Kaposi’s Sarcoma-Like Tumors in a Human Herpesvirus 8 ORF74 Transgenic Mouse

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The product of human herpesvirus 8 (HHV-8) open reading frame 74 (ORF74) is related structurally and functionally to cellular chemokine receptors. ORF74 activates several cellular signaling pathways in the absence of added ligands, and NIH 3T3 cells expressing ORF74 are tumorigenic in nude mice. We have generated a line of transgenic (Tg) mice with ORF74 driven by the simian virus 40 early promoter. A minority (approximately 30%) of the Tg mice, including the founder, developed tumors resembling Kaposi’s sarcoma (KS) lesions, which occurred most typically on the tail or legs. The tumors were highly vascularized, had a spindle cell component, expressed VEGF-C mRNA, and contained a majority of CD31+ cells. CD31 and VEGF-C are typically expressed in KS. Tumors generally (but not always) occurred at single sites and most were relatively indolent, although several mice developed large visceral tumors. ORF74 was expressed in a minority of cells in the Tg tumors and in a few other tissues of mice with tumors; mice without tumors did not express detectable ORF74 in any tissues tested. Cell lines established from tumors expressed ORF74 in a majority of cells, expressed VEGF-C mRNA, and were tumorigenic in nude mice. The resultant tumors grew rapidly, metastasized, and continued to express ORF74. Cell lines established from these secondary tumors also expressed ORF74 and were tumorigenic. These data strongly suggest that ORF74 plays a role in the pathology of KS and confirm and extend previous findings on the tumorigenic potential of ORF74.

Human herpesvirus 8 (HHV-8), also known as Kaposi’s sarcoma (KS) herpesvirus, is the most recently identified human herpesvirus (7). Extensive epidemiological evidence points to a necessary role for HHV-8 in the etiology of KS (7, 14, 22, 26, 33, 34, 44, 51). KS is an angio proliferative disease, and KS lesions are rather complex; they contain spindle cells, which are thought to be of endothelial origin (23, 24), and infiltrating inflammatory cells surrounding vascular spaces. HHV-8 is found in most of the spindle cells in mature KS lesions (12, 49), and infection with HHV-8 precedes and predicts the onset of KS (14, 26, 34, 42, 51). HHV-8 is also implicated in the etiology of several lymphoproliferative diseases, including primary effusion lymphomas and multicentric Castleman’s disease (6, 37).

Although infection with HHV-8 is clearly necessary for development of KS, the mechanisms of HHV-8 pathogenesis are not well understood. HHV-8 appears to be a very inefficient pathogen; many more people are infected than develop KS (1, 2, 15, 29). In early-stage KS lesions, only a small fraction of the cells are infected (5, 12), and in late stages only a small minority of the spindle cells undergo lytic phase viral gene expression, even though most are infected (8, 10, 12, 48, 49). Infiltrating lymphocytes and monocytes can also be infected (5). Several lines of evidence suggest that HHV-8 pathogenesis in KS is mediated indirectly by paracrine factors produced by cells that undergo lytic phase viral replication but that act on uninfected or latently infected cells. Treatment with drugs that target lytic replication has been reported to result in regression of KS (19, 27, 30), although this has not been a universal finding. Cultured primary endothelial cells can be transformed and converted into spindle cells by infection with HHV-8 (9, 13, 35). Following transformation of dermal microvascular endothelial cells, all cells express the latency nuclear antigen LANA1 and a small minority of the cells spontaneously enters the lytic cycle (9). Interestingly, after transformation of bone marrow endothelial cells, only a small minority of the cells is infected at any given time (13), suggesting a paracrine mechanism of transformation.

If HHV-8 paracrine factors indeed play a role in KS pathogenesis, two groups of viral gene products may be involved. HHV-8 encodes several gene products related to secreted cellular proteins, including a homologue of interleukin-6 and three homologues of the β-chemokine macrophage inflammatory protein 1 (32, 38), which could act on uninfected or latently infected cells. The virus also encodes gene products with homology to cellular signal transduction proteins; these might induce expression of a variety of cytokines and related molecules. One of the latter gene products is related to the cellular chemokine receptor CXCR2 and is encoded by open reading frame 74 (ORF74) (3, 20). Indeed, ORF74 has been shown to spontaneously activate several intracellular signaling pathways, including phospholipase C–PI 3-kinase–AP1 and PI 3-kinase–Akt–NFκB (3, 4, 31, 36, 39, 46), to induce the synthesis of proinflammatory cytokines and chemokines (4, 39, 47) and to elicit chemotaxis of monocytes and lymphocytes (39). ORF74-
transfected NIH 3T3 cells form fibrosarcomas when injected into nude mice (4). Transgenic (Tg) mice with an ORF74 transgene regulated by the human CD2 promoter develop tumors closely resembling KS lesions (52). Interestingly, ORF74 expression in these Tg mice is restricted to T cells and NK cells and occurs in only a minority of cells in the tumors, indicating that tumorigenesis is likely caused by paracrine mechanisms. We describe a line of Tg mice in which ORF74 is expressed from the simian virus 40 (SV40) early promoter- enhancer and characterize the tumors observed in these mice, confirming and extending the results of Yang et al. (52). We also describe a tumorigenic cell line derived from one of the tumors.

**MATERIALS AND METHODS**

**Mice.** The ORF74 coding region was amplified by PCR from an ~14-kbp fragment of the HHV-8 genome contained in λEMBL3 (20) by using primers ORF74a1 (5'-cgaatgagatcttgatttgagctgg-3') and ORF74b1 (5'-cgcaagtgaacctgcgtggc-3'). The primers introduced cleavage sites for EcoRI and BglII (underlined) at the 5' and 3' ends, respectively, of the coding region. The initiation codon for ORF74 is shown in bold letters. The PCR fragment was digested with EcoRI and BglII and cloned into the cognate sites of the expression vector pSG5 (Strategene, La Jolla, Calif.), which contains an SV40 early promoter- enhancer and poly(A) signal and the second intron of rabbit β-globin. After amplification in Escherichia coli, the majority of bacterial plasmid sequences were removed by digestion with XbaI and NdeI and the 2.9-kbp insert containing ORF74 was gel purified and microinjected into fertilized one-cell C57BL/6J eggs as described by Lacy et al. (25). The experimental protocol was approved by the University of Maryland Biotechnology Institute Institutional Animal Care and Use Committee. Several weeks after pups were born, DNA was purified from 1- to 1.5-cm tail snips by overnight digestion at 55°C in proteinase K sodi um dodecyl sulfate, followed by phenol and phenol-chloroform extraction and ethanol precipitation. The presence of the transgene was investigated by PCR amplification using primers ORF74a1 and ORF74b1 and verified by Southern blotting using the full-length insert labeled with 32P by nick translation. Mouse β-globin primers SF144 (5'-caatacgctcaacagcagagggc-3') and SF145 (5'-cttgaggtgctggtcagctc-3') were used in PCRs to verify DNA integrity. Two males testing positive for transgene were obtained from one litter and bred with normal C57BL/6J females to obtain F1 transgenic mice. Athymic nude mice or non-Tg control littermates were used for tumor transplantation studies. Cells (generally 5 x 10^6) were injected intradermally into the side. In some experiments, naked DNA (10 μg) was injected intramuscularly in 0.2 ml of sterile phosphate-buffered saline (PBS) in a 25-gauge needle.

**Expression of the transgene.** RNA was purified from mice with RNAzol (Tel-Test, Friendswood, Tex.). Solid tissue was snap frozen in liquid N2 and ground in a mortar and pestle prior to RNA extraction. Transgene RNA expression was assayed by reverse transcription-PCR (RT-PCR) with primers 334 (5'-aagctgctgattgccgaag-3') and 332 (5'-tgctgcagcgggtctgacag-3'), which span a β-globin intron in the pSG5 vector. Consequently, they generate a PCR product of 628 bp from RNA, compared with a 1.2-kbp product from DNA. The size of the products on gels thus indicates whether amplification is from DNA or RNA. RNA was reverse transcribed into cDNA with Superscript II RNase H- reverse transcriptase (Invitrogen, Carlsbad, Calif.) using the antisense primer. PCR (35 cycles) was performed with Bio-X-ACT DNA polymerase (Bioline, London, U.K.) and M-MLV reverse transcriptase (Invitrogen, Carlsbad, Calif.) by using the antisense primer. The amplification products were deparaffinized in xylene, incubated in citrate buffer (10 mM, pH 6.0) for antigen retrieval by microwaving, preblocked with 10% normal goat serum-1% bovine serum albumin (HSA)-0.05% Tween 20 in PBS for 30 min, and probed with a 1:1,000 dilution of rabbit antipeptide polyclonal antiseraum against a KS herpesvirus ORF74 epitope between amino acids 4 and 16 ([Y]EDLTFLDDDES[G]) (8). Detection was with a biotinylated goat anti-rabbit immunoglobulin (lg) antibody followed by streptavidin-biotin complex and peroxidase system with diaminobenzidine chromagen development and a hematoxylin counterstain, as previously described (8). ORF74 expression was detected, using the same antibody, with adherent cells grown directly on microwell slides and suspension cells grown on polylysine-coated microscope slides and fixed in absolute methanol (8). CD31 surface antigen expression was analyzed by staining 4-μm slices from snap-frozen tumor sections (30). Sections were reacted to a mouse monoclonal antibody to CD31 (clone JC 70A; Dako, Glostrup, Denmark) or an irrelevant antibody as an isotype control, labeled with Texas red-conjugated goat anti-mouse IgG, and examined in an Axioskop microscope.

**Cell lines.** Cell lines were developed from tumors or from tail tissue from a normal mouse by exploiting unmixed pieces of skin or tumor tissue into PBS under sterile conditions. After three washes in PBS, the tissue was transferred to a 25-cm² tissue culture flask and maintained for 30 days in Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal bovine serum and 10 U of penicillin-streptomycin/ml at 37°C in 5% CO2. The medium was changed every 3 days. Cells migrating from the tissue and adhering to the plastic were collected by trypsinization (0.025% trypsin, 0.1 mM EDTA) and centrifugation. After two washes with PBS, the cells were suspended in medium at 5 cells/ml and 200-μl aliquots were transferred into U-bottomed microtiter plates. Wells with single cells were selected and cultured for 8 to 10 days, at which point the cells (now numbering ~30,000) were cultured in 24-cm² tissue culture flasks.

**RESULTS**

**Identification and characterization of ORF74 Tg mice.** Following microinjection of purified insert DNA into eggs and implantation into pseudopregnant females, seven pups were born from a single mother. Of the seven, two males (animals 383 and 379) tested positive for the presence of the transgene, as determined by PCR amplification of tail snip DNA. This was confirmed by Southern blotting (Fig. 1) after digestion with EcoRI, which cuts within the transgene once. The presence of a fragment of the same apparent size (2.9 kbp) as the insert DNA suggests that the integrated transgene consisted of several tandem copies. The larger fragment (5.1 kbp) likely includes the junction of the transgene with the host genome. By 7 months of age, mouse 383 developed tumors on the tail and on the foot (Fig. 2A and B). Animal 379 did not develop detectable tumors and died of apparently unrelated causes at the age of 19 months.

Both mice were bred with normal females to produce F1 transgenic animals. Out of a total of 58 offspring from animal 383, 20 tested positive by PCR and Southern blotting or by PCR alone. Five of the transgene-positive animals developed tumors like those of the founder on their tails or feet, generally at more than 1 year of age (although one occurred at 5 months). Two of these animals also had internal tumors in the abdominal cavity. One animal without any external tumors also developed a similar internal tumor at more than a year of age. One of the animals (animal 116) with a tail tumor was mated to produce an F2 generation. Of 15 pups, 5 tested positive for the presence of the transgene. One developed two tumors on the tail and a large tumor behind the ear at 7 months of age. Another developed a tumor on the foot and an internal ab-
dominal tumor at 1 year of age. This animal (mouse 2840) was mated and produced three Tg pups out of a total of nine. One of these developed an internal tumor at the age of 6 months; the other two remained free of tumors at 9 months. Of 18 offspring from animal 379, 7 tested positive for the presence of the transgene. None developed the characteristic tail and foot tumors seen with animals from the 383 line. These data are summarized in Table 1. 

The violaceous nodular tumors on the tail (Fig. 2A) and foot (Fig. 2B) were firm and about 0.5 to 1 cm in diameter and were reminiscent of KS in humans. The black spot on the tumor surface in Fig. 2A corresponds to a hemorrhagic crust over an ulceration. Histological examination revealed that cells within the tumors were arranged in a cord-like pattern and were partly spindle shaped (Fig. 2D). The vast majority of these cells tested positive by immunofluorescent staining for the presence of CD31 (Fig. 2E), an endothelial cell marker that is expressed on spindle cells in human KS lesions (45, 50).

Expression of ORF74 in Tg tumors. RNA was purified from tumors and other tissues from Tg mice and RT-PCR was used to detect expression of transcripts from the ORF74 transgene, using the primers described in Materials and Methods. These primers bind on opposite sides of the β-globin intron of the transgene, as illustrated by the arrowheads in Fig. 1B; consequently, DNA is amplified as a 1.2-kbp fragment that can be discriminated from the 628-bp fragment generated from RNA. As shown in Fig. 3A, lane 1, a 628-bp fragment was readily detected in tumor tissue from mouse 470 as well as in tumors from five of five other mice (data not shown). No 1.2-kbp fragment was evident, indicating that the DNA corresponding to that fragment size was not present. When the RT step was omitted, there were no detectable products (data not shown), further establishing that the detected signals were from RNA. ORF74 RNA was also detected in apparently normal tissue (leg) of mouse 470 (Fig. 3A, lane 2) but was undetectable in three other tissue samples (Fig. 3A, lanes 3 to 5). In another Tg mouse with tumors (animal 2840), ORF74 RNA was detected in three of three apparently normal tissue samples (data not shown). In contrast, ORF74 transcripts were not detectable in four of four tissue samples from a Tg mouse with no tumors (animal 2830) (Fig. 3A, lanes 6 to 9) or in leg muscle tissue from three other Tg mice without tumors (data not shown). The lower bands in Fig. 3A are from RT-PCRs with 18S RNA primers that were preloaded onto the gel as a control for RNA integrity.

Expression of ORF74 protein was clearly evident in a minority of the cells in tumors. Figure 4 shows relatively high levels of expression in a few cells in an external tumor from behind the ear, as detected by immunostaining. Other cells expressed lower levels of ORF74, while many cells tested negative. As can be seen, the tumor contained areas composed of spindle cells mixed with areas that did not contain spindle cells. Figure 4A shows expression of ORF74 protein in cells both within the spindle cell areas and outside them. Figure 4B, taken at a higher magnification, shows expression of ORF74 in the cytoplasm of spindle cells. Figure 4C, taken at the same magnification, shows expression in the cytoplasm of nonspindle cells.

VEGF-C mRNA was expressed in tumor tissues (Fig. 3B, lanes 1 and 2) and in apparently normal tissues expressing ORF74 from a Tg mouse with a tumor (Fig. 3B, lane 3), which is consistent with an earlier report that HHV-8 infection of endothelial cells induces VEGF-C protein expression (28) and modestly upregulates VEGF-C mRNA expression in endothelial cells (40). VEGF-C RNA was not detected in other tissues not expressing ORF74 from the same Tg mouse (Fig. 3B, lane 5), tissue from a Tg mouse with no tumors (Fig. 3B, lane 4), or tissue from a normal mouse (Fig. 3B, lane 6). Transfection of mouse fibroblasts with ORF74 did not induce expression of VEGF-C mRNA (data not shown), indicating that expression of VEGF-C may be secondary to tumorigenesis in the mouse rather than directly induced by ORF74. VEGF-A protein expression is also induced by infection with HHV-8 (28). VEGF-A mRNA was detected in samples of tissues expressing ORF74 but was also detectable in other samples not expressing ORF74 (data not shown). RNA quality was verified for all tested samples by amplification with 18S RNA primers.

Establishment of tumorigenic cell lines from Tg mice. Clonal cell lines were developed from a tumor from ORF74 Tg mouse 116 (GR1 cell line) and from tail tissue of a normal (non-Tg) mouse (GR5) (Table 2) as described in Materials and Methods. The ORF74 transgene in Tg mice. (A) DNA was prepared from the following: a tail snap from founder mouse 383 (lane a); a tail snap from a non-transgenic littermate (lane b); cell lines GR3 (lane c) and GR4 (lane d), derived from serially transplanted tumors of nude mice (Table 2); cell line GR1 (lane e), derived from a Tg mouse primary tumor; or cell line GR5 (lane f), derived from a non-Tg mouse. DNA was digested with EcoRI and analyzed by Southern blotting, as described in Materials and Methods, with a probe consisting of the SV40 promoter, poly(A) signal, and β-globin intron are represented as boxes. The small arrowheads indicate the positions of the primers used for RT-PCR assays of the transgene.
Methods. The tumorigenic potential of the Tg tumor-derived cell line, GR1, was evaluated by injecting $5 \times 10^6$ cells into 10 nude mice, all of which rapidly developed tumors. As shown in Fig. 2C, aggressive tumors appeared within 3 weeks of inoculation. The tumors showed extensive ulceration with a darkened appearance. The tumors in nude mice tended to metastasize to other sites and rapidly killed the animals. As with the primary tumors in the Tg mice, ORF74 and VEGF-C mRNA were expressed at the primary site of inoculation as well as in a few other tissues (data not shown). GR1 cells were also moderately tumorigenic in immunocompetent mice; one of four non-Tg normal littermates developed a rapidly growing tumor within 2 weeks after similar injections. No tumors were obtained by similar inoculation of the control cell line, GR5, into 10 nude mice. However, GR5 cells transfected with ORF/pSG5 caused tumors within 10 days. A cell line derived from this tumor, GR6, was also tumorigenic in nude mice (Table 2).

TABLE 1. Tumor occurrence in ORF74 transgenic mice

<table>
<thead>
<tr>
<th>Generation (parental mouse no.)</th>
<th>No. of Tg pups (total no.)</th>
<th>No. of mice with tumors</th>
<th>Location of tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>F₁ (383)</td>
<td>20 (58)</td>
<td>6</td>
<td>Tail or foot (5), internal (3)</td>
</tr>
<tr>
<td>F₂ (116)</td>
<td>5 (15)</td>
<td>2</td>
<td>Tail or foot (2), ear (1), internal (1)</td>
</tr>
<tr>
<td>F₃ (2840)</td>
<td>3 (9)</td>
<td>1</td>
<td>Internal (1)</td>
</tr>
<tr>
<td>F₁ (379)</td>
<td>7 (18)</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

*F₁, F₂, and F₃ generations are indicated. Numbers in parentheses are the parental Tg mouse identification numbers. Mouse 383 was the founder and father of mouse 116; mouse 116 was the father of mouse 2840. Mouse 383 had tail and foot tumors, mouse 116 had a tail tumor, and mouse 2840 had a foot tumor and an abdominal tumor.

Numbers indicate the number of Tg pups compared to the total number born.

Numbers indicate the number of Tg mice developing tumors at the locations described.

Two F₂ mice (descended from parental mouse 383) and one F₃ mouse (descended from parental mouse 116) had both internal and surface tumors.
Cell lines were derived from the tumors originating from transplanted GR1 cells. These were serially passaged as tumors through nude mice 2362, 2363, and 2364, from which cells lines GR2, GR3, and GR4, respectively, were derived (Table 2). All four cell lines retained tumorigenicity in nude mice. Southern blots of EcoRI digests of DNA from cell lines GR1, GR3, and GR4 showed the same pattern of hybridization to an ORF74 probe as did a digest of DNA from the founder mouse (Fig. 1; compare lane a with lanes d, e, and f), indicating that the transgene remained grossly intact. These data are summarized in Table 2. A PCR product from the transgene from one of the cell lines was sequenced and found to be identical to the original insert used for the transgene.

ORF74 mRNA was expressed in the GR1 cell line, as shown by RT-PCR (Fig. 3A, lanes 10 and 11) and Northern blotting (not shown). ORF74 protein was detected by immunostaining in the majority of cells in the cell lines (Fig. 5C) but not in normal controls (Fig. 5B). Uninduced BCBL-1 cells are shown for comparison (Fig. 5A); a few BCBL-1 cells, presumably undergoing spontaneous lytic phase viral replication, gave highly positive results, while the majority of cells gave slightly positive or negative results. Tumor cell lines GR1 and GR3 but not the normal GR5 cell line tested positive for VEGF-C mRNA by RT-PCR (Fig. 3B, lanes 7 to 9). VEGF-C mRNA levels in GR3 cells were almost 10-fold higher than in GR5 cells, as judged by real time RT-PCR, whereas VEGF-A mRNA levels were approximately the same (Fig. 5D).

**DISCUSSION**

We have constructed a Tg mouse in which ORF74, a chemokine receptor homologue encoded by HHV-8, is expressed from an SV40 early promoter. One of two founder animals developed tumors on the tail and foot. A minority of second- and third-generation animals developed similar tumors, generally (but not always) at more than 1 year of age. In some cases, internal tumors associated with viscera also occurred in the abdominal cavity. Similar tumors have been reported in Tg mice in which ORF74 expression is regulated by a T-cell-specific promoter (52). The tumors were highly angiogenic and tested positive for the presence of CD31, an endothelial cell-specific marker typically present on spindle cells in KS lesions in humans (45, 50). Tumors also tested positive for the presence of VEGF-C, which is expressed in KS lesions (45), although the expression was likely characteristic of the tumor type rather than directly induced by ORF74.

The pattern of transgene expression is interesting. We did not detect ORF74 mRNA in tissues from transgenic animals without tumors. We infer that the SV40 promoter is generally silenced in these animals. This would be expected if expression of the transgene in utero was lethal. A protein such as ORF74, capable of constitutively activating multiple signaling pathways, would be likely to interfere with embryogenesis, and transgenic embryos would reach term only if expression were repressed. In the case of transgenic animals with tumors, ORF74 transcripts were readily detected in the tumors and in some tissues without tumors. However, they were not detectable in other tissues even by relatively sensitive RT-PCR. How ORF74 expression becomes activated is not clear, but infrequent stochastic events may lead to activation of the promoter. Cells that express ORF74 and survive may become tumorigenic following further events or may be able to cause tumors directly. Alternatively, tumorigenesis may follow the genera-

**TABLE 2. Characteristics of cell lines derived from ORF74 transgenic mouse tumors**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Derivation</th>
<th>Tumorigenicity</th>
<th>ORF74 DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR1 (116)</td>
<td>Tail tumor of Tg mouse 116</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GR2 (2362)</td>
<td>Tumor in nude mouse injected with GR1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GR3 (2363)</td>
<td>Tumor in nude mouse injected with GR2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GR4 (2364)</td>
<td>Tumor in nude mouse injected with GR3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GR5 (2380)</td>
<td>Normal tail of normal mouse</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>GR6 (164)</td>
<td>Tumor of nude mouse injected with GR5 and transfected with ORF74/pSG5</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

*The numbers in parentheses designate the mice from which the cell lines were derived.

*b Cell lines originated from tumors or tail explants as indicated.

*c, cells formed tumors in nude mice; –, cells did not form tumors in nude mice.

*d, +, Tg DNA was detected by PCR and Southern blot assays; –, Tg DNA was not detected.
FIG. 4. Expression of ORF74 protein in Tg tumors. Sections from a KS-like tumor behind the ear were analyzed by immunostaining with a rabbit polyclonal antipeptide antiserum to ORF74 as described in Materials and Methods. (A) ORF74 expression both inside and outside areas containing spindle cells (magnification, ×25). (B) Expression in cytoplasm of spindle cell (magnification, ×40). (C) Expression in cytoplasmic area of a nonspindle cell (magnification, ×40).
tion of a critical mass of cells expressing ORF74. Expression of ORF74 RNA in apparently normal tissue may represent activation of expression in normal cells that in time would result in tumor formation or it may represent cells that are metastatic from the primary tumor but have not yet formed tumors. There appears to be some advantage for the generation of tumors on the extremities (tail, leg), although such an advantage appears not to be absolute.

Only a small minority of cells expresses ORF74 in the KS-like tumors in Tg mice reported by Yang et al. (52). Expression in these Tg mice was limited to T cells, suggesting that T cells were driving tumorigenesis by paracrine mechanisms affecting endothelial cells not expressing the transgene. We also find that ORF74 is highly expressed in a small minority of tumor cells, as evidenced by immunohistochemistry. Our interpretation regarding transgene expression in our mice is that random activation and persistent expression led to the proliferation of some of these cells and subsequently to formation of tumors, either by further proliferation of the cells expressing ORF74 or by a paracrine effect on other cells. The scattered pattern of expression of ORF74 in the tumor suggests that the latter interpretation is more likely to be correct.

Clonal cell lines from these tumors were highly tumorigenic in nude mice and to a lesser degree in normal mice. ORF74 was expressed in the majority of cells in these clonal lines, suggesting that some of the tumor cells expressing ORF74 maintain stable expression. The transplant tumors could be serially transplanted without loss of malignancy and often metastasized to distant sites, suggesting that they evolved to a more malignant phenotype than the primary tumors. This may be comparable to the course of KS in humans, in which KS lesions begin as sites of inflammatory hyperproliferation but in some cases appear to progress to clonal malignancies (11, 18, 41).

What is the mechanism of tumorigenesis caused by ORF74? Bais et al. and others have shown that ORF74 induces the expression of proinflammatory cytokines and chemokines (4, 39, 47) and cell adhesion molecules (39) secondary to its activation of transcriptional factor signaling pathways such as NFkB. Furthermore, supernatants from endothelial cells ex-
expressing ORF74 activate NFκB in endothelial cells not expressing ORF74 (39), suggesting that its effects may be amplified in vivo by paracrine mechanisms. A recent study by Holst et al. (21) showed that transgenic mice expressing ORF74 mutants that fail to signal constitutively do not develop KS-like lesions. ORF74 signaling is modulated by several chemokines (16, 17, 43), most notably Groα (an agonist) and IP-10 (an inverse agonist). Interestingly, mice that are transgenic for ORF74 mutants that can signal constitutively but are refractory to regulation by exogenous chemokines also generally fail to develop KS-like lesions (21). This suggests that cellular factors, whose expression may be dysregulated by ORF74, play an important role in KS pathogenesis.

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