. Because PC-3 cells are androgen-independent and lack AR expression [16], the androgen-dependent XMRV Gag protein level was presumably below detectable limits (Fig. 3A). In contrast, Gag protein was detected by western analysis in LNCaP cells chronically infected with amphotropic MLV (Fig. 3A), showing that the

182 amphotropic MLV expression was independent of androgen.

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4. Discussion

In this study, we found that XMRV infection converts the androgen-dependent phenotype of LNCaP cells to androgen independence, and it reduces AR expression. This effect seems to be relatively specific to XMRV, as it was not observed with amphotropic MLV infection of the same cells. Consistently, it has been reported that XMRV activates tumor growth and invasiveness of LNCaP cells [17,18,19], but androgen-dependence of LNCaP cell proliferation was not analyzed in these studies. Many human cancer cells have been transplanted into nude mice, but prostate cancer cells are preferentially infected with xenotropic MLVs [2]. The XMRV-mediated androgen-independent growth of prostate cancer cells may explain the propensity for XMRV infection observed in prostate cancer cells.

It has been reported that androgen-independent LNCaP cells spontaneously appear during culture in the absence of androgen [14,20]. However, XMRV-infected LNCaP cells became androgen-independent even in the presence of androgen. Because the XMRV infection suppressed AR protein expression, DHT cannot induce growth activation. Indeed, less than 3 months after the XMRV infection, growth of the infected LNCaP cells was not activated even in the presence of DHT. Then, spontaneous alterations inducing androgen-independent growth might be selected in the XMRV-infected LNCaP cell culture. Because the expression of XMRV proteins was suppressed in chronically XMRV-infected LNCaP cells, the viral proteins are not necessary for maintenance of the androgen-independent state. This observation supports the above speculation. If so, the XMRV infection did not directly induce the androgen-independent growth. However, the abrogation of AR

expression by the XMRV infection triggered the conversion to androgen-independent growth.

How does XMRV reduce AR expression? Amphotropic MLV infection inhibited growth of LNCaP cells. Thus, though the XMRV infection did not clearly suppress the cell growth, XMRV proteins may be slightly toxic to LNCaP cells. Because XMRV expression is androgen-dependent, lower level of AR expression induces lower amount of XMRV proteins. Due to this mechanism, LNCaP cells expressing AR at lower level might be selected during the culture. Further study is required to understand the mechanism by which XMRV infection abrogates AR protein expression.

The expression of XMRV was reduced in the androgen-independent XMRV-infected LNCaP cells. However, xenotropic MLVs are constitutively expressed in the androgen-independent CWR22Rv1 prostate cancer cells [9] and in several human cancer cell lines other than prostate cancer [21,22,23]. These results indicate that expression of these xenotropic MLVs is independent of androgen.

As a clinical problem, most prostate cancer patients treated with combined androgen blockage (CAB) therapy develop castration resistant prostate cancer (CRPC) [24]. Growth of the prostate cancer cells is androgen-dependent in the first stage, and thereafter androgen-independent cancer cells are selected during CAB therapy. The androgen refractory mechanisms are explained by the following hypotheses [24]: i) mutations in or enhanced expression of the AR gene; ii) mutated AR is activated by other steroids; iii) mutated AR is activated by other signals, e.g., peptide growth factors or cytokines; or iv) an AR bypassing pathway is activated. Mechanisms of the fourth case are not completely understood, and it is to this category that XMRV-induced conversion belongs. This is

because AR expression is significantly reduced in XMRV-infected LNCaP cells. The mechanism of AR-deficient CRPC development in human patients may be similar to that of the XMRV-induced LNCaP androgen independence. Elucidation of the mechanism by which XMRV induces androgen-independent growth of LNCaP cells would contribute to a more complete understanding of CRPC development and novel therapies for human prostate cancer patients.

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316 **Figure legends**

317 Fig. 1 XMRV infection converts the phenotype of LNCaP cells from androgen-dependent to
318 androgen-independent. (A-D) Growth of uninfected, chronically XMRV-infected, and
319 amphotropic MLV-infected LNCaP cells cultured in media supplemented with or without the
320 androgen dihydrotestosterone (DHT) and/or the androgen receptor antagonist bicalutamide.
321 (E) Effects of DHT and bicalutamide on growth of uninfected and XMRV-infected LNCaP cells
322 were analyzed. Cells were counted after 6 days in culture. In all the panels, the data
323 presented are average values ± SD from two independent experiments performed in triplicate.
324 The concentrations of DHT used in the experiments were (+) 10 nM, or (–) 0 nM; and of
325 bicalutamide, (+) 10 μM, or (–) 0 μM.

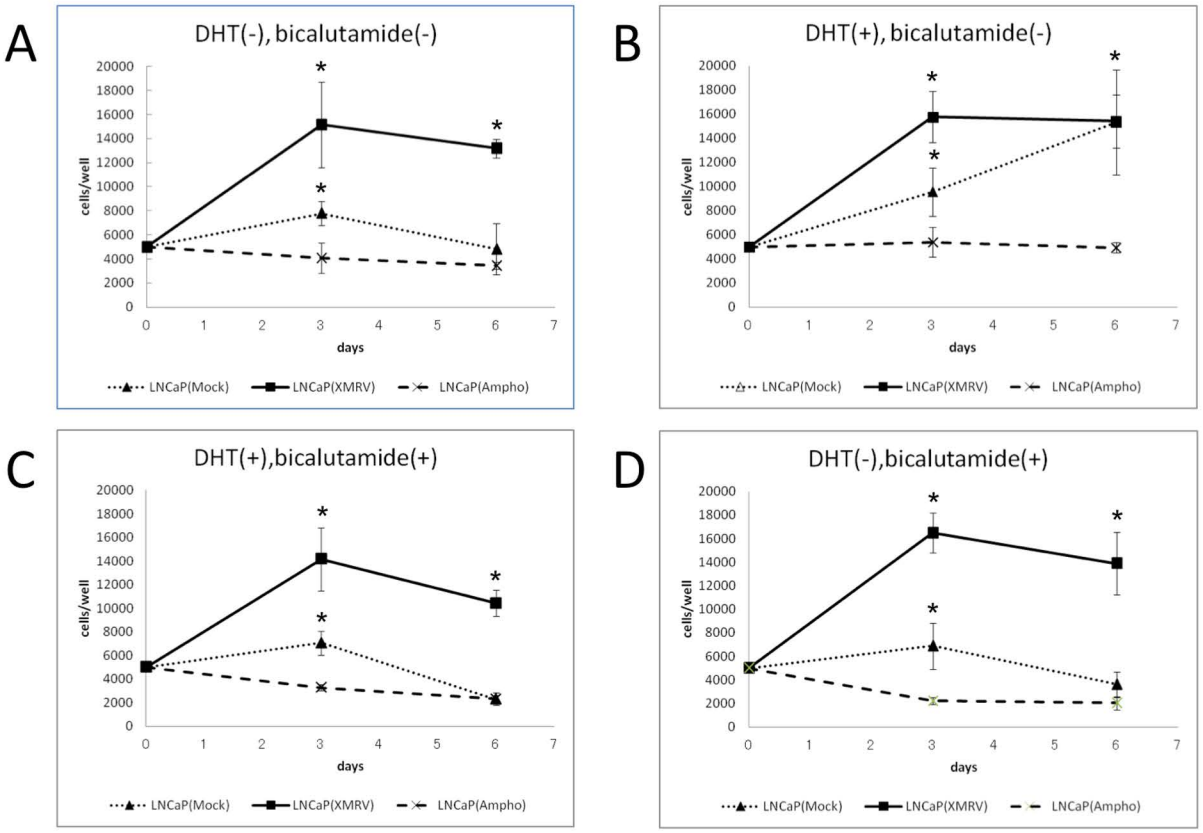
326

327 Fig. 2 XMRV infection results in reduced androgen receptor (AR) protein and mRNA expression. (A)
328 Results of western blot analysis of cell lysates from uninfected, XMRV-infected (at three time
329 points after infection, indicated below the chart), and amphotropic MLV-infected LNCaP cells.
330 Levels of AR (lower panel) and β-actin protein expression (internal positive control, in upper
331 panel) are shown. (B) AR and GAPDH mRNA expression levels from uninfected and
332 XMRV-infected LNCaP cells analyzed by semiquantitative RT-PCR. Arrow heads indicate
333 predicted sizes of the PCR products. (C) PSA protein expression was analyzed in HeLa, 293T,
334 PC-3, uninfected LNCaP, and XMRV-infected LNCaP cells by western blot. As control,
335 dynamin expression was also examined.

Fig. 3 XMRV expression was decreased in parallel with XMRV-induced conversion of LNCaP cells from androgen-dependent to –independent growth. Western blot analysis of MLV Gag (panel A) and Env (panel B) protein levels were evaluated in uninfected, amphotropic MLV-infected, and XMRV-infected LNCaP, and PC-3 cells. As a positive internal control, β-actin protein expression levels were also analyzed.

Fig. 4 XMRV transcription was decreased in XMRV-infected LNCaP cells. (A) XMRV sequences integrated into LNCaP cell genomes were detected by PCR (left panel). Equal amounts of genomic DNAs (500 ng) were analyzed (right panel). (B) Levels of XMRV env mRNA was quantified by RT-PCR in XMRV-infected LNCaP cells. (C) XMRV env mRNA levels was measured in uninfected and XMRV-infected PC-3 cells (right panel). As control, GAPDH mRNA was analyzed in the same samples (left panel). Arrow heads indicate predicted sizes of the PCR products.

Fig.1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C
DHT(+),bicalutamide(+)
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LNCaP(XMRV)
LNCaP(Ampho)

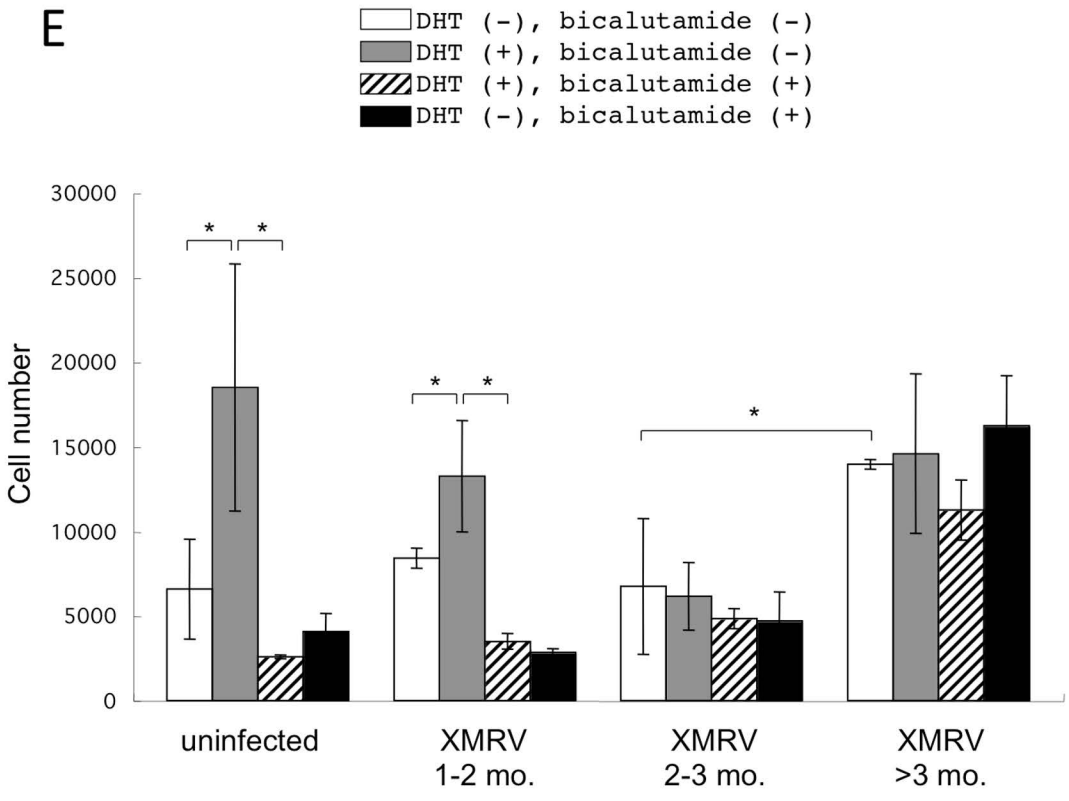
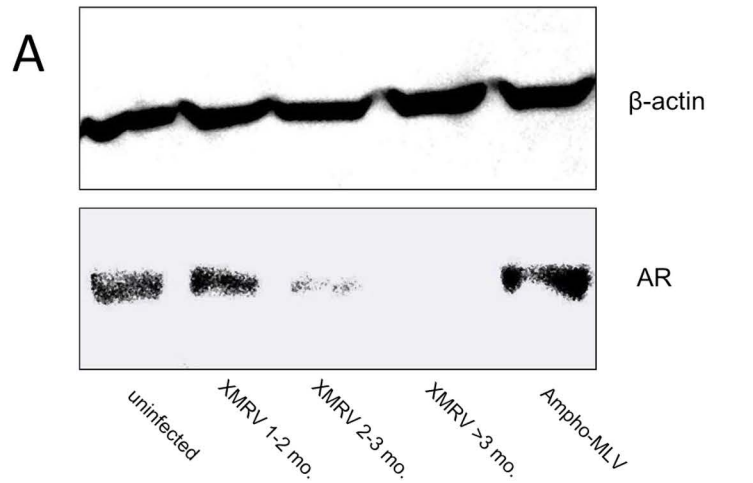

E
DHT (-), bicalutamide (-)
DHT (+), bicalutamide (-)
DHT (+), bicalutamide (+)
DHT (-), bicalutamide (+)
Cell number
uninfected
XMRV 1-2 mo.
XMRV 2-3 mo.
XMRV >3 mo.

Fig.2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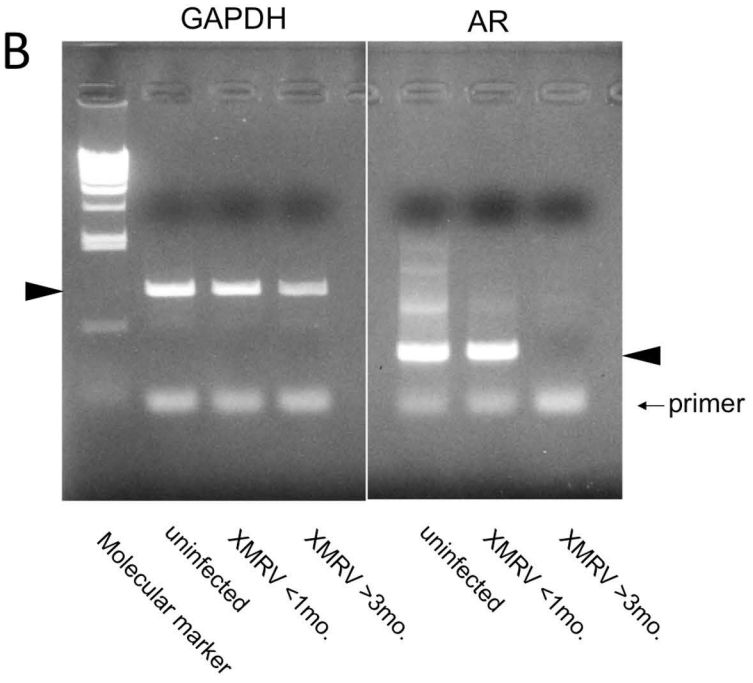
XMRV 2-3 mo.
XMRV >3 mo.
Ampho-MLV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uninfected
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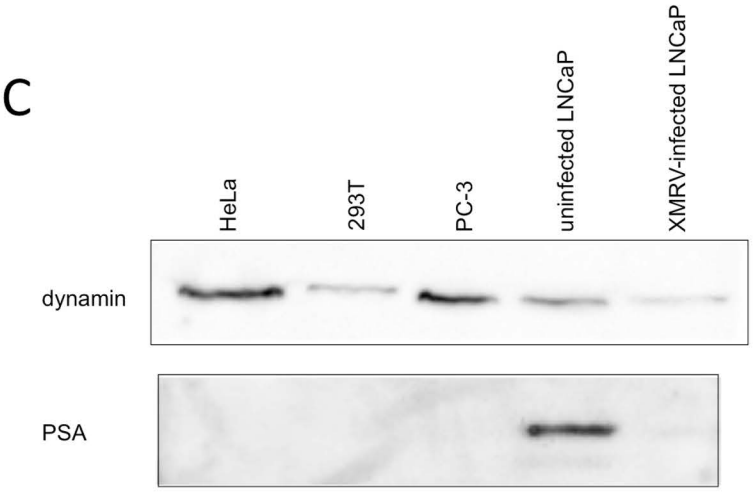

C
HeLa
293T
PC-3
uninfected LNCaP
XMRV-infected LNCaP
dynamin
PSA

Fig.3

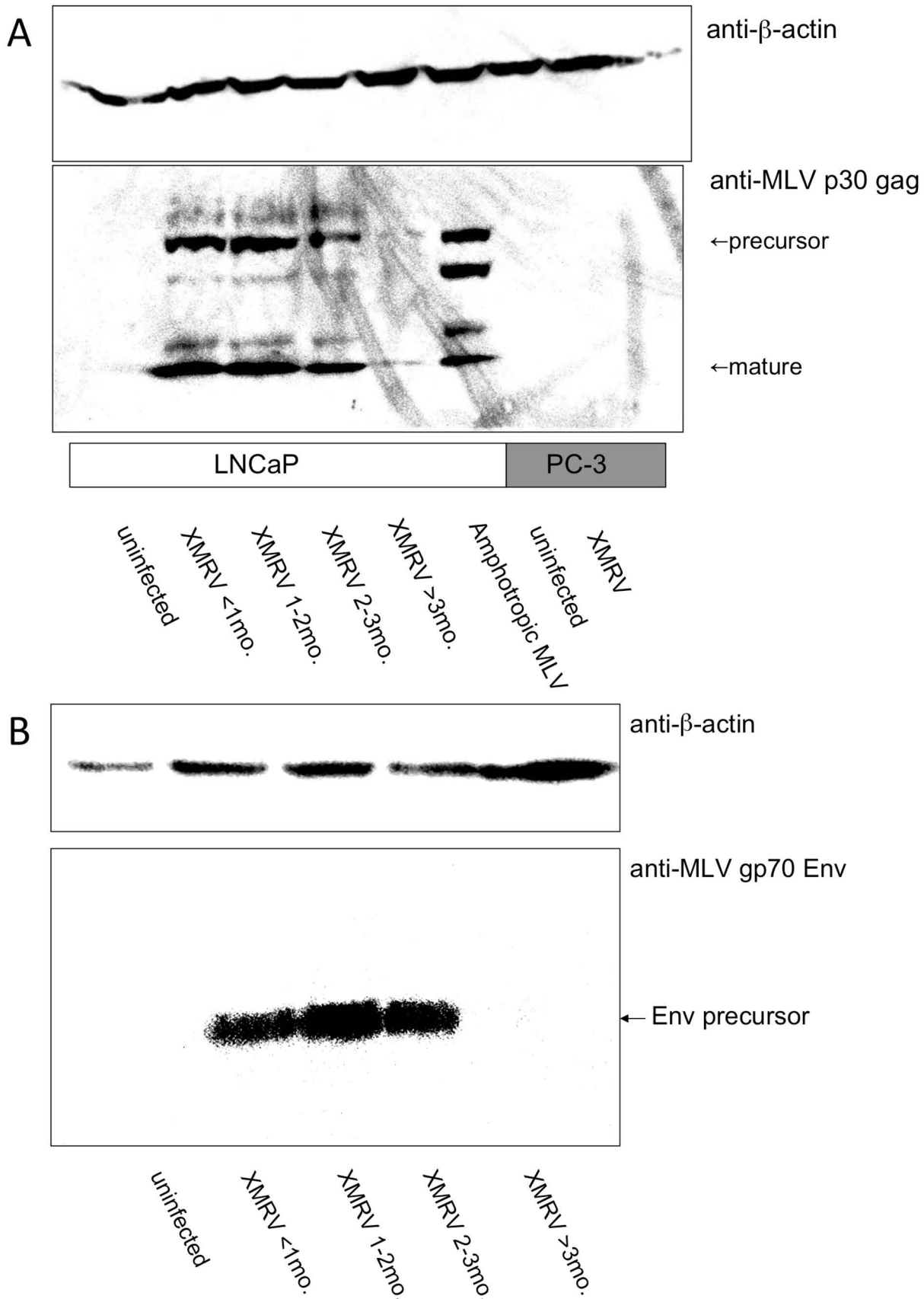
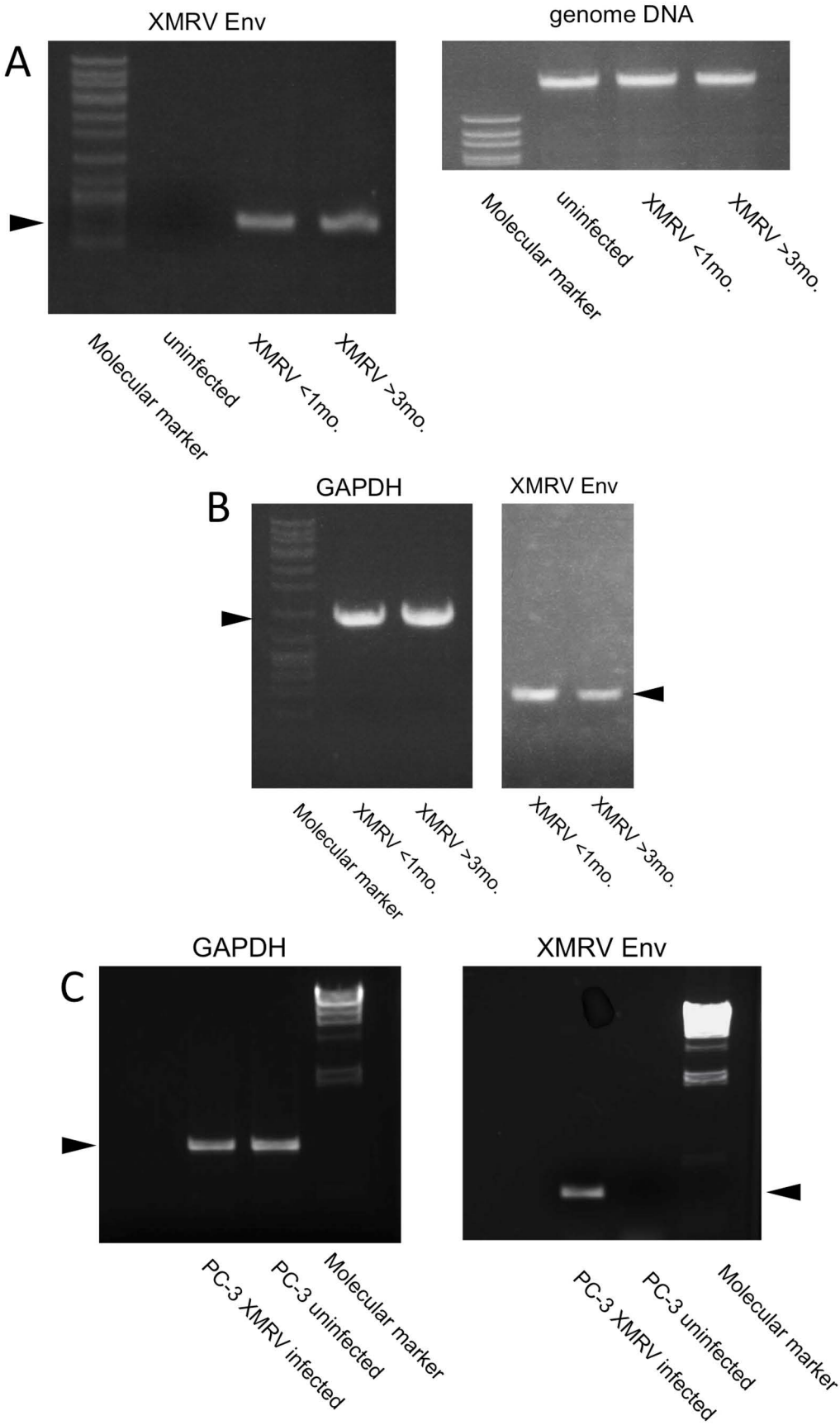

A
anti-β-actin
anti-MLV p30 gag
←precursor
←mature
LNCaP
PC-3
uninfected
XMRV <1mo.
XMRV 1-2mo.
XMRV 2-3mo.
XMRV >3mo.
Amphotropic MLV
uninfected
XMRV
B
anti-β-actin
anti-MLV gp70 Env
← Env precursor
uninfected
XMRV <1mo.
XMRV 1-2mo.
XMRV 2-3mo.
XMRV >3mo.

Fig.4


A
XMRV Env
Molecular marker
uninfected
XMRV <1mo.
XMRV >3mo.
genome DNA
Molecular marker
uninfected
XMRV <1mo.
XMRV >3mo.
B
GAPDH
Molecular marker
XMRV <1mo.
XMRV >3mo.
XMRV Env
XMRV <1mo.
XMRV >3mo.
C
GAPDH
PC-3 XMRV infected
PC-3 uninfected
Molecular marker
XMRV Env
PC-3 XMRV infected
PC-3 uninfected
Molecular marker