Retinoblastoma (RB) Gene Structure and Function in Primary Canine Osteosarcoma Cell Lines

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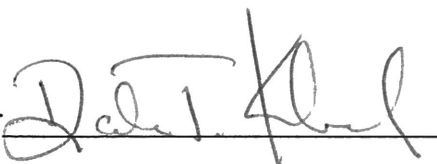
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Summary

Tumor suppressor genes are an important factor in the transformation of normal to cancerous cells. The retinoblastoma susceptibility gene (Rb), when deleted, removes an important cell growth negative regulator and thus subjects the cell to continuous DNA replication. The objectives in this study are to establish canine osteosarcoma cell lines from primary explants and to compare their Rb gene expression to positive and negative human and canine cell line controls via immunoblotting and Northern analysis. The gene itself will also be examined using Southern analysis. Immunoblotting with two different anti-human Rb antibodies yielded no cross-reactivity with canine proteins. Attempts to optimize the heterologous recognition of canine proteins were not successful. Experiments are planned to develop specific anti-canine Rb reagents for characterization of canine samples. Initial Northern analyses of canine Rb expression utilizing a human cDNA were inconclusive and will require further optimization for proper interpretation. The human Rb cDNA has previously been used to describe canine Rb restriction fragment length polymorphisms (RFLPs) in genomic DNA extracted from canine tumors. Optimal Southern analysis of Rb RFLPs in primary canine osteosarcoma cell lines is in progress but has not been completed.

Introduction

Oncogenes and Tumor Suppressor Genes

Cancerous cell lines have several characteristics that are reflective of their altered phenotype. One prominent feature is the loss of contact inhibition. Normal cells discontinue growth upon completion of a confluent monolayer, but the loss of this regulation allows cancer

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cells to aggregate and eventually create a tumor. A second characteristic is the ability to grow in semi-solid media, indicative of the potential of the tumor cells to metastasize via a hematogenous route. Last, cancerous cells will form solid tumors when injected into immunodeficient nude mice.

The genesis of a cancerous phenotype involves much change in a normal cell. Conversion of a normal cell to a tumor cell is called transformation, and may involve two types of genes. Oncogenes, when activated, participate in conversion of a normal cell to a malignant one. This type of gene can act in a heterozygous state by producing abundant, active, or mutant protein. A second type of gene is called a tumor suppressor gene or a recessive oncogene. The loss of function of this gene is associated with the emergence of cancerous behavior in a cell. The retinoblastoma susceptibility gene (Rb) is the tumor suppressor gene addressed in this study.

Retinoblastoma and Osteosarcoma

Retinoblastoma is a rare cancer found in retinoblasts, the precursors of retinal cells. It occurs at a frequency of 1 in 20,000 births (McFall). This disease in humans presents itself in two forms. One third of the cases are the familial form, which usually presents itself as multifocal tumors in both eyes and requires that one faulty Rb allele be inherited from one parent. Incidentally, the "recessive" title of the Rb gene is questionable, as inheritance of one defective allele leads to a high frequency of the second, somatic, mutation. After these individuals are treated for retinoblastoma lesions, they are more likely to suffer secondary tumors because all the cells in the individual already have at least one impaired Rb gene. The sporadic form makes up the other two-thirds of retinoblastoma cases and is usually only found

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as one tumor in one eye; these tumors originate when both Rb alleles suffer alterations as separate somatic events. Loss of Rb gene function in tumors other than Rb include osteosarcoma, small cell lung cancers, some breast carcinomas, and some bladder cancers (Marshall).

The retinoblastoma susceptibility gene was first detected in 1978 when Francke noted the absence of the 13q14.1 somatic band of some retinoblastoma tumors. (Francke). This finding was pursued by Friend in 1986 when he detected a homozygous deletion in two retinoblastomas (Friend). Several recessive oncogenes, or tumor suppressor genes, have been found since 1978, including p53, WT1, and Rb (Marshall). When Friend cloned the Rb gene in 1986, he observed that it was a large gene with twenty-seven exons in 200 kb of DNA. The resulting 4.7 kb mRNA product encodes a 110 kd nuclear protein, whose function is the regulation of DNA synthesis. The Rb gene product oscillates between hypophosphorylated and phosphorylated forms. Levine states that Rb molecular weight ranges from 105 to 110 kd, depending on phosphorylation. Activation is achieved by hypophosphorylation of p110RB following the mitotic phase of the cell cycle. Once activated, p110RB binds DNA to prevent commencement of a new cell cycle. Phosphorylation and inactivation of p110RB coincides with dissociation of the protein from DNA during the synthetic phase of the cell cycle. Hence p110RB plays an essential role in cell cycle regulation; without this protein, DNA would replicate continuously (Marshall).

Cells transformed *in vitro* by DNA tumor viruses display a lack of cell cycle regulation attributed to binding and functional blockade of p110RB action. Transforming proteins from viruses such as adenovirus, SV40, polyoma virus BK, and human papilloma virus display similar

p110RB binding sequences (Green). Combined with other insults to the genome, the altered Rb tumor suppressor gene may contribute to tumor formation.

As mentioned above, individuals suffering from the familial form of retinoblastoma are likely to have secondary neoplasms, most commonly osteosarcoma (OS). Some osteosarcoma tumors display a deletion at the Rb locus. This information leads one to hypothesize that some OS cases originate by the same mechanism as retinoblastoma (Cavenee).

Comparative Features of Canine Osteosarcoma

The most commonly occurring skeletal tumor in dogs is osteosarcoma, with a frequency of 7 in 100,000 canines a year. Incidence of canine osteosarcoma and its human counterpart has been associated with regions of increased mutation in the tumor suppressor retinoblastoma (Rb) susceptibility gene. This finding in dogs was a result of Southern blot analysis of clinical samples of OS tumor DNA compared to DNA from normal canine tissues. RB deletions were observed in human tumor tissue positive controls and in canine tumor tissue, but not in normal canine samples. Table 1 shows other similarities between canine and human osteosarcoma. Frequency of the disease is higher among the canine population, and Southern analysis shows that Rb deletions are present in a higher percentage of canine OS samples (90%) than in human OS samples (35-40%) (Personal communication). Our goal is to extend the usefulness of the canine model to the molecular level by establishing and characterizing primary canine OS cell lines. Manipulation of Rb in these cells will form the basis for long-term studies of Rb function *in vivo* in the canine. We hope to eventually utilize the canine as a comparative therapeutic model for gene therapy in animals and people.

Table 1. Comparative features of human and canine osteosarcoma.

	Human	Canine
Incidence	Annual rate of 1.1/100,000	Annual rate of 7.9/100,000
Age	Bimodal peaks; major peak 10—25 years; minor peak 50—60 years (superimposed on Paget's disease)	Major peak 6—9 years (40—52 years human equivalent) with a minor peak at 1—2 years (12—14 years human equivalent)
Genetic factors	Occasional familial tendencies. Higher incidence as a second tumor in retinoblastoma patients	Familial tendencies in certain large and giant breeds
Site	75% in long bones	77% in long bones
Clinical findings	Pain and swelling. Rapidly progressive with early lung metastases	Same
Radiological features	Aggressive bone lesion including osteolytic and osteoblastic changes	Same
Histopathological features	Presence of sarcomatous tumor and direct formation of tumor osteoid	Same
Therapy	Surgical excision and chemotherapy	Surgical excision

Specific Objectives

1) To establish at least two canine osteosarcoma (OS) cell lines from primary clinical samples using basic tissue culture techniques.

2) To determine if deletions of the Rb gene exist in the primary cell lines and in commercially available cell lines using Southern analysis.

3) To characterize expression of Rb mRNA and protein in all cell lines using Northern analysis and immunoblotting techniques.

Materials and methods

Cell lines and reagents

In order to provide a basis for comparative analysis, a human fetal fibroblast cell line (HEPM) and canine renal cell line (MDCK) were used as positive controls. Human retinoblastoma cell lines Y79 and WERI-Rb which had partial and complete Rb deletion, respectively, were used as negative controls. Four primary osteosarcoma cell lines were established in the fall of 1993. OS1093B and OS1093L were taken from the same seven-year-old German Shepherd and established from the bone tumor and metastasis to the lung, respectively. OSCJ93L was explanted from the lung of a twelve-year-old German Shepherd mix. OS1027B was taken from bone tissue of a one-year-old Mastiff. OS2.4 was a previously established primary osteosarcoma cell line, and D17 is a commercial canine osteosarcoma cell line. The following cell lines were obtained from American Type Culture Collection (Rockville, MD): D17 (CRL #6248), MDCK (CRL #6253), Y79 (HTB #18), WERI-Rb (HTB #169), and CCRF-CEM (CCL #19). HEPM (CRL #1486) cells were a gift from Dr. Robert Burghardt.

Retinoblastoma cDNA was obtained from Weinburg, Friend, and Dryja at the Massachusetts Eye and Ear Infirmary as the 3.8R plasmid in the HB-101 strain of *E. coli*. Antibodies were obtained from Oncogene Science (mouse anti-recombinant Rb Ab-4), Pharmingen (mouse anti-human Rb 14001A), and BioRad (goat anti-mouse alkaline phosphatase conjugated secondary antibody). An alkaline phosphatase assay kit was obtained from Sigma (#104-LS).

Cell culture

Cell types and their culture conditions are described in Table 2.

Table 2. Cell lines and growth conditions.

Cell Line	Base Media	% FBS	Description
HEPM	DMEM	5	Human fibroblast, positive control
MDCK	MEM	10	Canine kidney, positive control
Y79	RPMI	15	Human retinoblastoma, negative control
WERI-Rb1	RPMI	15	Human retinoblastoma, negative control
CCRF-CEM	RPMI + 8-Azaguanine	10	Human leukemia, negative control
D17	MEM	10	Canine osteosarcoma
OS2.4	DMEM	10	Canine osteosarcoma, primary
OSCJ93	DMEM	20	Canine osteosarcoma, primary
OS1093B	DMEM	20	Canine osteosarcoma, primary
OS1093L	DMEM	20	Canine osteosarcoma, primary
OS1027B	DMEM	20	Canine osteosarcoma, primary

All cells were grown in 5% CO_2, 95% O_2 at 37° C. Primary cultures of canine osteosarcomas were established from explants as described (Freshney). Briefly, tumor tissue was excised from animal and placed into DMEM media containing 10% fetal bovine serum and 1X antibiotic. In a sterile environment, tumor tissue was minced finely and allowed to incubate

at 37° in the same media. Non-adherent and/or poorly dispersed tissue pieces were removed from the dish 24 hours after initiation of culture. Cells were maintained as described above.

Extraction of Protein

Cells were harvested by trypsin/EDTA digestion, rinsed, centrifuged for 5 minutes at 800 g, and resuspended in tumor solubilization buffer (50 mM Tris-Cl, 50 mM KCl, 5 mM EGTA, 5 mM $MgCl_2$, 2% CHAPS, 0.1 mM Leupeptin, 0.2 mM PMSF, 10 mM DTT). Samples were then centrifuged at 800 g for five minutes and the supernatants were collected. Protein from these lysates were quantified using the Modified Lowry technique (Ausebel).

Characterization of Primary Samples

The enzyme alkaline phosphatase was used as a marker for bone-derived cells in order to characterize the four primary cell lines and previously established OS2.4. D17 canine osteosarcoma cells were used as a positive control for the alkaline phosphatase assay (Sigma #104-LS), and CCRF-CEM human leukemia cells were used as a negative control. Cell lysates were added to an alkaline phosphatase substrate and spectrophotometrically quantitated for enzyme activity. $Absorbance_{410}$ values were converted to Sigma Units/ml by comparison to Calibration Curve 1.

Calibration Curve One. Correlation between A_{410} of sample and serum alkaline phosphatase.

Alkaline Phosphatase Calibration Curve
March 1, 1994 by Julia Warren

$$y = -3.3737e\text{-}3 + 7.7568e\text{-}2x \quad R^2 = 0.999$$

Absorbance (410 nm) vs. Serum Alkaline Phosphatase (Sigma Units/mL)
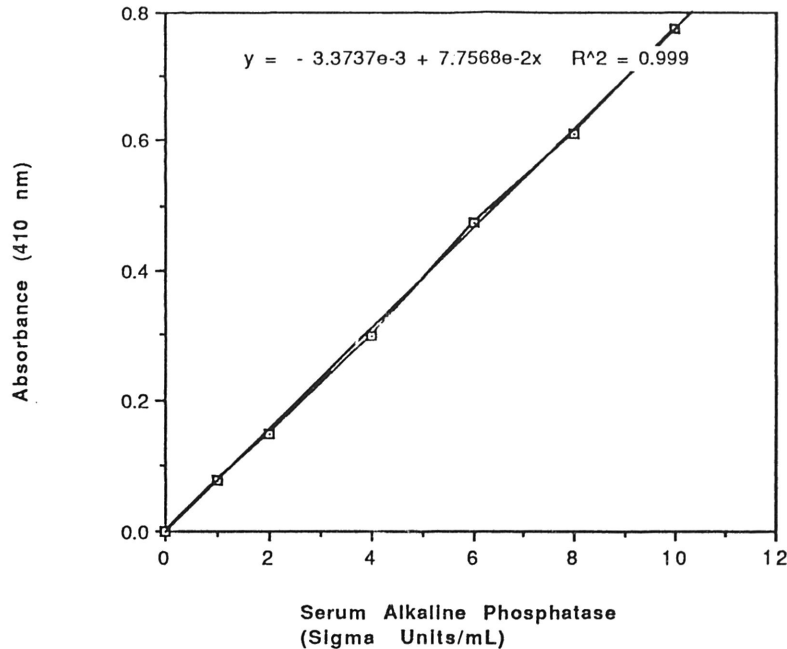
Immunoblotting of SDS-PAGE gel

Cell protein lysates were electrophoresed in an 8% polyacrylamide gel at 30V overnight and subsequently transferred to Immobilon-P Membrane (Millipore) at 100mA overnight. When transfer was complete, the membrane was blocked overnight in Blotto (50 mM Tris-HCl, 2 mM $CaCl_2$, 80 mM NaCl, 5% Carnation nonfat dry milk, 0.2% Nonidet P-40, 0.03% sodium azide). The primary antibody (anti-Rb Ab-4) was added in indicated concentrations, respectively, and allowed to incubate overnight. After three rinses with fresh Blotto, the alkaline phosphatase-conjugated blotting grade affinity purified goat anti-mouse IgG (H+L) secondary antibody (BioRad) was applied to each panel at a concentration of 1:3000 (3.33×10^{-4} ug/ml) and allowed to incubate for two hours at room temperature. The membranes were then washed with Buffer A (50 mM Tris-HCl, 2 mM $CaCl_2$, 80 mM NaCl), and allowed to develop with BioRad alkaline

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phosphatase conjugate substrate solution overnight. An alternative anti-Rb primary antibody (anti-Rb 14001A) was also examined in the above fashion.

RNA Extraction

Total RNA was extracted as described (Liu, Batt, and Carmichael) from all cell lines Briefly, cell monolayers were rinsed and lysed with Solution A (10mM EDTA, pH 8.0, 1% SDS). Cell lysate was collected, 100 ug of Proteinase K was added, and the sample was allowed to incubate at 45° C for 30 minutes. Solution B (10 mM EDTA, pH 8.0, 0.1 M sodium acetate) and water-saturated phenol were used to extract the RNA. RNA was precipitated with 5M NaCl and ethanol and resuspended in DEPC-treated water. Extracted RNA was diluted 1:20 in 0.5% SDS in DEPC-treated water and observed spectrophotometrically at 260 and 280 nm. The 260/280 ratio should be between 1.6 and 2.0 for acceptable purity. Quantity was determined by the following calculations (Ausebel):

ug RNA = (A_{260})(extinction coefficient)(dilution factor)(sample volume in ml.)

extinction coefficient = 40 ug/ml

dilution factor = 20 (5 ul/100 ul)

sample volume in ml = 100 ul - 5 ul = 0.095 ml

DNA Extraction

Genomic DNA was extracted from commercial as well as primary cell lines as described (Ausebel). Cells were harvested by trypsin/EDTA digestion, rinsed, centrifuged at 800g for 5 minutes and resuspended in digestion/lysis buffer STE (100 mM NaCl, 10

mM Tris-Cl, pH 7.5, 1 mM EDTA). Proteinase K (100 ug/ml) and 0.1% SDS were also added

before the sample was allowed to incubate at 55°C overnight. Phenol, phenol/chloroform

isoamyl alcohol (25:24:1), and chloroform isoamyl alcohol (24:1) extractions followed. Samples

were then ethanol precipitated and dissolved in TE buffer (10 mM TRIS-Cl, pH 8.0, 1 mM

EDTA). DNA was spectrophotometrically quantitated as described above, except extinction

coefficient is 50 ug/ml (Ausebel).

Probe Labelling

Probe was labelled using the Random Primers kit (Boehringer Mannheim). 3.8R human

cDNA, which hybridizes to the 3' end of the human Rb gene, was labelled using the BRL

Random Primers DNA Labeling System. 100 ng of DNA was added to 1x TE to a final volume

of 23 ul and denatured in a boiling water bath. 2 ul each of dATP, dTTP, and dGTP, 15 ul

Random Primers Buffer Mixture, and 5 ul of [alpha-^{32}P]dCTP were added on ice and mixed

thoroughly. 1 ul Klenow fragment was added and the mixture was allowed to incubate at room

temperature for one hour. 50 ul stop buffer was added and the mixture was then separated using

filtration and centrifugation. 2 ul of labelled probe were removed and counted.

Blotting of DNA and RNA

DNA samples were digested simultaneously with EcoRI and PstI before being

eletrophoresed on a 1% agarose gel at 70V for five hours. RNA samples were electrophoresed

on a 1% agarose formaldehyde gel at 50 V for four hours. Gels were transferred overnight to

GeneScreen Nylon membranes (NEN Research Products), pre-hybidized with Rapid Hyb buffer

(Amersham Life Science) at 65°C for fifteen minutes, and probed with 1×10^6 cpm [alpha-^{32}P]-labelled 3.8R probe (specific activity of 2.8×10^{12}) for two hours. Three low stringency washes followed. DNA membranes were washed with a 2X SSC, 0.1% SDS solution once for ten minutes at room temperature, and twice with the same solution for twenty-five minutes at 65°C. RNA membranes were treated with a 1X SSC, 0.1% SDS solution once for fifteen minutes at room temperature and twice with the same solution for thirty minutes. The membranes were placed in heat-sealed bags and exposed to X-ray film for 48 hours.

Results

Hallmarks of transformed phenotype

As mentioned above, the transformed phenotype is rather unique, with the most pronounced characteristic being loss of contact inhibition. Figure 1 shows a photomicrograph of canine osteosarcoma (D17) cells in culture. Upon exhaustion of culture surface area, normal cells stop dividing. Transformed cells, as seen in this figure, have lost this form of growth regulation.

Characterization of primary cell lines

Alkaline phosphatase is an enzyme that is present in high amounts in hepatic, renal, and bone-derived tissue. Since osteosarcoma is a bone tumor, it should express this protein at a significantly higher level than fibroblasts or other cancers we might have cultured from our primary tumor explants. The results of the alkaline phosphatase assay are shown in Table 3.

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Table 3. Alkaline Phosphatase assay results.

Cell line	Role	Activity/cell (Sigma Units/ml)
CCRF-CEM	Negative control	0.000
D17	Positive control	6.097×10^{-8}
OS2.4	Canine Osteosarcoma, primary	2.392×10^{-7}
OSCJ93L	Canine Osteosarcoma, primary	2.185×10^{-8}
OS1093L	Canine Osteosarcoma, primary	0.000
OS1093B	Canine Osteosarcoma, primary	0.000
OS1027	Canine Osteosarcoma, primary	2.296×10^{-7}

Detection of the Rb protein

Using immunoblotting techniques, we assessed the presence of Rb protein (p110RB) in canine and human cell lines. Figure 2 shows immunoblots of three dilutions of primary antibody with HEPM and MDCK lysates (25 ug protein/lane). At all dilutions, anti-RB Ab-4 (Oncogene Science, see Materials and methods) recognized proteins of the proper molecular weight in the human sample, but no canine proteins were detected. To increase the possibility of detection of canine protein, a second gel with increased protein loads was prepared. Figure 3 shows three panels in which 25, 50, and 100 ug of both HEPM and MDCK lysates were detected with 1ug/ml, 0.5 ug/ml, and 0.1 ug/ml of anti-RB Ab-4, respectively. Again signal was only observed for the human RB protein. A second antibody, anti-Rb 14001A (Pharmingen, see Materials and methods) was tested for canine cross-reactivity. Figure 4 shows an immunoblot with ascending concentrations of 25, 50, and 100 ug of HEPM and MDCK protein lysates that was incubated with anti-Rb 14001A (2 ug/ml). Note there was no signal at all. To guarantee the quality of the antibody, we repeated the blot using fresh primary antibody along with a lysate of human leukemia cells (Molt 4) that served as a positive control. Figure 5 shows dot blots of 50 ug each of Molt 4, HEPM, and MDCK detected with anti-Rb Ab-4, an anti-heatshock protein

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70 as a positive control, and two dilutions of anti-Rb 14001A. The anti-Rb 14001A antibody detected the positive control, so an SDS-PAGE gel was prepared. Figure 6 shows panels loaded with 100 ug each of lysate from HEPM, MDCK, and Molt 4. India ink staining showed that relatively equal amounts of protein in each sample were loaded and that the transfer was efficient. The immunoblot showed that while the Pharmingen antibody did indeed detect one positive control, Molt 4 lysate, but it did not detect the human lysate (HEPM) or the canine cell line (MDCK).

Detection of the Rb message

Expression of the Rb gene can be evaluated by measuring the presence and size of the Rb mRNA message. Northern analysis was the technique chosen for this task. Figure 7 shows extracted RNA after it was separated on a formaldehyde/agarose gel and transferred overnight. Note the presence of the 28S and the 18S rRNA. These sharp bands are indicative of good quality RNA because these fragments have not been degraded. The samples include a human fetal fibroblast cell line to serve as a positive control (HEPM), a representative canine kidney positive control (MDCK), two human retinoblastoma cell lines for negative controls (Y79, WERI-Rb), two established canine osteosarcoma cell lines (OS2.4, D17), and four primary canine osteosarcoma cell lines (OSCJ93, OS1093L, OS1093B, OS1027). After hybridization with ^{32}P-labelled human Rb cDNA, the membrane was processed by direct counting (BetaGen) and for autoradiography. Neither detection method revealed any specific hybridization signal.

Detection of the Rb gene

To assess the presence, absence, or alteration of the Rb gene in all cell lines, we used Southern blotting techniques. Figure 8 shows an agarose gel containing approximately 10 ug of digested DNA per lane; this gel was transferred to Nytran membrane and subsequently hybridized. Hybridization of ^{32}P-labelled human Rb cDNA with this membrane was not successful. No specific hybridization signals were noted.

Discussion

In preliminary characterization of the four primary osteosarcoma cell lines, alkaline phosphatase was used as a marker to suggest a bone-derived cell origin. Canine osteosarcoma cell line D17 was used as a positive control and human leukemia cell line CCRF-CEM was used as a negative control. After noting that cells tend to dedifferentiate in culture and the length of time that D17 cells had to lose that marker, we decided that its alkaline phosphatase value was the lower limit. The newly-established primary cell lines were expected to express the marker in higher amounts if they were indeed canine osteosarcoma cells. Only one of the four new primary cell lines showed convincing alkaline phosphatase activity. Further characterization of these cell lines is planned.

In these experiments we attempted to detect both the Rb gene and its expression in primary canine osteosarcoma cell lines. We began by investigating immunoblotting as a means of detecting Rb protein p110RB. However, our results indicated that neither of the two available antibodies would cross-react adequately with canine protein. With further optimization, the

antibodies may have shown minimal cross-reactivity but this was not deemed sufficient for assessment of Rb deletions or characterization in the canine.

Initial efforts to detect Rb mRNA were inconclusive. Northern analysis demonstrated some degree of hybridization of the probe, but the signal-to-background ratio was unacceptable. Ausebel suggests this may be due to incomplete washing of the membrane. Low stringency conditions were used to wash the membrane because we felt that hybridization of the human probe and the canine sample would be optimized under these circumstances. However, a higher stringency may be necessary to remove background hybridization. Satisfactory results will require further work.

Initial attempts to detect Rb RFLPs in canine cell lines were unsuccessful. After the first DNA gel was run, transferred, and blotted, we saw no signal. Assuming the probe to be at fault, a fresh 3.8R fragment was isolated from uncut plasmid and labelled again. In addition, a second DNA gel (Figure 8) was prepared. When no signal was observed the second time, the Random Primers kit was replaced. Figure 9 is an image of the third Southern analysis. While the DNA gel appeared to have a satisfactory smear in most lanes, the blot indicates the 3.8R probe did not display any specific binding. This could be due to a number of reasons. The DNA quality could have been unsatisfactory. Spectrophotometric analysis showed all the DNA was of high quality and purity, however, the DNA was simultaneously digested with two restriction enzymes. As mentioned above, the smear was even and should have included the fragment to which the 3.8R probe hybridizes. As discussed above, low stringency conditions were applied to allow hybridization in a heterologous system. On one hand, this should have encouraged detection of any hybridization. However, it also accounted for moderately high non-

specific binding of probe. Non-specific binding may have been compounded by an inadequate prehybridization time. High background can be due to non-specific binding of the probe to nucleic acid sites on the membrane surface. Prehybridization involves incubation of the membrane with a blocking solution before addition of the probe to block these sites and thus reduce background signal. Our Southern blot techniques suggested a blocking duration of fifteen minutes prior to addition of probe, and this amount of time may be inadequate. Further refinement of hybridization technique is necessary to complete this study. Analysis of Rb in canine cell lines can then be compared with Southern analyses previously accomplished using genomic DNA from canine tumor tissue.

Conclusion

The Rb gene and its expression are difficult to characterize in canine because the reagents used to seek the Rb gene and its products are largely human in origin. Proper characterization will require the synthesis of an anti-canine Rb antibody and a 3.8R canine cDNA probe. However, these experiments are beyond the scope of this project. Cell lysates, extracted RNA, and extracted DNA from my experiments have been preserved at -80°C and are available for future experiments to characterize the canine Rb gene and its expression.
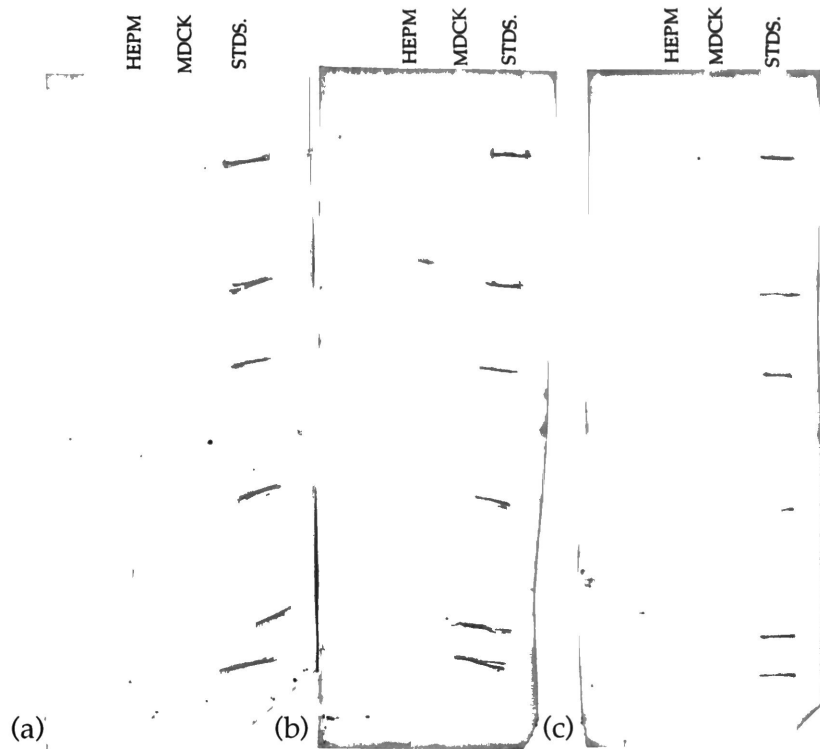
Figure 1: Photomicrograph of cultured canine osteosarcoma cells (D17). Cells were grown in monolayer culture and photographed using an inverted phase camera. Magnification is 4X.

Figure 2: Immunoblots of human (HEPM) and canine (MDCK) cell lysates. Protein (25 ug/ml) was processed for SDS-PAGE and immunoblotted using anti-Rb Ab-4 primary antibody at 1 ug/ml (a), 0.5 ug/ml (b), and 0.1 ug/ml (c) followed by alkaline phosphatase conjugated second antibody. Molecular weight standards are indicated (STDS).
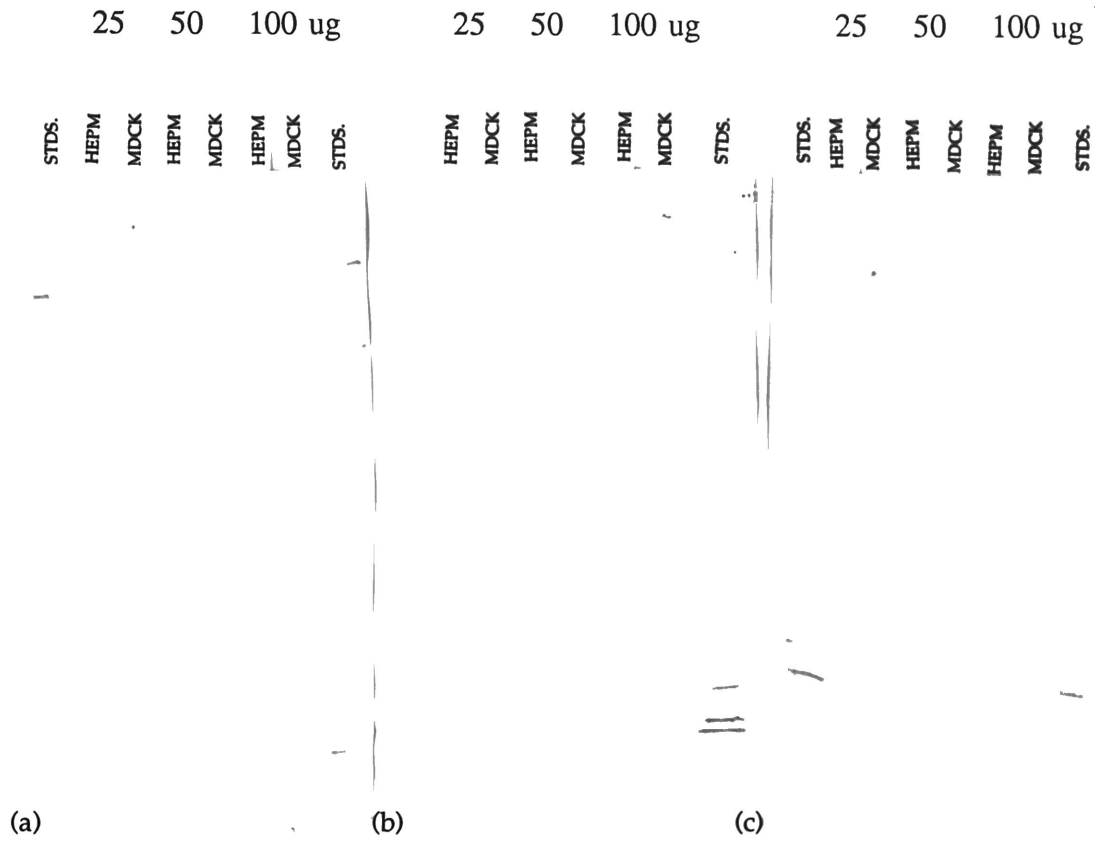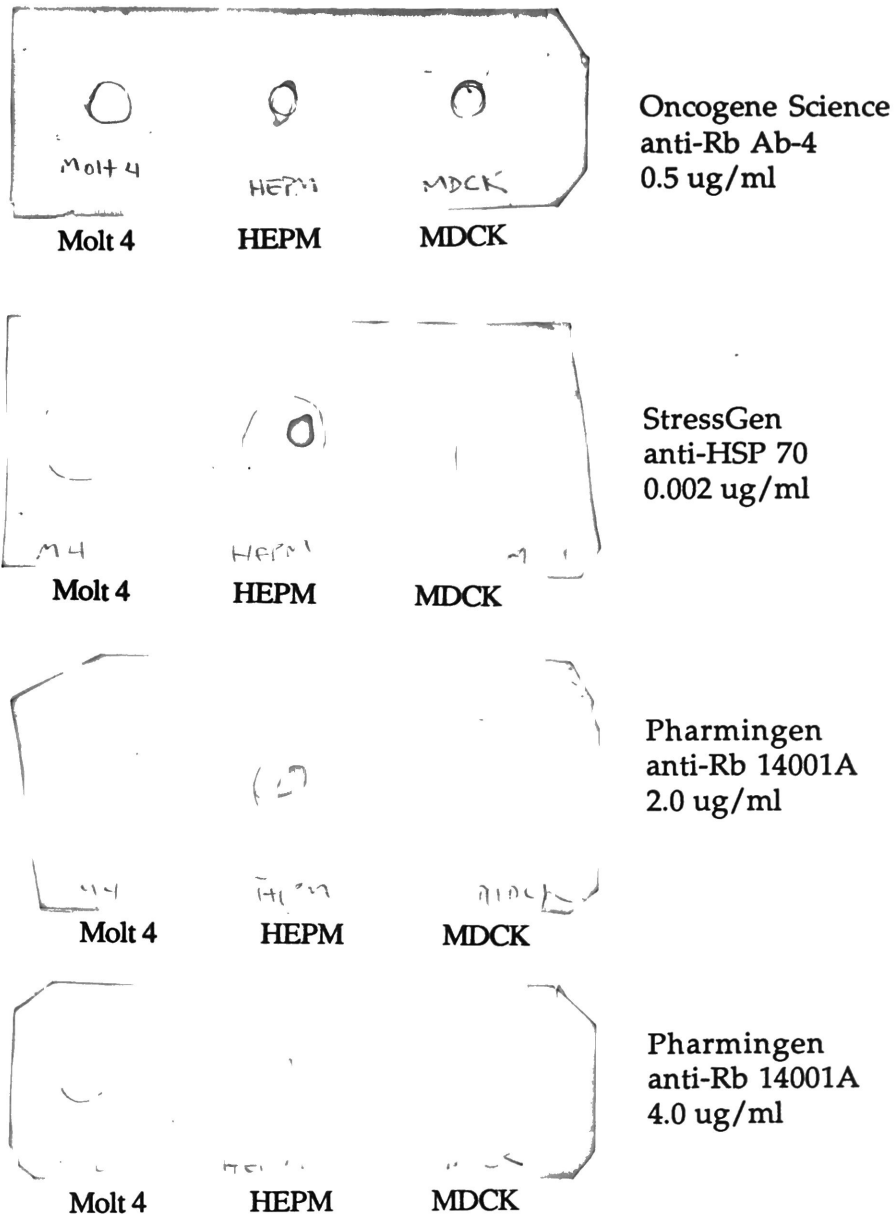
Figure 3: Immunoblots of human (HEPM) and canine (MDCK) cell lysates. Protein (25, 50, and 100 ug/lane) was processed for SDS-PAGE and immunoblotted using anti-Rb Ab-4 primary antibody at 1 ug/ml (a), 0.5 ug/ml (b), and 0.1 ug/ml (c) followed by alkaline phosphatase conjugated second antibody. Molecular weight standards are indicated (STDS).

25   50   100 ug

STDS.   HEPM   MDCK   HEPM   MDCK   HEPM   MDCK   STDS.

Figure 4: Immunoblot of human (HEPM) and canine (MDCK) lysates. Protein (25, 50, and 100 ug/lane) was processed for SDS-PAGE and immunoblotted using anti-Rb 14001A primary antibody and 4 ug/ml followed by alkaline phosphatase conjugated second antibody. Molecular weight standards are indicated (STDS).

Figure 5: Dot immunoblots of human (Molt 4, HEPM) and canine (MDCK) cell lysates (50 ug/dot) using the antibodies and concentrations indicated followed by alkaline phosphatase conjugated second antibody.
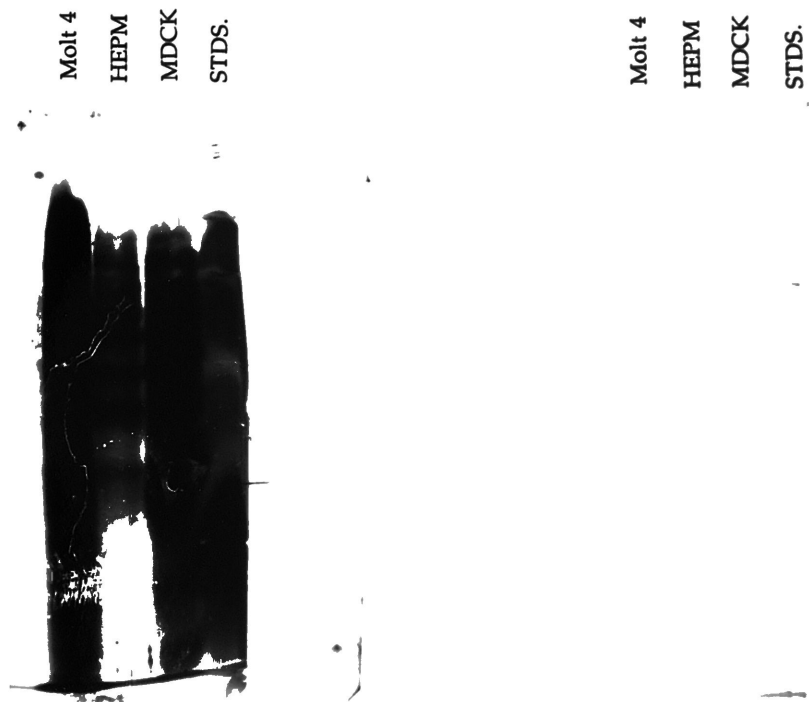
Figure 6: India ink stain and immunoblot of human (Molt 4, HEPM) and canine (MDCK) cell lysates. Protein (100 ug/lane) was processed for SDS-PAGE and stained with India ink (a) or immunoblotted (b) using antiRb 14001A followed by alkaline phosphatase conjugated second antibody. Molecular weight standards are indicated (STDS).
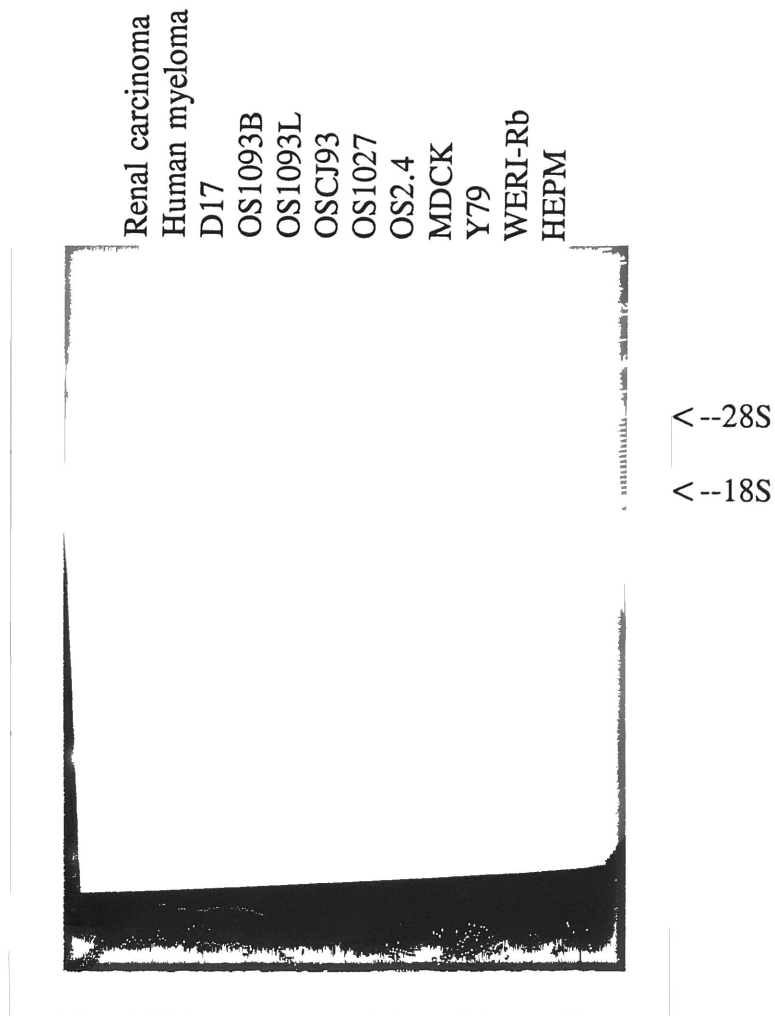
Figure 7: Immobilized RNA on Nytran membrane prior to hybridization. RNA (10 ug/lane) was electrophoresed on an agarose and formaldehyde gel and allowed to transfer to membrane overnight. Human fibroblast (HEPM), canine kidney (MDCK), human retinoblastoma (Y79, WERI-Rb), canine osteosarcoma (D17, OS2.4), primary canine osteosarcoma (OSCJ93, OS1093L, OS1093B, OS1027), human myeloma, and renal carcinoma cell lines are represented. 28S and 18S rRNA bands are indicated.
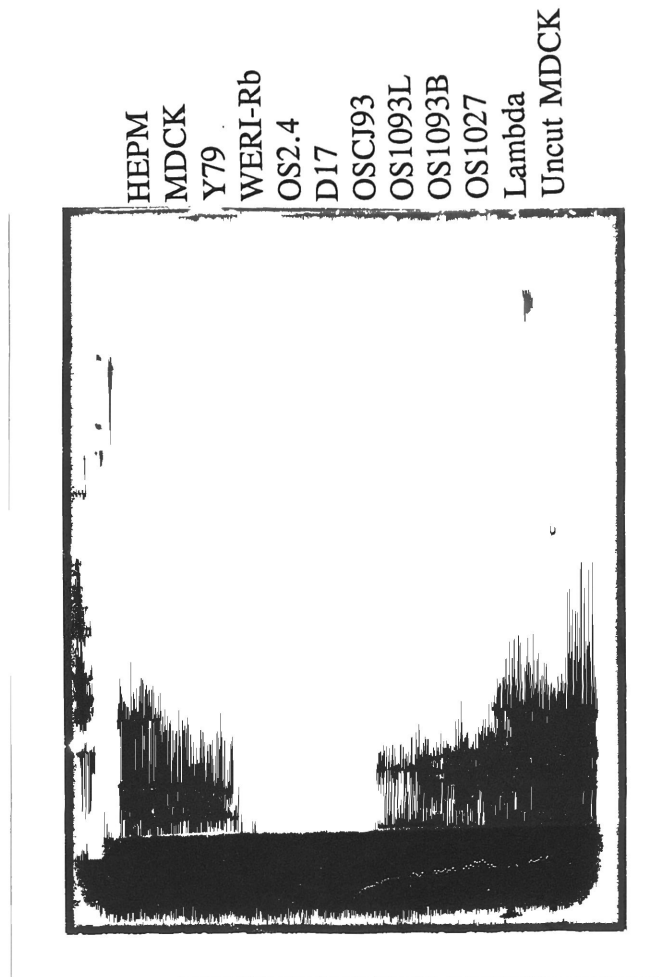
Figure 8: Electrophoretic separation of genomic DNA. Genomic DNA (10 ug/lane) from human fibroblast (HEPM), canine kidney (MDCK), human retinoblastoma (Y79, WERI-Rb), canine osteosarcoma (D17, OS2.4), and primary canine osteosarcoma (OSCJ93, OS1093L, OS1093B, OS1027) was extracted, quantitated, and digested with EcoR1 and Pst1 and electrophoresed.
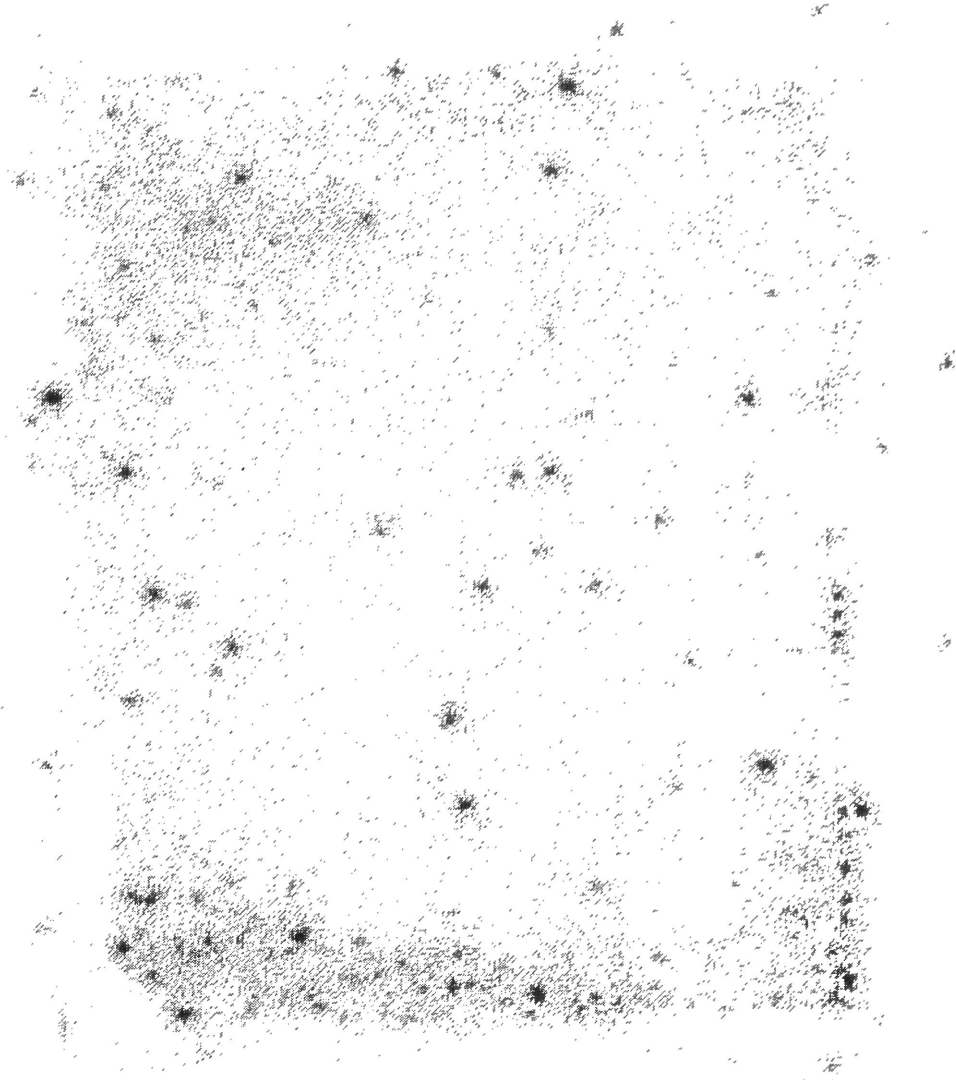
Figure 9: Image of Southern blot of genomic DNA using 3.8R probe. DNA from Figure 8 was transferred to Nytran membrane overnight and hybridized to 3.8R human cDNA probe (specific activity 2.8 x 10^{12}) for five hours. Membrane was washed three times under low stringency conditions before this image was taken with the BetaGen.

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References

Ausebel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl,K. 1988. Current protocols in molecular biology, vols. 1 and 2, John Wiley and Sons, New York, New York.

Cavenee, W.K., Koufos, Al, and Hansen, M. 1986. "Recessive mutant genes predisposing to human cancer." *Mutation Research.* 168: 3-14.

Francke, U. 1978. "Retinoblastoma and chromosome 13." *Birth Defects* 12: 131-137

Freshney, R.I. 1987. Culture of animal cells (2nd ed.), Alan R. Liss, New York, New York.

Friend, S. Hl., Bernards, R., Rogelj, S., Weinberg, R. A., Rapaport, J. M., Albert, D. M., and Dryja, T. P. 1986 "A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma." *Nature* 323: 643-646.

Green, M.R. 1989. "When the products of oncogenes and anti-oncogenes meet." *Cell.* 56: 1-3.

Kochevar, D.T., Kochevar, J. and Garret, L. 1990. "Low level amplification of c-sis *and c-myc* in a spontaneous osteosarcoma model." *Cancer Letters.* 53: 213-222.

Levine, A.J. 1992. "Tumor Suppressor Genes, the Cell Cycle, and Cancer." *Cancer Surveys.* vol 12. Cold Spring Harbor Laboratory Press.

Liu, Z., Batt, D., and Carmichael, G. 1994. "An improved rapid method of isolating RNA from cultured cells by SDS-acid phenol/chloroform extraction." *BioTechniques* 16: 56-7.

Marshall, C.J. 1991. "Tumor Suppressor Genes." *Cell.* 64: 313-26.

McFall, Rosemary et. al. 1977. "Characterization of a new continuous cell line derived from a human retinoblastoma." *Cancer Research.* 37: 1003-1010.