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Original Paper

Varying Sensitivity of Human Mammary Carcinoma Cells to the Toxic Effect of Parvovirus H-1

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We have previously demonstrated lysis of non-established cultures of human mammary carcinoma cells by parvovirus H-1, which has little effect on the proliferation of corresponding normal cultures. In the present study, we examined this effect in a number of breast-tumour specimens and found them to differ as to the amplitude of their response to parvoviral attack. We first investigated whether the differences in cell sensitivity to parvovirus infection reflected the differentiation level of the initial tumour. Among the biochemical and anatomopathological indicators of original tumour differentiation, the presence of oestrogenic receptors (ER) was found to have a predictive value as to the sensitivity of derived cultures to the cytopathic effect of H-1 virus. The ER^+ tumour-derived cultures showed an incresed sensitivity to the lytic effect of H-1 virus compared with the ER^- tumour-derived cultures, in spite of similar average proliferation rates for the two types of cultures. The proliferation rate was more heterogeneous among ER^+ tumour-derived cultures and, in this group, the faster growing cultures were also the most sensitive. This observation was corroborated by the study of established cell lines retaining ER expression under *in vitro* culture conditions. Oestradiol was found to increase the sensitivity of these cells to the parvovirus in parallel with induction of proliferation. This effect appeared to be mediated by ER activation, since it was not observed in the ER-negative cell line MDA-MB-231. These data point to the importance of hormonal influences and cellular parameters, notably differentiation and proliferation, in determining the extent to which human cancer cells can be targets for the cytopathic effect of parvoviruses. © **1997 Elsevier Science Ltd.**

Key words: H-1 parvovirus, oncolysis, human mammary epithelial cells, differentiation, proliferation

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INTRODUCTION

PARVOVIRIDAE ARE small, single-stranded DNA viruses that infect a wide range of host species, including humans who are notably infected by the pathogenic B19 virus. The low genetic complexity of parvoviruses implies that they depend heavily on exogenous factors to complete their lytic cycle. In the case of autonomous parvoviruses like the H-1 virus, the helper factors are provided by the host cell and are expressed as a function of cell proliferation and differentiation [1]. The antineoplastic properties of autonomous parvoviruses are especially interesting. *In vivo* studies indicate that parvovirus-infected animals may be protected against spontaneous carcinogenesis and tumour induction by viruses, chemical carcinogens or transplanted trans-

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formed cells. Parvoviruses can also induce regression of established tumours in animals. *In vitro*, parvoviruses have been shown to preferentially kill a number of transformed cells compared to the corresponding normal parental cells [2].

We have previously shown that parvovirus H-1 strongly interferes with the proliferation of short-term breast-tumour-derived cultures. This effect is specific to neoplastic cells, H-1 virus infection having little influence on the growth rate of normal breast tissue [3].

Mammary tumours are heterogeneous, ranging from slowly progressing to very aggressive. They thus provide an interesting sampling of human neoplastic lesions [4]. We therefore chose to examine whether such differences between tumours *in vivo* influence the response to parvoviral attack *in vitro*. This investigation led us to study in particular the role of oestrogen receptors (ERs) and their stimulation in determining the sensitivity of breast cancer cell lines to the H-1 virus.

MATERIALS AND METHODS

Human breast carcinoma short-term tissue cultures

Primary mammary carcinomas from patients having received, prior to surgery, neither radiotherapy, chemotherapy nor tamoxifen therapy, were processed for primoculture [3], in minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS), 5% tryptose phosphate broth, insulin (5 μg/ml), cholera toxin (0.1 μg/ml), epidermal growth factor (10 ng/ml) and hydrocortisone (0.5 μg/ml). Subconfluent primary cultures were dissociated with trypsin and viable cells were counted with a haemocytometer, using the Trypan blue exclusion test.

Infection of primocultures with H-1 virus

One day after subculturing (approximately 22 days after initiation of the primary culture), the cells were either mock-treated or infected with H-1 virus at a multiplicity of infection (MOI) of 3 plaque-forming units (PFU) per plated cell. After 5 days, the cells were harvested and counted. In some cases, it was possible to extend the experiment, i.e. harvested cells were re-seeded, cultured and collected on the 13th day after H-1 virus infection.

Determination of growth and survival rates in primocultures

The cell growth rate was calculated as the ratio of the numbers of harvested cells to seeded cells, with correction for the plating efficiency (17.5%, as extrapolated from the value measured for four of the cultures). The survival rate was defined as the ratio of the growth rate recorded in H-1 virus-infected cultures to that recorded in mock-treated cultures, and it was expressed in percentages.

Established breast cancer cell lines and media

MCF-7, T47D and ZR75-1 are metastatic breast carcinoma cell lines that retain, to a variable extent, ER expression in culture. MDA-MB-231 is a metastatic breast carcinoma cell line that does not express ERs.

Cell lines were routinely maintained in red phenol-free MEM supplemented with 2 mM glutamine, 40 μg/ml gentamicin, 1% non-essential amino acids (Gibco, Belguim) and 7.5% FCS that was depleted of steroid hormones by treatment with dextran-coated charcoal.

Determination of the effect of oestradiol on the proliferation of breast carcinoma cell lines

Before assessing the effect of oestradiol on growth, cell cultures kept in hormone-depleted medium were supplemented, or not, with oestradiol (E2, Sigma, Belguim) at 10^{-11} M or 10^{-10} M concentration, and further incubated for 48 h. E2 was dissolved in 100% ethanol and added to the medium at 1:1000 dilution. Control medium was supplemented with solvent alone. On day 0, cultures were collected and replated in the same medium at a density of 10^5 cells per 3 cm dish. The medium was changed every other day and cells were harvested, stained with Trypan blue and counted with a haemocytometer on days 2, 3, 4 and 5.

Determination of the effect of oestradiol on the survival of breast carcinoma cell lines after H-1 virus infection

On day 0, cell cultures kept in hormone-depleted medium were supplemented with either oestradiol (10^{-11} M or 10^{-10} M) or solvent alone, further incubated for 24 h, collected and replated in the same medium at a density of 10^5 cells per 3 cm dish. On day 2, cells were either mock-treated or infected with H-1 virus in phosphate-buffered saline (PBS) at an MOI of 10 PFU per cell. At the indicated times after infection, cells were harvested with trypsin/EDTA and cell number and viability were determined by staining with Trypan blue and counting with a haemocytometer. The survival rate was calculated from the number of living cells in infected cultures and expessed as a percentage of the corresponding value in uninfected cultures.

RESULTS

Varying sensitivity of short-term tumour-derived cell cultures to H-1 virus

To see whether different original tumours can be distinguished from each other by their responsiveness to parvovirus attack *in vitro*, we examined 19 short-term cultures derived from breast carcinomas differing with regard to the patient's age, the clinical stage, the histological type and biochemical parameters. H-1 virus was tested for its ability to inhibit the growth of these cultures, a phenomenon that we could previously assign to both killing and cytostatic effects of the virus and that was expressed as a survival factor representing the relative number of infected versus mock-treated cells [3]. Breast tumour-derived cultures were sensitive to the toxic effect of H-1 virus (mean survival on day 5 after infection 68%), but survival rates showed a wide distribution, ranging from 21% to 90%. Survival after H-1 virus infection correlated neither with the patient's age nor with the size, axillary-node involvement or histological type of the original tumour (data not shown).

Parvoviruses are known to depend on host-cell differentiation for their own replication and thus for inducing cell disturbances [5]. Therefore, we first examined whether the heterogeneity of breast tumours regarding their sensitivity to H-1 virus correlated with the extent of their differentiation. Two types of criteria can be used to characterise the differentiation status of the initial tumour. Breast cancers can be distinguished on the basis of biochemical criteria, including expression of oestrogen receptors and DNA content, or of morphological criteria quantified by means of scores such as the Scarff, Bloom and Richardson score (SBR score, applicable to breast ductal carcinomas only) [6] and the Nottingham score (an adaptation of the SBR score, appli-

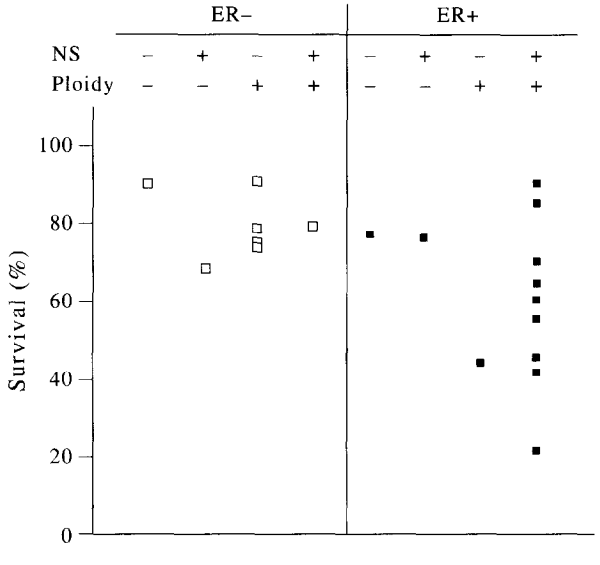

Figure 1. Survival of carcinoma-derived cultures 5 days after H-1 virus infection, as a function of the original tumour's differentiation. Carcinomas were classified according to the presence (+) or absence (–) of ER, Nottingham scores (NS) of I and II (+) or III (–), and euploid (+) or aneuploid (–) DNA contents.

cable to a larger group of breast tumours [7]. The Nottingham histoprognostic score I to III is based on the percentage of tubule formation, the degree of nuclear pleomorphism and the mitotic count. A score of I corresponds to the most differentiated pattern (1 out of 19 tumours in our series). While ER expression allows the response to hormone therapy to be predicted, it is also used, together with the DNA content, as a general differentiation marker to identify tumours with low or high differentiation levels and with poor or good prognosis [8]. The validity of these parameters as differentiation markers was further confirmed in our system by their good correlation with the Nottingham score: 90% of the euploid ER^+ tumours included in the present study had a score of I or II, while 66% of the other tumours had a score of III.

As shown in Figure 1, the primocultures derived from euploid breast tumours did not differ significantly from the aneuploid tumours, as far as their survival 5 days after H-1 virus infection was concerned. In the same manner, there was no significant difference in survival after infection between highly-differentiated tumour-derived cultures, defined by a Nottingham score of I or II, compared to poorly differentiated ones defined by a Nottingham score of III. Even taken together, these two parameters failed to account for the differences in sensitivity to H-1 virus observed among breast tumour-derived primocultures. However, the third parameter, the presence or absence of ERs, was found to correlate with a significant difference in cell sensitivity to viral attack (Figure 1). The ER^- tumour-derived cultures were relatively resistant to H-1 virus (mean survival of $80 \pm 6\%$), compared with their ER^+ equivalents (mean survival of $62 \pm 11\%$, given with a 95% confidence interval). If less than 67.5% survival was chosen as the criterion for sensitivity to H-1 virus (corresponding to the mean sensitivity of all cultures), the ER^- group included only resistant tumours, whereas the ER^+ group contained a majority of sensitive tumours (7/12). Moreover, all the sensitive tumours belonged to the ER^+ group (Figure 1). The same difference in survival between ER^+ and ER^- tumour-derived cultures was also observed on day 13 after H-1 virus infection (data not shown). The ER status or original breast tumours thus proved to have a predictive value for their sensitivity to H-1 virus, as assessed under *in vitro* conditions. The ER^+ group was significantly more sensitive to viral attack than the ER^- group (Student's *t*-test $P < 0.02$). ER expression alone did not provide an absolute measure of sensitivity, since 41% of the most differentiated tumours (ER^+) were relatively resistant to parvovirus infection (Figure 1).

Besides differentiation, proliferation of the host cell is also known to be important to confer permissiveness for parvoviruses [9]. We thus wondered whether the difference observed between the H-1 virus sensitivities of ER^- and ER^+ tumour-derived cultures might be assigned to varying growth rates. This did not appear to be the case since the sensitisation of ER^+ versus ER^- tumours could still be detected when comparing pairs of cultures that exhibited similar relative cell numbers at the two time points (days 5 and 13) when a difference in survival was noted (Figure 2). This result does not rule out that proliferation yield may

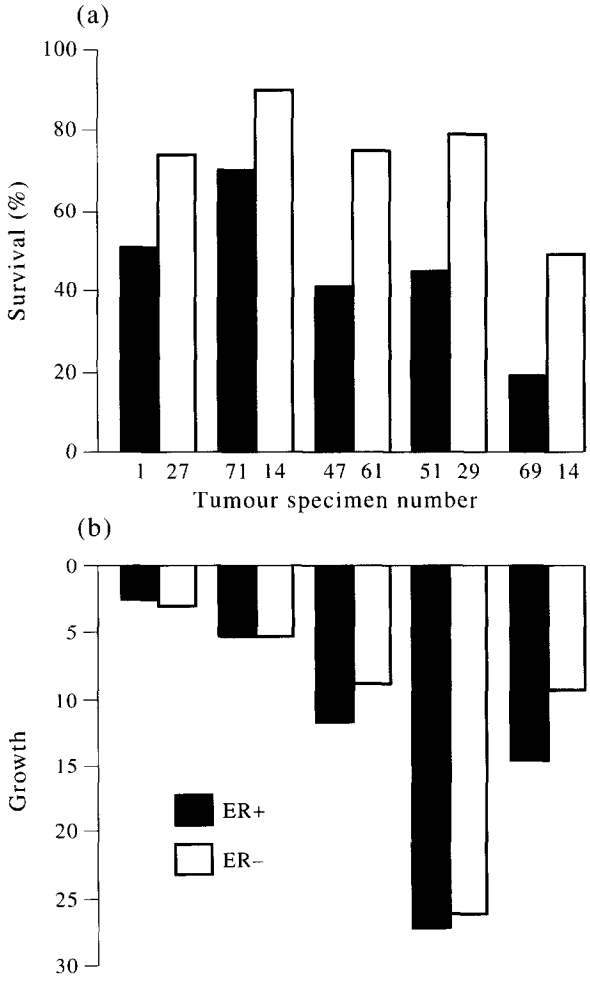

Figure 2. Comparison of ER^+ and ER^- tumour cultures for their sensitivity to H-1 virus (a) and growth rate in the absence of virus (b). The growth of uninfected cultures and the survival were measured 5 days (tumours 1, 27, 71, 14, 47, 61, 51, 29) or 13 days (tumours 69, 14) after mock or H-1 virus infection.

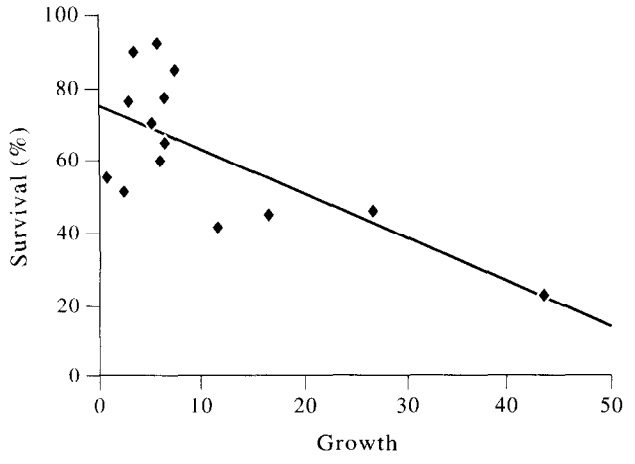

Figure 3. Survival of ER$^+$ breast tumour-derived short-term cultures to H-1 virus infection, as a function of the growth rate of corresponding uninfected cultures. Cell culture growth and survival were measured 5 days after mock or H-1 virus-infection, respectively.

constitute a positive determinant of neoplastic mammary cell sensitivity to H-1 virus. The ER$^-$ breast cancer-derived primocultures showed a quite uniform behaviour, as far as survival to the virus and proliferation of uninfected cells were concerned. Important variations were observed among ER$^+$ tumour-derived cultures, regarding both survival and proliferation, with a clear tendency of cell survival after H-1 virus infection to decrease when the growth rate of uninfected cultures increased (Figure 3).

Modulation of the sensitivity of breast carcinoma cell lines to H-1 virus

We used ER$^+$ breast cancer cell lines to determine whether stimulation of the oestrogenic receptor by oestradiol may influence cell survival after parvovirus infection. For this purpose, oestradiol was tested for its effect on the sensitivity of MCF-7, T47D and ZR75-1 cells to H-1 virus. These human mammary carcinoma cell lines retain ER expression, a quite uncommon and interesting feature, since the great majority of breast-derived cultures lose ER expression after the first passage (data not shown and ref. 10) while recovering it after transplantation into nude mice (G. Leclercq, Université Libre de Bruxelles). The level of ER expression, measured by immunochemistry in oestradiol-depleted medium, was found to vary among the cell lines, being high in MCF-7 (165 fmole/mg protein), intermediate in ZR75-1 (43 fmole/mg protein) and low in T47D cells (5 fmole/mg protein).

In order to study the possible interconnection of ER expression and sensitivity to parvovirus H-1, ER$^+$ cells were exposed to physiological concentrations of oestradiol. The E2 hormone is known to the stimulate growth of ER$^+$ MCF-7 cells [11], while inducing morphological modifications which notably include an increase in the number and length of microvilli [12]. E2 is also required for MCF-7 tumour growth in nude mice [13]. In agreement with these reports, physiological oestradiol concentrations proved able to stimulate the growth of MCF-7 and T47D cells (Figure 4). This effect was dose-dependent within the range of E2 concentrations used. No significant change was observed in the growth of ZR75-1 cells, in agreement with a previous report showing that levels higher than physiological

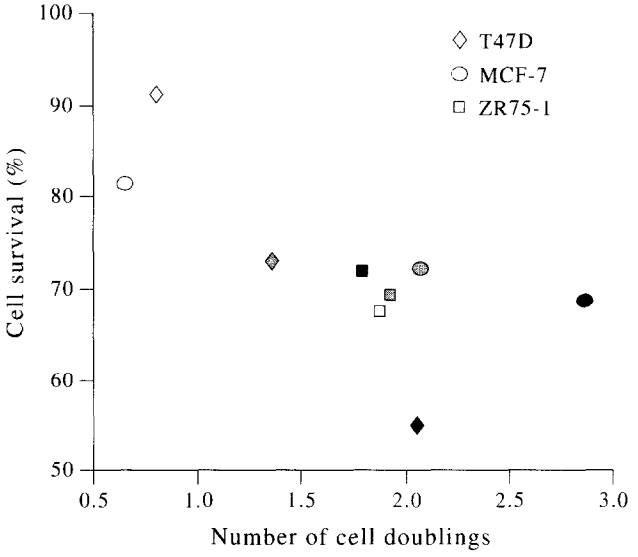

Figure 4. Survival after H-1 virus infection of established cell lines, as a function of their growth rate in different E2-containing media. Open symbols, E2-depleted medium, shaded symbols, medium containing 10^{-11} M E2; solid symbols, 10^{-10} M E2. Average values from three (T47D, MCF-7) or six (ZR75-1) independent experiments done in duplicate.

E2 concentrations are required to enhance the proliferation of these cells [14].

The survival of T47D cells was significantly lower in medium supplemented with 10^{-11} M and 10^{-10} M oestradiol than in hormone-depleted medium, as illustrated in Figure 4 for the 48 h post infection time point ($P < 0.01$, Student's t-test). The difference was still higher when survival was measured at 72 h post infection ($P < 0.001$, Student's t-test) (data not shown). As shown in Figure 4, T47D cell sensitisation to parvovirus H-1 was E2 dose-dependent and correlated with the number of mock-infected population doublings induced by oestradiol ($P < 0.05$). A similar, although less pronounced sensitisation of MCF-7 cells to the virus was found to take place in relation to the growth stimulation caused by increasing E2 concentrations. In contrast, the survival of ZR75-1 cells to H-1 virus was little affected by oestradiol treatment, in keeping with their poor ER expression and the lack of responsiveness of their proliferation to the E2 doses used. Furthermore, involvement of the ER signalling pathway in the above-mentioned effects of E2 on the ER$^+$ T47D and MCF-7 cell lines was supported by the hormone inability to modulate the growth rate and H-1 virus sensitivity of the ER$^-$ cell line MDA-MB-231, tested under the same conditions (data not shown).

DISCUSSION

Non-established cultures of human breast carcinoma cells are inhibited in their proliferation by parvovirus H-1, but differ with regard to the amplitude of their sensitivity to virus attack. To complete their lytic cycle, autonomous parvoviruses depend on cellular factors, some of which are expressed as a function of the differentiation status of the host cells. Animal development comprises stages of resistance to parvoviruses (the embryonic and adult periods) and a window of greater sensitivity (the fetal and neonatal periods) [15]. The developmental switch from resistance to

sensitivity was studied in teratocarcinoma stem cells which can be induced to differentiate *in vitro*. A pluripotent mouse teratocarcinoma stem cell line displayed resistance to infection with the H-1-related Minute Virus of Mice (MVM), whereas 16-day-old whole-embryo cultures from the same strain of mice were sensitive. When this teratocarcinoma cell line was induced to differentiate *in vitro*, a fibroblast-like cell type arose which proved sensitive to MVM [5, 15].

In keeping with these data, a correlation was observed in the present work between the differentiation status of the original mammary tumours and the extent of the cytotoxic effect of parvovirus H-1 on corresponding primocultures. One of the biochemical markers of the differentiation of mammary tumours, the presence of oestrogen receptor, was found to have a predictive value with regard to the hypersensitivity of the derived cultures to the cytopathic effect of H-1 virus. It should be stated, however, that this criterion constitutes a statistical rather than absolute indicator of tumour cell responsiveness to H-1 virus, since some differentiated (ER^+) tumour cultures were quite resistant to the parvovirus. No significant correlation was observed between the sensitivity of mammary tumour cells to H-1 virus and either anatomopathological features (Nottingham score) or ploidy of the original tumours. The sensitisation of ER^+ tumour primocultures to H-1 virus could not be assigned to a difference in their growth rate compared with the ER^- group, arguing for an effect of differentiation on the permissiveness of neoplastic mammary cells to the virus.

Exposure of ER^+ breast cancer cell lines to physiological doses of oestradiol was found to increase their sensitivity to the cytotoxic effect of H-1 virus. Through its interaction with oestrogen receptors, oestradiol stimulates cell growth [11] and induces a number of phenotypic changes such as the production of tumour growth factor α, progesterone receptor, cathepsin D and pS2 protein [10, 16, 17]. It is at present a matter of speculation whether any of these changes has a direct or indirect effect on the outcome of parvovirus infection. Parvovirus replication shows an absolute requirement for the proliferation of host cells [5]. A parallel could be drawn in this work between the sensitisation of ER^+ mammary carcinoma cell lines to H-1 virus in the presence of oestradiol, and an increase in the growth rate of corresponding uninfected cultures. This correlation was especially clear for the T47D cell line, but also applied to a lesser extent to the faster-growing MCF-7 line in which the H-1-sensitising effect of oestradiol may be partly masked by the rapid outgrowth of surviving cells. Given the known dependence of parvovirus replication on cellular factor(s) expressed during the S phase [18], the growth-stimulating effect of oestradiol may contribute to the sensitisation of ER^+ cells to H-1 virus. Yet, the correlation observed between the hyperproliferation and H-1 hypersensitivity of oestradiol-treated cells may be coincidental, and the hormone may potentiate the virus cytopathic effect through other differentiation-related cellular changes as well.

Irrespective of the mechanism involved, the capacity of oestradiol for sensitising ER^+ neoplastic mammary cells to H-1 virus is reminiscent of the recently reported enhanced killing of transformed rat fibroblasts by parvovirus MVMp in the presence of thyroid hormone T3. It should also be stated that expression of the T3 receptor TRHα is upregulated by MVMp in these cells [19, 20], in contrast with the ER which failed to be induced by H-1 virus in the human mammary cell lines analysed in the present study (data not shown). TRHα belongs to the same steroid receptor superfamily, but not to the same group as ER [21] and is also considered a biochemical marker of differentiation [22]. Steroid hormone receptors are intracellular transcription factors existing as inactive apoproteins in the cytoplasm or nucleus. Upon interaction with their respective hormonal ligands, the receptors are activated and can bind to DNA elements (hormone response elements, HRE) and induce transcription of *cis*-linked genes. In the absence of ligand, THRα but not ER appears to bind to DNA and thereby to cause gene silencing [21]. The fact that the sensitivity of two different cell systems to parvoviruses is modulated by distinct steroid hormones known to control cell proliferation and differentiation points to the interrelation of the latter processes with cell permissiveness to parvovirus infection. Further studies are needed to unravel the molecular mechanism(s) by which the steroid hormone signalling pathways regulate the expression and/or activity of cellular factor(s) involved in the accomplishment of the parvovirus life-cycle and as a consequence of cell disturbances.

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