Chapter 2
Modeling Brain Tumors Using Avian Retroviral Gene Transfer

Tod D. Holland and Eric C. Holland

Abstract  RCAS/tv-a is a system for postnatal cell-type-specific gene transfer. It is used for the modeling of gliomas and medulloblastomas. This system provides a combination of lineage tracing from the cell origin with oncogenesis induced by mis-expression of specific genes. The genes that are most potent at inducing tumors are those that encode components of signal transduction and undifferentiated cells are most capable of serving as the cell of origin. The system effectively generates tumors with the histologic characteristics of human disease. Mice bearing RCAS/tv-a-induced brain tumors are currently being used for preclinical trials to understand the biology of therapeutic response in the various cell types that make up gliomas and medulloblastomas.

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E.C. Holland (✉)
Departments of Neurosurgery, Neurology, Surgery and Cancer Biology and Genetics, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA
e-mail: hollande@mskcc.org

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2.1 History

The history of retroviruses and our understanding of cancer are intertwined and illuminated by some of the most important scientific breakthroughs in the last century. In 1908, Danish scientists Vilhelm Ellerman and Oluf Bang discovered an infectious pathogen that could be transmitted from one chicken to another, a non-cellular pathogen capable of causing cancer. The agent was able to pass through filters designed for cells and bacteria, leading them to the observation that a non-cellular pathogen was inducing cancer (Ellerman and Bang, 1908). A few years later, in 1911, Peyton Rous from Rockefeller University made a related observation by identifying a similar pathogen, naming it the Rous sarcoma virus (RSV). Peyton Rous was awarded the Nobel Prize for his discovery, the first identifiable retrovirus (Rous, 1911).

Between 1911 and 1960, many other cancer-causing viruses were identified and cataloged. The viruses were named after either the species that were their hosts or the tumor types that they caused. It became apparent, even before the mechanisms were understood, that these viruses were highly specific to the target hosts that they are able to infect. This newfound study of cancer-causing viruses brought about an understanding of this universal phenomenon that could be observed in mammalian and avian organisms. The concept that cancer was caused by an infectious agent was a natural thought process that followed the discoveries of multiple external agents responsible for disease throughout the eighteenth and nineteenth centuries, including the vaccination work against smallpox by Edward Jenner in 1796. The concept of cancer being caused by an infectious agent had the unwanted consequence of sending researchers on the wrong track, since we now know that most human cancers are not caused by viruses but rather from “within” (see below).

The structure and function of DNA, proposed by James Watson and Francis Crick in 1953, postulated that information flowed in a set pattern that would begin from DNA transcribed to RNA, which would be the blueprint to form proteins. This central dogma was widely accepted to be invariant in every organism and implied that something that changed a cell’s phenotype (such as a virus) would necessarily require that its genetic information be stored in the form of DNA (Watson and Crick, 1953). However, in 1959, Howard Temin found the first exception to this rule while doing research for his PhD thesis on RSV and other viruses that would soon be compiled into a family known as Retroviridae. His viral stocks were absent of DNA and appeared to use RNA as their genome, implying that retroviruses did not replicate themselves in the set pattern described in the Central Dogma. As a graduate student, Temin had managed to prove that there were viruses that could replicate through a process he called reverse transcription. The reason it was called as such was due to the fact that replication seemed to occur in reverse, using a single strand of RNA to form double-stranded DNA (Temin and Rubin, 1958). Furthermore, before Temin’s work, it was unclear whether a virus was a quantal unit or a solution with transforming potential. He proved that viruses were actually particles capable of transforming the phenotype of an infected
cell and all of its progeny. Temin also proved the existence of the enzyme that was responsible for reverse transcription, which he called reverse transcriptase. This enzyme was isolated by David Baltimore, who shared the Nobel Prize with Howard Temin for this work (Temin, 1964).

The nature of the transforming potential of retroviruses became evident after the technology to identify the specific sequence of nucleic acids was invented. In the 1970s, Harold Varmus, Mike Bishop, and colleagues identified the general structure of retroviral genomes as having two repeated sequences on the ends and three viral genes, \textit{gag}, \textit{pol}, and \textit{env}. However, in the case of the tumor-forming viruses, there was another gene downstream of \textit{env} that turned out to be responsible for the induction of the transformed phenotype. In the case of RSV, the gene was called \textit{Src} and referred to as an oncogene because it caused cancer (Stehelin et al., 1976). Further experiments showed that this fourth gene was different from virus to virus, and unlike the other viral genes, the oncogene was closely related to a cellular gene found in the cells of the host animal that the virus previously infected. These oncogenes appeared to be stolen from the host genome and then expressed either in the wrong cell type or in an unregulated manner by the virus leading to the formation of cancer (Kurth, 1983). The realization that our own genes caused cancer and that cancer was a product formed from within us changed the way we think about this disease and led to Varmus and Bishop being awarded the Nobel Prize for their work (Bishop, 1983).

The collection of genes expressed as oncogenes from the known retroviruses was then catalogued, and the functions of the normal cellular homologues (proto-oncogenes) were identified. These cellular proto-oncogenes were found to encode the components of signal transduction pathways involved in both the proliferation and the inhibition of apoptosis. These gene products cover the entire spectrum from overexpression of ligands such as PDGF (v-SIS) to mutant active receptors such as EGFR (v-erbB), to constitutively active downstream components such as Ras and Akt, and transcription factors such as Myc, Jun, and Fos (Pech et al., 1989). Because of their natural ability to function as tumor-inducing agents, retroviruses became prime candidates for the creation of genetically engineered tumorigenic vectors that could be designed to transfer any gene and test its ability to induce tumor formation. In the 1990s, RSV was turned into a viral vector by Steve Hughes. The \textit{Src} gene was replaced by any gene of interest that was less than 2.5 kb (due to packaging constraints) and became known as the replication-competent ALV splice acceptor, or RCAS in short (Petropoulos and Hughes, 1991).

2.2 RCAS

The most commonly used version of RCAS is based on the subgroup A avian leukosis (ALV) viruses. The cell surface receptor that permits ALV-A subgroup infection of host cells is limited to birds (Coffin et al., 1997). The genetic locus
that encodes the receptor for subgroup A viruses is referred to as tv-a, or tumor virus-a. This gene was cloned from quail cells by Bates, Young, and Varmus using expression cloning from quail DNA into mouse cells and identified by its ability to confer susceptibility to RCAS infection (Bates et al., 1993). When expressed in mammalian cells, this gene product, tv-a, allows the infection by RCAS vectors and subsequent expression of whatever gene is inserted downstream of the \textit{env} gene. Furthermore, due to splicing differences between avian and mammalian cells, the mRNAs that encode the viral gene products \textit{gag}, \textit{pol}, and \textit{env} are unstable and produce essentially no protein. The overall result is receptor-mediated gene targeting to mammalian cells engineered to express tv-a. Federspiel and Hughes were the first to demonstrate the specificity of this system in transgenic mice. They showed that RCAS vectors expressing the marker gene alkaline phosphatase would specifically infect myocytes of mice transgenic for expression of tv-a from a muscle-specific promoter (Federspiel et al., 1994).

2.3 Retroviral Life Cycle

The life cycle of a retrovirus is comprised of a series of steps through which the virus replicates itself and its genetic information (Figure 2.1 and Color Plate 4). Genetically engineered retroviruses are called “ecotropic” when they can only infect host cells of the same species as the natural hosts of the wild-type virus and “xenotropic” when they can only infect cells from species that differ from its natural host. “Amphotropic” viruses are capable of infecting both host cells and cells of other species (Katen et al., 2001). It is largely due to the polytropic characteristics of amphotropic viruses that diseases can transfer from one species to another. Two examples of cross-species disease transfer are seen in the HIV virus (monkey to human) and the virus that causes SARS (avian to human) (Marx et al., 2001). Retroviruses have two identical copies of single-stranded RNA, called a capsid and an outer envelope comprised of glycoproteins used in the docking of the virus to the host cell. The envelope glycoproteins interact in a highly specific manner, allowing them to dock only to a corresponding receptor protein on the membrane of the cell. For example, avian sarcoma and leucosis viruses and mouse mammary tumor virus infect in a species-specific manner (DeLarco and Todaro, 1976).

The binding of the virion to the cell membrane marks the first stage in viral replication. Following the binding stage, the viral glycoproteins fuse to the cell membrane without undergoing endocytosis. Upon successful completion of the envelope–membrane fusion, the nucleocapsid (the viral equivalent of a nucleus) makes its way into the cytoplasm of the host cell. Deoxynucleoside triphosphates (dNTPs) enter the nucleocapsid, where the process of reverse transcription is carried out by Temin and Baltimore’s famous enzyme, reverse transcriptase. Reverse transcriptase, as well as several other proteins, begins the conversion from the virus’ single-stranded RNA to a piece of double-stranded DNA copy (Baltimore, 1970).
The DNA is synthesized beginning at the U3 region of the strand, which holds the enhancer and promoter elements. The R region at the 5' LTR contains the transcription start site, and the RNA processing signal lies at the 3' end. Proviral transcription gives rise to a single primary transcript, some of which will be spliced in order to produce alternative subgenomic messenger RNAs (Coffin et al., 1997). The viral transcripts will be exported out of the nucleus and

Fig. 2.1 Retroviral virion (A) binds to the target cell via interaction of envelope protein with its receptor (B). The nucleocapsid enters the cytoplasm and RNA genome (F) is converted to double-stranded DNA (C), which is integrated into the genome as a provirus (G) and transcribed (D, H). The proviral transcripts are translated into viral proteins and full-length transcripts serve as the viral genome of the next generation of virus (E) (see Color Plate 4)
synthesized into proteins. These viral proteins will be transported along the secretory pathway to the packaging site and along with two copies of the DNA (Mulligan, 1993).

Upon synthesizing this DNA molecule, it is transported directly into the nucleus of the host cell. From this stage, integrase (coded by the pol gene) allows for the integration of the DNA into one of many possible sites. The extensive number of integration sites is directly related to the size of the host cell’s genome. The newly integrated viral DNA, now called a provirus, is transcribed using the host cell’s RNA polymerase, generating mRNA and genomic RNA molecules (Mikkers and Berns, 2003). It is at this stage that the machinery of the host cell translates the viral mRNA copied from the provirus to glycoproteins and nucleocapsid proteins, identical to that which initiated the procedure (Mulligan, 1993).

The nucleocapsids assemble with genomic RNA to generate several progeny nucleocapsids, also identical to the original retrovirus that catalyzed the infection. The new progeny nucleocapsids interact with membrane-bound viral glycoproteins, restoring the progeny to their full form. The host-cell membrane buds out and the progeny virions are expelled to begin the entire process again. The only difference between the initial infecting agent and its second-generation progeny is the sheer number of virions that can be produced from a single pathogen infecting a single cell (Mikkers and Berns, 2003).

### 2.4 Tv-a Transgenic Mice

In order for RCAS to be able to effectively infect mice cells, it is essential that they express the avian tv-a receptor (Federspeil et al., 1994). This is achieved by genetically altering the mouse cells so as to make them express the receptor used by the retrovirus, either by in vitro transfection or by creating a transgenic mouse. As noted above, the tv-a expression in mammals does not occur, so infection with RCAS vectors will only happen if the mammal is transgenically modified to express tv-a in order for susceptibility to RCAS to occur. Usually, there is no obvious phenotype expressed by tv-a expression, because its only effect is to force susceptibility to RCAS (Fisher et al., 1999). Several transgenic mice have been generated that express tv-a from a variety of tissue-specific promoters, including those from the GFAP (glial fibrillary acidic protein) and nestin genes which direct expression of tv-a in brain tissue (Holland and Varmus, 1998, Holland et al., 1998) (Figure 2.2 and Color Plate 5).

The cell type that is infectable is defined by the promoter that drives the tv-a gene. This gives the opportunity to control the cell of origin in RCAS/tv-a modeling experiments. For example, the first tv-a transgenic mice used for brain tumor modeling drove the tv-a gene from the GFAP promoter. The second line drove tv-a from the nestin promoter (Holland et al., 1998). Comparison between the two demonstrated that properties of the two cell populations in
vivo controlled their ability to form tumors from one oncogenic stimulus or another. Generally speaking, less differentiated cells are more capable of serving as the cell of origin for gliomas in an otherwise wild-type background. Loss of tumor suppressor genes changes the spectrum of tumors that form and enables some cell types to form tumors that were otherwise not able to do so (see Section 2.5.2).

2.5 Modeling Brain Tumors with RCAS/tv-a

The main advantage of somatic cell gene transfer is modeling gain of function mutations as these are the genes that are typically carried on the RCAS vector. As noted above, the neutral experiment done by nature of identifying genes that cause cancer when carried on retroviruses identified a predominance of genes encoding signal transduction components. This is the case for experimental modeling of tumor formation with these vectors. Both gliomas (Dai and Holland, 2001) and medulloblastomas (Rao et al., 2004) are modeled effectively by gene transfer of genes encoding signaling components involved in cell–cell interaction and differentiation and development of the CNS.
2.5.1 Gliomas

There have been a significant amount of knowledge and information gained on the development of medulloblastomas and gliomas in recent years. Gliomas probably form from stem cells or progenitors and are driven by the signaling pathways that drive normal development in the CNS such as PDGF and downstream effectors such as RAS and Akt. The most potent oncogene in the formation of gliomas is the PDGFB ligand (Dai et al., 2001). Several systems including RCAS/tv-a have shown that the histology of glial tumors induced by PDGF is that of a diffuse glioma with oligodendroglioma characteristics, including oval-shaped cells with the “fried egg” white halo appearance and the full secondary structures of Scherer. There appears to be a correlation with the amount of PDGF expression and the grade of the tumors, ranging from either low-grade gliomas to those having anaplastic features such as pseudopalisading necrosis and microvascular proliferation (Shih et al., 2004). The bulk of the cells in these tumors shows low Akt activity and GFAP expression consistent with their counterparts in humans. The activation of Akt by infection with constitutively active Akt leads to the formation of cells with more astrocytic histology with irregular nuclei, cytoplasm staining for GFAP, and astrocytic morphology (Uhrbom et al., 2002).

The activation of the MAP Kinase pathway by constitutively active Ras in and of itself is not capable of forming gliomas from either GFAP- or nestin-expressing cells using the RCAS/tv-a system. However, coinfection of nestin but not GFAP-expressing cells with both constitutively active Ras and constitutively active Akt leads to astrocytic gliomas (Holland et al., 2000).

2.5.2 Loss of Function, Knockouts, and Cre/lox

Loss of tumor suppressor genes are common events in the progression of gliomas in humans. Although the RCAS system is designed for gain of function experiments, it can be adapted to additionally investigate the consequences of tumor suppressor loss as well. The easiest approach is to cross the tv-a transgenic mice to mice strains with germline deletion of the tumor suppressors in question and compare the tumor formation seen with RCAS vectors to mice wild type for that tumor suppressor.

Mice deficient in the tumor suppressor locus INK4a/Arf are particularly susceptible to brain tumor formation. Tv-a transgenic mice with an ink4a/arf –/– background form gliomas at a considerably higher frequency than wild-type mice upon RCAS-mediated PDGF gene transfer. Furthermore, the histology of the tumors that form in this background shows that they are of higher grade than if INK4a/arf is intact. The data indicate that loss of ink4a/arf in humans during glioma progression is likely to enhance the oncogenic effects of PDGF signaling and lead to malignant histology (Figure 2.3 and Color Plate 6).
In the case of Ras-driven oncogenesis, loss of *ink4a*/arf enables Ras to form tumors that are similar to those obtained with the co-activation of the Akt and Ras pathways (Uhrbom et al., 2002).

In a similar way, the p53 tumor suppressor has been investigated by crossing tv-a transgenic mice to a p53-deficient background. Tumor protein 53, or p53, is important in multicellular eukaryotic organisms, and its main functions are cell cycle regulation and apoptosis control. Due to its role in maintaining the rate at which cells undergo mitosis, it functions as a tumor suppressor involved in the prevention of cancerous malignancies. p53 has been called the “guardian of the genome” for its role in conserving genetic stability by preventing genomic DNA damage (Lane, 1992). When this gene is knocked out, the mouse becomes susceptible to various types of gene mutations, thereby deactivating and removing the “guardian angel” effect that p53 has over the DNA (Xu et al., 2001). Loss of p53 results in increased penetrance and grade in RCAS/tv-a-induced gliomas. The effect is most pronounced in tumors derived from a nestin-expressing cell of origin.

Homozygous loss of some tumor suppressors, which are critical in glioma progression, such as PTEN, is embryonically lethal, rendering the above approach obsolete (Penninger and Woodgett, 2001). In this case, conditional knockout of the locus can be achieved with RCAS gene transfer. This process

Fig. 2.3 Glioma formation with the RCAS/tv-a system. (A) Survival curves for mice of different genetic backgrounds infected with RCAS-PDGF demonstrating cooperativity between gain of function oncogenes and loss of tumor suppressors. (B) Whole mount H&E-stained section of mouse brain harboring large high-grade glioma. (C) T1 with contrast and T2-weighted MRI scans of a necrotic contrast enhancing high-grade glioma (courtesy of Brian Ross U. Mich.). (D) H&E section of high-grade tumor demonstrating microvascular proliferation and pseudopalisading necrosis (green arrows) (see Color Plate 6)
uses cre-lox recombination with the site-specific gene recombinase cre that catalyzes recombination between two specific and identical sites in the DNA, called the lox p sites. The core sequence flanked by the lox sites (or floxed) is then deleted when the two lox sites are recombined. The PTEN gene was floxed by knocking in a floxed version of PTEN to the endogenous locus resulting in a functional PTEN allele that could be deleted by cre expression. The RCAS vector has also been engineered to carry the cre recombinase, and cells from mice with the floxed PTEN allele that are infected with RCAS-cre subsequently delete PTEN (Hu et al., 2005). Loss of PTEN in this context enhances glioma formation in both PDGF- and Ras-driven gliomas derived from RCAS/tv-a technology.

2.5.3 Medulloblastomas – SHH Signaling

Medulloblastomas probably arise from stem cells in the rhombic lip and external granule cell layer of the brain, and at least some of these tumors are driven by the SHH signaling pathway that gives rise to formation of the normal cerebellum (Weiner et al., 2002). Mouse models of medulloblastomas have been generated by activating this pathway using several methods such as SHH overexpression, loss of PTC, and activation of SMO. In addition, loss of function of the DNA damage repair pathways contributes substantially to these SHH medulloblastomas such as loss of p53, Lig4, or Chk2.

Medulloblastomas are modeled using the RCAS/tv-a system. Originally, it was recognized that myc was insufficient to cause these tumors but that myc overexpression created nests of undifferentiated cells (Fults et al., 2002). Then SHH gene transfer with RCAS vectors into nestin-expressing cells of the rhombic lip created medulloblastomas in a minority of mice (Rao et al., 2004). This incidence of medulloblastoma formation was increased by several genes including Myc, Akt, and IGF2 (Rao et al., 2003). Although the incidence of tumors formed is increased by additional gene transfer, the incidence of medulloblastomas remains significantly below that seen with PDGF gene transfer to generate gliomas in this modeling system (Hambardzumyan et al., 2008).

It is possible that cell types other than nestin-expressing cells can serve as the cell of origin for these tumors. For example, math-1-expressing cells of the external granule cell layer can give rise to medulloblastomas using other genetic modification strategies (Yang et al., 2008). This is a good example of how various modeling strategies give different complementary results. It is likely that there are several cell types that can give rise to brain tumors and the development of further strains of transgenic mice with tv-a driven by other CNS cell population-specific promoters is expected to permit to derive models for any tumor type in the brain.
2.6 Imaging, Stem Cell Niches, and Preclinical Trials

The use of RCAS/tv-a-induced brain tumors in preclinical trials has been made possible by the development of several imaging technologies that allow each mouse to serve as its own control (Momota and Holland 2005). Bioluminescence imaging (BLI) has been developed in other systems to follow the fate of cells in vivo noninvasively. The most commonly used source of light production for BLI is the firefly luciferase gene that can be expressed from a tissue-specific promoter or a promoter that responds to a signaling pathway that is critical to the biology of the tumor. The mice are then injected with the substrate for luciferase, luciferin, and the enzyme cleaves luciferin in an ATP-dependent manner. The light is quantified by a CCD camera and is a direct and noninvasive readout of the activity of the promoter that drives luciferase expression (Momota et al., 2005).

BLI was used to generate a reporter mouse that would read out cell cycle proliferation using the E2F1 gene promoter. This mouse emitted light from PDGF-driven gliomas allowing identification of mice with tumors and a noninvasive measure of therapeutic effect (Uhrbom et al., 2004). A second reporter line was generated that expressed luciferase from a Gli responsive promoter which is a downstream readout of SHH signaling. Not surprisingly, this background gives off light from SHH-driven medulloblastomas as well (Figure 2.4 and Color Plate 7). The strength of such reporter systems is in showing the activity of pathways not previously known to be activated in a given tumor type. In this case, PDGF-induced gliomas also produced light; the tumors produce the SHH ligand from trapped and reactive astrocytes (Becher et al., 2008).

Several trials have been performed with the mice harboring RCAS-induced gliomas and medulloblastomas. In PDGF-induced gliomas, the effect of PDGFR and mTOR inhibition was shown to result in cell cycle arrest but not death (Uhrbom et al., 2004). Temozolomide had a modest effect on survival of these tumors and proliferation, but little immediate effect on the histological appearance of the tumors (Momota et al., 2005, McConville et al., 2007). Similar to what is seen in human gliomas, a radiation dose of 2 Gy had little effect on any cell type in these tumors and increasing the dose to 10 Gy was needed to achieve an effect, which was mainly cell cycle arrest.

In contrast to the relative resistance of glioma to radiation, medulloblastomas in a wild-type p53 background show substantial killing with 2 Gy and cell cycle arrest only in the perivascular niche. These surviving cells activate Akt in the process of arresting and blockade of Akt prior to radiation sensitizes these cells resulting in fewer perivascular cells surviving radiation (Hambardzumyan et al., 2008).

Cells with stem cell properties have been shown to exist in the perivascular structures of both medulloblastomas and gliomas (Calabrese et al., 2007). In the RCAS/tv-a models of these tumors, resistant cells with stem-like properties also exist in the same region allowing these models to serve as excellent systems for understanding the importance of such cells in the biology of these tumors and
their response to treatment. Furthermore, RCAS/tv-a models for brain tumors provide the ability to modulate the genetics of the specific cell types and treat the tumors with therapies that parallel the treatments given to humans (Hambardzumyan et al., 2007).

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