Published in final edited form as:

Curr Protoc Pharmacol. ; 72: 14.37.1–14.3712. doi:10.1002/0471141755.ph1437s72.

Overview of transgenic glioblastoma and oligoastrocytoma CNS models and their utility in drug discovery

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Abstract

Many animal models have been developed to investigate the sources of CNS tumor heterogeneity. In this unit, we review a recently developed CNS tumor model using *piggyBac* transposon system delivered by *in utero* electroporation in which sources of tumor heterogeneity can be conveniently studied. Their applications in CNS tumor study and drug discovery are also reviewed.

Keywords

central nervous system; tumor heterogeneity; *piggyBac* transposon; in utero electroporation; glioblastoma multiforme; anaplastic oligoastrocytoma; atypical teratoid rhabdoid tumor,

Introduction

Extensive genetic and phenotypic diversity exists not only between tumors (inter-tumor heterogeneity) but also within individual tumors (intra-tumor heterogeneity) (Burrell et al., 2013). Inter-tumor heterogeneity occurs between tumors arising from same organ, leading to classification of different tumor subtypes. Intra-tumor heterogeneity occurs within individual tumors in which tumor cells often differ in features such as size, morphology, and membrane composition as well as behaviors such as proliferation rate, cell-cell interaction, metastatic proclivity, and sensitivity to chemotherapy (Heppner, 1984; Martelotto et al., 2014; Marusyk et al., 2012; McGranahan and Swanton, 2015). Two, not mutually exclusive general models have been proposed to explain inter-tumor heterogeneity (Visvader, 2011). The genetic mutation model proposes that different genetic mutations lead to different tumor formation, whereas the cell of origin model explains different tumors as arising from different cell types (Blanpain, 2013; Fisher et al., 2012; Visvader, 2011).

Primary malignant central nervous system (CNS) tumors represent about 2% of all cancers but account for a disproportionate rate of morbidity and mortality (Buckner et al., 2007). Each year around 13,000 people in United States will die from primary malignant CNS tumors. Gliomas including glioblastoma multiforme are the most common malignant brain

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tumors. Current treatment regimens for malignant CNS include surgery, resection of tumor body followed by radio- and chemotherapy (Buckner 2007). CNS tumors are highly heterogeneous (Faury et al., 2007; Haque et al., 2007; Sturm et al., 2012; Verhaak et al., 2010). Experimental evidence supports both the cell of origin and genetic mutation model to explain the origin of inter-tumor heterogeneity. It is thought that different GBM subtypes have distinct cellular origins. Animal studies show that oligodendrocyte progenitor cells (OPCs) can be the cell of origin for proneural subtype GBM (Lei et al., 2011; Liu et al., 2011). Knockdown of NF1 and p53 using RNAi in astrocytes or neurons induced GBMs that matched human mesenchymal subtypes, while the same RNAi in neural progenitors induced GBMs matching human neural subtypes (Friedmann-Morvinski et al., 2012). It has been reported that 3 distinctly different CNS tumor types, primitive neuroectodermal tumor (PNET), high grade gliomas and atypical teratoid rhabdoid tumor (ATRT) like tumor, can be induced by infection of postnatal mouse neural stem cells with virus containing V12HRAS and c-MYC depending on the combination and sequence in which oncogenes are introduced (Hertwig et al., 2012). Similarly, Jacques *et al* (2010) showed that different combinations of conditional genetic deletions in p53, Retinoblastoma protein (Rb) and Phosphatase and tensin homolog (PTEN) in mouse subventricular zone neural stem cells induced formation of either PNET (primitive neuroectodermal tumor) or glioma: recombination loss of PTEN/p53 gave rise to gliomas whereas deletion of Rb/p53 or Rb/p53/PTEN generated primitive neuroectodermal tumor (PNET) .

To fully explore the causes of tumor diversity, it is desirable to have an animal model in which both the cell of origin and genetic insult can be conveniently and independently manipulated. To achieve this, we have recently developed a CNS rat tumor model in which multiple oncogenes can be expressed in selected cell populations at different times in brain development (Chen et al., 2014a; Chen et al., 2014b). In this unit, necessary background information regarding this model is introduced, followed by a review of the salient features of each tumor type induced by this approach and the potential applications of these tumor models for CNS tumor study and drug discovery.

Somatic transgenesis in cerebral cortex with *in utero* electroporation of *piggyBac* transposon system

Most human cancers are caused by accumulation of sporadically arising somatic mutations. In order to more accurately model human cancers, tools for inducing genetic alterations found in human cancers in somatic cells are needed. *In utero* electroporation is originally developed to deliver plasmids DNA into neural progenitors in developing forebrain (Figure 1 A,B) (Fukuchi-Shimogori and Grove, 2001; LoTurco et al., 2009; Saito and Nakatsuji, 2001; Tabata and Nakajima, 2001) and now is being used to transfect various cell types in many brain regions including pyramidal neurons and glia in neocortex (Figure 1C, D) (Chen et al., 2014b; Chen and LoTurco, 2012), ganglion eminence derived interneurons (Borrell et al., 2005), interneurons in hippocampus (Borrell et al., 2005; Chen et al., 2014b) and olfactory bulb (Borrell et al., 2005; Chen et al., 2014b), neurons in hypothalamus (Haddad-Tovoli et al., 2012) and Purkinje cells in cerebellum (dal Maschio et al., 2012). It is effective for delivering multiple plasmids DNA (Maher and LoTurco; Saito and Nakatsuji, 2001) and has

been adapted for both gain-of function and loss-of-function mutation studies to study neuronal development and function (Bai et al., 2003; Centanni et al., 2012; Centanni et al., 2014; Fukuchi-Shimogori and Grove, 2001; LoTurco et al., 2009; Saito and Nakatsuji, 2001; Tabata and Nakajima, 2001). Applications of *in utero* electroporation (IUE) in glia are hindered by the fact that conventional plasmids remain episomal and are lost or inactivated during cell division (Chen and LoTurco, 2012; Chen et al., 2014c; Siddiqi et al., 2014) (Figure 1A-C). This limits the application of IUE in experiments where labeling of complete lineage of neocortical progenitors are desired. One way to circumvent the loss or inactivation of episomal plasmids is to enable transgenesis of neocortical progenitors using a DNA transposon system such as *piggyBac*.

DNA transposons are genetic elements that can change their location within the genome, many of which have been engineered to become useful molecular biology tools for cellular transgenesis (Chen and LoTurco, 2012; Ivics and Izsvak, 2005; Kawakami, 2007). The *piggyBac* transposon was isolated from the genome of the cabbage loop moth *Trichoplusia ni* in the 1980s (Cary et al., 1989). Unlike other DNA transposons, the *piggyBac* transposon specifically transposes into TTAA sites in the host genome (Elick et al., 1996; Fraser et al., 1996). In order to utilize the *piggyBac* transposon, it has been engineered to a binary *piggyBac* plasmid system including a helper plasmid encoding for the enzyme, *piggyBac* transposase (PBase), and donor plasmid bearing the 5' and 3' terminal repeats, the recognition sites for the PBase enzyme (Wu et al., 2007). Transgenes of interest flanked by the *piggyBac* terminal repeats on the donor plasmid will transpose into the host genome via the action of PBase.

By using *IUE* to deliver combinations of PBase helper (for example, CAG-PBase) and donor plasmid expressing fluorescent reporter (for example, PBCAG-eGFP), transgenesis of the complete lineage of neocortical neural progenitors can be achieved, resulted in labeling of neurons, astrocytes, oligodendrocytes, oligodendrocytes precursors as well as olfactory bulb interneurons (Figure 1C) (Chen and LoTurco, 2012; Chen et al., 2014c; Siddiqi et al., 2014). This *piggyBac IUE* approach has been adapted to gain of function experiments (Chen et al., 2014b; Chen and LoTurco, 2012; Glasgow et al., 2014), clonal labeling of glia cells (Siddiqi et al., 2014) (Fig. 1), selectively labeling of particular cells types in the neocortical progenitor lineage (Chen et al., 2014c) (Figure.1D) and tracking lineage of subpopulations of neocortical progenitors (Siddiqi et al., 2014). *PiggyBac IUE* provides a faster and more convenient approach to introduce somatic cellular transgenesis to cells of interest in brain regions including cerebral cortex (Chen et al., 2014c), hippocampus (Chen et al., 2014c), striatum (Borrell et al., 2005), olfactory bulb (Borrell et al., 2005; Chen and LoTurco, 2012; Chen et al., 2014c), hypothalamus (Haddad-Tovoli et al., 2012) and cerebellum (dal Maschio et al., 2012) and can be used to assess effects of different genetic mutations and cell of origin on tumor heterogeneity.

***PiggyBac IUE* approach induced CNS tumors**

Genetic abnormalities frequently found in GBM patients involve genetic elements that control genomic stability, cell cycle as well as signaling transduction pathways (Rich and Bigner, 2004). Common signal transduction pathways involving Ras, and Akt (Feldkamp et

al., 1997; Kulik and Weber, 1998) are frequently found hyperactive in GBM. RAS is activated in almost all cases of GBM and AKT is activated in 70% of GBM tumors (Guha et al., 1997; Holland et al., 2000). Combined activation of Ras and AKT have been used to induce glioma in many animal models of glioma (Guha et al., 1997; Holland et al., 2000; Holmen and Williams, 2005; Marumoto et al., 2009; Uhrbom et al., 2002).

We have recently developed a new glioma model in Wistar rat using *piggyBac in utero* electroporation approach to direct expression of oncogenic HRasV12/AKT in different cell populations in cerebral cortex (Figure 2A) (Chen et al 2014a). The *piggyBac* plasmids used in this model include: 1) *piggyBac* helper plasmid GLAST-PBase that expresses the *piggyBac* transposase in radial glial progenitors via the glutamate aspartate transporter (GLAST) promoter (GLAST-PBase) and thereby drives transposon integration into the genomes of radial glia progenitors; 2) oncogenic donor plasmid in which HRasV12 and AKT expression is under the control of one of three different promoters [CAG, myelin basic protein (MBP), or glial fibrillary acidic protein (GFAP); Fig. 2A]; 3) a multicolor system of donor plasmids with the CAG promoter driving expression of eGFP, monomeric red fluorescent protein (mRFP), or CFP to create multicolor clonal labeling (Siddiqi et al., 2014). *PiggyBac* donor and helper plasmid mixture was *in utero* electroporated into lateral cerebral ventricles of E14-E15 Wistar rat embryos. After the animals were born, tumor assessment was performed at various postnatal days. Multicolor labeling of tumor cells allows visualization of tumor cell clonality and revealed significant clonal expansion and clonal mixing of tumor cells (Figure 2B-E). This model system affords the opportunity for directed oncogene expression, clonal labeling, and addition of tumor-modifying transgenes. Glioblastoma multiforme, anaplastic oligoastrocytoma and atypical teratoid rhabdoid like tumor can be induced by targeted expression of oncogenic HRasV12/AKT in different cell populations with 100% induction efficiency.

Glioblastoma multiforme

Over expression of CAG donor plasmid condition (PBCAG-HRasV12/AKT) resulted in large aggregates of fluorescently labeled cells invading striatum and the ventricular and subventricular zones of neocortex by the day of birth (P0) (Figure 3B). Necrosis appeared as early as postnatal day4 (P4). In contrast, aggregates of proliferative cells were not apparent in the GFAP donor plasmid condition (PBGFP-HRasV12/AKT) in the first few days after birth (P2; Fig. 3D), but instead normally differentiating neurons were apparent a few days after birth with only scattered cells outside of the neuronal cortical plate. By the beginning of the second postnatal week (P9), masses of proliferating cells seemed in the GFAP donor plasmid conditions. These masses were often of a single clonally labeled color and were present in areas centered within white matter and the subpial zone (Fig. 3 E).

A CAG donor plasmid transfected brain hemisphere was significantly smaller than a non-transfected brain hemisphere, whereas both transfected and non-transfected hemisphere in GFAP donor plasmids transfected brain had comparable size (Fig. 3A). This observation may arise due to expression of HRasV12/AKT under the control of CAG promoter induces extensive cell death in neocortical progenitors, resulting in the smaller brain hemisphere.

Histopathologically, both CAG and GFAP donor plasmids induced tumors that recapitulate the histopathological features of human GBMs including the presence of necrotic foci surrounded by "pseudopalisading" tumor cells, cells with highly pleomorphic cytologic appearance and increased mitotic activity, and astroglial cells with extensive process that were positive for GFAP, vimentin and MAP2c. Interestingly, vascular proliferation was not observed in CAG donor plasmid induced GBMs but frequently observed in GFAP donor plasmid induced GBM.

Oligoastrocytoma

In contrast to GFAP donor plasmid induced GBM formation, overexpression of HRasV12/AKT in oligodendrocytes using MBP donor plasmid (PBMBP-HRasV12/AKT) induced anaplastic oligoastrocytoma (OA) in all electroporated animals. Normally differentiating neurons rather than aggregates of fluorescently labeled proliferative cells were apparent a few days after birth in MBP donor plasmid condition (P3; Fig. 3F). By the end of the first postnatal week (P7), masses of proliferating cells were observed populating neocortex and striatum (Figure 3G). Tumors induced by MBP donor plasmids contain prominent "honeycomb"-like cell clusters with round isomorphic nuclei located centrally to perinuclear halos resembling cytological features of human oligodendroglioma and a small fraction of astroglial components.

Atypical teratoid rhabdoid like tumors

In utero electroporation confers high multi plasmids co-transfection efficiency (Maher and LoTurco; Saito and Nakatsuji, 2001) which allows for introduction of multiple transgenes including oncogenes, modifying genes and fluorescent reporters (Figure 2A). By co-electroporating tumor inducing CAG donor plasmids with another modifying *piggyBac* donor plasmid encoding neurogenic basic helix loop helix (bHLH) transcription factor Neurogenin2 (Ngn2, PBCAG-Ngn2), Neurodifferentiation1 (NeuroD1, PBCAG-NeuroD1), or episomal plasmid encoding Ngn2 (CAG-Ngn2), it is possible to induce a highly malignant pediatric atypical teratoid rhabdoid like tumor(ATRT like) tumor which resembles the histopathological features of human atypical teratoid rhabdoid tumors but retained nuclear INI 1 expression. ATRT is extremely aggressive malignant rhabdoid tumor that generally occurs in infants and young children. Loss of function in the INI1 gene is believed to be a significant cause of ATRT in humans (Biegel et al 2006). Aggregates of fluorescently labeled cells were visible at birth; tumor cells expanded rapidly into very large tumors with street like necrosis invading the entire cerebral hemisphere by 3 weeks after birth (Fig. 3 H-J) resulting in essentially 100 % tumor-associated mortality by 30 days of age.

Applications of the *piggyBac* IUE CNS tumor model

Regulation of glioma subtypes

It is increasingly clear that tumorigenesis is the convergence of genetic mutation and developmental context (Chen et al., 2014a; Chen et al., 2014b; Glasgow et al., 2014; Visvader, 2011). However, how developmental and cellular context influences tumor subtype generation is unclear. Using *piggyBac* IUE induced tumor model, Glasgow et al (2014) demonstrated that specific interactions between oncogenes and developmental regulators of

glial sub-lineages influence the generation of glioma subtypes. Glasgow et al (2014) first generated a mouse oligodendroglioma mouse model using *in utero* electroporation of PBMBP-RasGFP. The authors further showed that co-electroporation of gliogenic transcription factor NFIA with oncogenic PBMBP-RasGFP converted oligodendroglioma to an astrocytoma like subtype of glioma. Considering the large cargo capacity of *piggyBac* (Ding et al., 2005) and high multi plasmids cotransfection efficiency of *in utero* electroporation (Maher and LoTurco; Saito and Nakatsuji, 2001), *piggyBac* IUE approach can be used to study molecular basis of glioma subtype specification (Glasgow et al., 2014). Furthermore, addition of luciferase expressing plasmids to tumor inducing plasmid mixtures, adapts this system to monitor tumor progression *in vivo* using bioluminescence imaging (Bhang et al., 2011; Uhrbom et al., 2004), making it a valuable tool for high throughput anti-cancer drug screening.

***In vivo* tumor imaging**

Upon combining the *piggyBac* multicolor clonal labeling system with tumor inducing donor plasmids, the resultant tumor cells can be labeled with distinct colors (Fig. 3A,B) (Chen et al., 2014b) making these animal models suitable for *in vivo* tumor imaging. In previous *in vivo* imaging studies, fluorescently labeled tumor cell lines were used to generate tumor models that could be imaged *in vivo* in live animals (Yamamoto et al., 2011; Yang et al., 2000; Yang et al., 2002). Even though xenograft mouse models provide convenient approach for modeling human tumor, the tumor host immunological responses is inhibited in xenograft tumors and many xenograft tumors do not recapitulate human histopathology or molecular character (Jones and Holland, 2011). In contrast, the multicolor tumor models induced by somatic transgenesis mediated by *piggyBac* IUE retain intact tumor-host immunological response and are molecular and histologically similar to human tumors (Table 1) (Chen et al., 2014b). Thus these tumor models provide a platform for tumor *in vivo* imaging study.

Evaluating sources for tumor heterogeneity

The *PiggyBac* transposon system is binary; it is possible to direct genomic integration in a subset of progenitors by the choice of promoter driving the expression of PBase, and to drive expression of the integrated transgenes in selected progeny by the promoter in the donor plasmid. It is also feasible to express oncogenes in lineages of subsets of neural progenitors and evaluate their contribution to tumorigenesis. It is still unclear that whether different tumor types or subtypes come from different neural progenitor populations or tumors generated from different subsets of neural progenitors have different behaviors. It has been shown that astrocytes derived from the glutamate–aspartate transporters positive (GLAST +) progenitors have larger clonal clusters than astrocytes from Nestin+ progenitors (Siddiqi et al., 2014). It is reasonable to ask whether astrocytes in GLAST+ progenitor lineage are more susceptible to tumor induction or tumors induced from astrocytes in GLAST+ progenitor lineage are more malignant. These questions can be addressed by selectively expressing oncogenes in specific cell types in the lineage of neural progenitor subpopulations using the *piggyBac* IUE approach.

Studying CNS tumors in other species

Unlike genetic engineered mouse models for brain tumor that are specific for mice, there is no species dependence for using the *piggyBac* IUE approach. Theoretically IUE can be performed in any species that with ventricles. Besides applications in rodents IUE has also been used in ferret (Kawasaki et al., 2012; Kawasaki et al., 2013). Moreover, the combination of *piggyBac*-IUE with existing transgenic mouse models could potentially broaden the utility of both approaches. For example, similar to viral vector mediated conditional gene modification in the brain (Kaspar et al., 2002), *in utero* electroporation of Cre encoding *piggyBac* donor plasmids in to transgenic mice with floxed alleles can also efficiently modify endogenous gene expression (Breunig et al., 2015).

Limitations and solutions

We used promoter fragments instead of complete MBP and GFAP regulatory elements to direct HRasV12/AKT expression in oligodendrocytes and astrocytes. One concern for using promoter fragments is that they sometimes do not recapitulate the endogenous promoter element activity. This is true with the mouse GFAP and rat MBP promoter fragments used in this dissertation. Even though, they showed enriched labeling of astrocytes and oligodendrocytes respectively, both promoters can label small fraction of other cell types (Figure 1D). One solution to this is to perform *piggyBac* IUE on transgenic mouse lines. By combining conditional *piggyBac* donor or helper plasmids in which the transgene of interest or PBase is flanked by loxP sites or FRT sites with transgenic Cre or Flp mouse lines, cell type specific expression or integration can be achieved (Awatramani et al., 2001). This will allow genomic integration and/or transgene expression in specific cell types in radial glia subpopulations designated by transgenic mouse lines.

Electroporation is most efficient when performed embryonically. Efficient postnatal electroporation can be achieved in young animal between the age of P0 and P3 (Wiesner et al., 2009). The efficiency drops dramatically beyond the age of P3. This limits the application of *piggyBac* IUE approach in experiments where postnatal/adult expression of gene of interest is required, especially in CNS tumors in which most oncogenic mutations occurred much later in life. Precise temporal control of gene expression can now be achieved through *piggyBac* based inducible gene expression systems gated by tetracycline (Li et al., 2013; Saridey et al., 2009). Therefore transgenes can be introduced embryonically but will not express until treated with tetracycline at desirable time depending on experimental need.

Conclusion

The *piggyBac in utero* electroporation approach induced CNS tumors model complements existing CNS tumor models and is valuable in both understanding basic CNS tumor biology and preclinical high throughput screening for anti-cancer drugs.

Acknowledgement

This work is supported by grants from NIH (R01HD055655 and R01MH056524) to J. LoTurco.

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Figure 1. Somatic transgenesis in cerebral cortex with *in utero* electroporation of *piggyBac* transposon system

A. Schematic demonstration of *in utero* electroporation procedure. Plasmid mixtures are delivered to the lateral ventricles of embryonic rat followed by electric pulses which drives the plasmids into neural progenitor lining the lateral ventricle wall.

B. Two days post electroporation, most transfected cells are residing the ventricular zone (VZ). The transfected cell demonstrated typical neural progenitor morphology with long basal process and short apical process contacting lateral ventricle.

C. IUE with conventional plasmid (CAG-mRFP, right) labeled neurons and *piggyBac* plasmid system (PBCAG-eGFP and CAG-PBase, middle and left) labeled cells within the neural progenitor lineage including neurons, astrocytes, oligodendrocytes as well as olfactory bulb interneurons.

D. Cell type specific labeling using *piggyBac* IUE. PBCAG-eGFP labeled all cells in the neural progenitor lineage (right). PBGFAP-eGFP and PBMBP-eGFP resulted in enriched labeling in astrocytes (middle) and oligodendrocytes (left) respectively.

Figure 2. Multicolor GBM

A. Summary of plasmids used in this study. The plasmids used here consist of piggyBac helper plasmid and a set of donor plasmid: fluorescent donor plasmids are used to label transfected cells; oncogene donor plasmid is used to induce glioma; Transcription factor donor plasmid is modifying plasmids that will modify existing tumor type. Transcription factor plasmid is episomal non-*piggyBac* plasmid encoding transcription factor. FP: fluorescent protein; TF: transcription factor.

B. Representative image of multicolor labeled GBM induced by CAG donor plasmids. Tumor clones were labeled with different colors. Large views of boxed areas were shown on the left (C-E). C, a tumor clone labeled by mRFP. D, a tumor clone labeled by GFP. E, mixture of tumor cell labeled by GFP and mRFP. F, 3D reconstruction of 5 tumor clones.

Figure 3. Tumors induced by expression of HRasV12/AKT

A. Representative images of brain transduced with different tumor inducing donor plasmids. B-J. Developmental time course of induced tumors. CAG donor plasmids induced GBM showed early onset. Abnormal cell proliferation can be seen at birth (B and B'). Neocrosis was apparent at the age of P7 (C and C'). GFAP donor plasmids (D-E) and MBP donor plasmids (F-G) induced tumors had relatively late onset. Cells were normal in the first week (D, D', F, F'). Early in the second week, abnormal cell proliferation started to appear. ATRT like tumors induced by CAG donor plasmids and Ngn2 expressing donor plasmid was highly malignant (H and I). At birth, massive cell proliferation was seen (H, H'). These tumor cells quickly spread to entire cerebral hemisphere (I). Extensive street like necrosis were frequently seen (J).

Table 1

Features of existing CNS tumor models

Animal models	Features	References
Orthotopic Xenograft	Tumor histology doesn't resemble human tumors Fast	(Candolfi et al., 2007; Jones and Holland, 2011)
Genetic Engineered Mouse Model (GEM)	Tumor histology resembles human GBM. Can't distinguish primary mutation and secondary mutation. Species dependent. Laborious	(Alcantara Llaguno et al., 2009; Jacques et al., 2010; Kwon et al., 2008; Persson et al., 2010; Swartling et al., 2012);
Somatic Engineered Mouse Model (SEM) -Viral vector	Controlled gene expression in particular organs, cell types and at specific developmental stages. Clonal origin and migration pattern is difficult to track. Not suitable for multigene delivery.	(Charest et al., 2006; Hambardzumyan et al., 2009; Holland et al., 2000; Marumoto et al., 2009; Uhrbom et al., 2002; Uhrbom et al., 1998; Wei et al., 2006)
Somatic Engineered Mouse Model (SEM) - <i>piggyBac IUE</i>	Controlled gene expression in cell types and at specific developmental stages Multigene delivery High efficiency	(Chen et al., 2014b; Wiesner et al., 2009)