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From Cell Biology to Tissue Engineering

Review

Facilitating tailored therapeutic strategies for glioblastoma through an orthotopic patient-derived xenograft platform

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Summary. Despite years of research into its pathobiology and continuing clinical trials for novel therapies, the prognosis for patients with glioblastoma (GBM) remains dismal. An important obstacle against treatment efficacy may be a high degree of intra- and inter-tumoral heterogeneity within GBMs, which may be caused by the presence of self-renewing GBM stem cells (GSCs). Recent advances in multi-omics technology introduce new possibilities for applying personalized strategies to GBM therapy. As drug discovery is accelerating with the transition from non-selective, cytotoxic therapy to a precision, targeted approach, the appropriate *in vivo* platform for GBM is critical for validating drug targets and prioritizing candidates for clinical studies, for co-development of companion diagnostics and, ultimately, for drug approval. Here we will describe GBM orthotopic patient-derived xenografts (PDXs) as more useful, clinically relevant resources for individually tailored strategies for GBM.

Key words: Glioblastoma, Personalized medicine, Tumor heterogeneity, Glioblastoma stem cells, Tumor microenvironment, Patient-derived xenografts, Orthotopic

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Introduction

Extensive tumor heterogeneity, invasion through brain parenchyma, and intrinsic resistance to treatment are distinctive hallmarks that contribute to poor prognosis of glioblastoma multiforme (GBM) (Thakkar et al., 2014). GBM is virtually incurable with conventional and targeted therapies, with newly diagnosed GBM patients showing a median survival of approximately 15 months, which falls to 5-7 months in cases of recurrent GBM (Hegi et al., 2008; Stupp et al., 2009). The number of therapeutic options is unfortunately limited when GBM recurs after standard treatment, including maximum tumor resection with concomitant temozolomide (TMZ) and radiotherapy (RT) (Wick et al., 2010; Chinot et al., 2014). In fact, initial trials with a diverse group of therapeutic agents that supposedly target various signal transduction pathways have been uniformly disappointing (Bastien et al., 2015).

The properties of GBM that distinguish it from other extra-cranial tumors need to be considered for development of effective strategies. GBMs diffusely invade into normal brain parenchyma and ultimately result in tissue edema and failure of treatment. Furthermore, they are common in elderly patients who show poor performance, and are found in nonexpendable parts of the cranium, thus limiting the treatment to modalities that do not cause extensive morbidity (Duffner, 2010; Sahebjam et al., 2012). Finally, delivering effective and sustained treatment to invading

cells without damaging the healthy brain tissue is a major challenge in GBMs because of impaired drug delivery to the central nervous system (CNS) due to the blood-brain barrier (BBB) (Neuwelt et al., 2011).

The inability of current preclinical systems to mimic the biology of human GBM in situ is increasingly cited as a key cause of low success rates for translation of drug discovery efforts to the clinic (Ellis and Fidler, 2010). Therefore, there is a compelling need for more reliable *in vivo* models in order to facilitate the elucidation of pathophysiology and for testing new drugs and therapies. Additionally, attempts to target drivers of tumor progression such as tumor invasiveness or angiogenesis can be carried out in a clinically relevant site by orthotopic transplantation. In this review, we discuss the importance of GBM orthotopic patient-derived xenografts (PDXs) in bridging the gap between translational studies and targeted therapeutics in terms of unmet clinical needs related to GBM tumor heterogeneity and specialized microenvironment.

Tumor heterogeneity and GBM stem cells (GSCs) in personalized treatment approaches to GBM

In the past two decades, advances in multi-omics technology have made possible the genome-wide evaluation of genetic and epigenetic changes in GBM (Beroukhim et al., 2007; Parsons et al., 2008; Verhaak et al., 2010). Deregulation of the core retinoblastoma (RB)/p53, phosphoinositide 3-kinase (PI3K)/AKT/phosphatase and tensin homolog (PTEN)/mammalian target of rapamycin (mTOR), and receptor tyrosine kinase (RTK)/RAS/RAF/mitogen-activated protein kinase kinase (MEK)/mitogen-activated protein (MAP; extracellular signal-regulated kinase [ERK]) pathways is an obligatory event in most GBM tumors (Cancer Genome Atlas Research, 2008; Parsons et al., 2008). Thus, ongoing efforts are attempting to target different nodes along these pathways with small-molecular inhibitors, antisense molecules, or monoclonal antibodies. Additional efforts include targeting individual actionable mutations such as deletion of the extracellular domain of EGFR (EGFRvIII)(Huang et al., 2007), or some proteins that are not mutated, but are central nodes for aberrant signaling pathways such as CCAAT/enhancer binding protein β (C/EBP) and signal transducer and activator of transcription-3 (STAT3) in the mesenchymal subtype of GBM (Carro et al., 2010).

Both tumor heterogeneity and presence of GBM stem cells (GSCs) may contribute to treatment-resistant and lack of treatment efficacy in GBM, resulting in negative results in clinical trials (Cancer Genome Atlas Research, 2008; Nickel et al., 2012). Recent integrated genomic analyses highlight the complex intra- and/or inter-tumor genetic heterogeneity in GBM (Verhaak et al., 2010; Sottoriva et al., 2013). Intra-tumoral heterogeneity is particularly challenging for therapy, since alternate signaling pathways overriding the inhibition of the targeted molecule are quickly activated in GBM, or the dramatic heterogeneity within the GBM tumors possibly allows the rapid selection of resistant clones (Bonavia et al., 2011; Patel et al., 2014). Roughly 10% of GBMs show amplification of multiple RTKs such that tumors are comprised of discrete cell populations, each harboring the amplification of a distinct RTK (Snuderl et al., 2011; Szerlip et al., 2012). The use of targeted drugs is associated with an inevitable increase in drug resistance or escape mechanisms in GBM as tumor heterogeneity is not fully considered in preclinical drug efficacy tests. Moreover, vascular heterogeneity and variations in the degree of hypoxia in combination with reprogramming of energy metabolism, confer another layer of complexity to micro-environmental variability in GBM (Turcotte et al., 2002; Griguer et al., 2005; Di Ieva, 2010, 2011), which can impact the distribution of chemotherapeutic drugs within the tumor or result in variable responses to anti-angiogenic therapies.

A large amount of phenotypic, morphological, and cellular heterogeneity in GBM is generated by a population of self-renewing GSCs (Singh et al., 2004), which contribute to tumorigenesis and treatment resistance (Salmaggi et al., 2006; Kang and Kang, 2007; Rich, 2007). Studies with GSCs have shown that the microenvironment holds the key to understanding how these cells retain their stemness (Calabrese et al., 2007; Christensen et al., 2011). GSCs preferentially associate with endothelial cells, and proliferate rapidly in presence of factors secreted from endothelial cells effectively producing orthotopic brain lesions upon implantation (Calabrese et al., 2007). Given their critical role in tumor initiation, propagation, and maintenance, direct targeting of GSCs within the tumor bulk is the key to effective GBM treatment. The heterogeneity of GSCs that drive GBM growth is also beginning to be appreciated (Gunther et al., 2008; Fine, 2009). Attempts are underway to find the aberrance of several development pathways implicated in GSC creation and maintenance, allowing treatments to specifically target pathways such as the interleukin 6 (IL6) / STAT3 pathway (Van Meir et al., 1990), mammalian target of rapamycin (mTOR) signaling (Jhanwar-Uniyal et al., 2013), Sonic hedgehog and Notch (Singh et al., 2004).

The necessity of orthotopic PDXs in preclinical GBM research

Pros of GBM PDX models

A potential reason for the lack of predictability in existing xenograft models is that they are based on established cancer cell lines (ECLs) derived from human GBMs (Huszthy et al., 2012). Xenografts derived from these ECLs generally show a homogeneous, undifferentiated histology, probably indicating the higher selection pressure of *in vitro* serum-containing conditions during extensive culturing. Consequently, these xenografts no longer retain the original molecular

characteristics of parental tumors and lack human stromal and immune cells which are important for tumor growth and invasion (De Wever and Mareel, 2003).

Invasive GBM cells infiltrate the surrounding brain parenchyma and escape surgical resection and local therapeutic modalities, and are considered a principle reason for tumor recurrence and mortality (Giese et al., 2003). As identification of the mechanisms governing GBM cell invasiveness is mandatory for the development of therapeutic strategies that inhibit tumor recurrence, the establishment of a highly invasive GBM preclinical model provides a deep insight into GBM cell invasiveness. However, U-87MG, which are commonly used human GBM ECLs propagated in serum supplemented medium, grew as well-differentiated tumors with a regular shape, sharply demarcated margins, and nodular areas that do not recapitulate the observed pattern of GBM growth in humans (Hashizume et al., 2010). Although we previously established highly invasive and stem cell-like subclones via four rounds of serial *in vivo* intracranial transplantations of U-87MG cells (Jin et al., 2011), several limitations of such artificial models still hamper our understanding of the potential invasiveness of GSCs.

The disadvantages of ECL xenografts have subsequently been overcome by the development of GBM PDXs, whereby human tumor tissue fragments removed surgically are directly injected into the brain of mice or are serially passaged subcutaneously in immunodeficient mice, to preserve intra-tumor heterogeneity due to good retention of GSCs (Jimeno et al., 2009; Yu et al., 2010; Joo et al., 2013; Rosfjord et al., 2014). Alternatively, primarily dissociated patient-derived cells (PDCs) or PDX cells can be cultured as nonadherent spheroids in growth factor-defined, serum-free medium prior to orthotopic transplantation (Huszthy et al., 2012; Tentler et al., 2012). PDXs share many advantages of ECL models including high penetrance and short latency *in vivo*, especially in PDXs from clinically aggressive cases such as recurrent GBMs (Joo et al., 2013). However, unlike ECLs, PDXs can predict clinical success faithfully and allow mechanistic studies of action by recapitulating the molecular diversity and cellular heterogeneity observed in patient tumors (Giannini et al., 2005; Hodgson et al., 2009; Joo et al., 2013; Yost et al., 2013). For instance, we previously demonstrated the utility of orthotopic implantation of GBM PDXs derived from acutely dissociated GBM cells as clinically relevant models based on the significant correlation between the invasiveness of parental and corresponding xenograft tumors (Fig. 1) (Joo et al., 2013). Furthermore, the development of these techniques has been critical in defining the functional heterogeneity in human GBMs and exploring the biological and therapeutic implications of GSCs (Nduom et al., 2012; Sottoriva et al., 2013; Wang et al., 2013; Stieber et al., 2014). As we continue to develop drugs that target GSCs, PDX models that facilitate the characterization of these cell populations will be at the forefront of cancer drug discovery. For example, TMZ showed a wider response range in GBM PDX models without prior serum-based culture than in human ECL models of GBMs, suggesting that these newer models may more accurately reflect the clinical efficacy of TMZ (Kitange et al., 2009; Hirst et al., 2013). Finally, these models provide a microenvironment including vessels and human stromal cells in early passages, offering increased clinical relevance for tumors with stromal involvement.

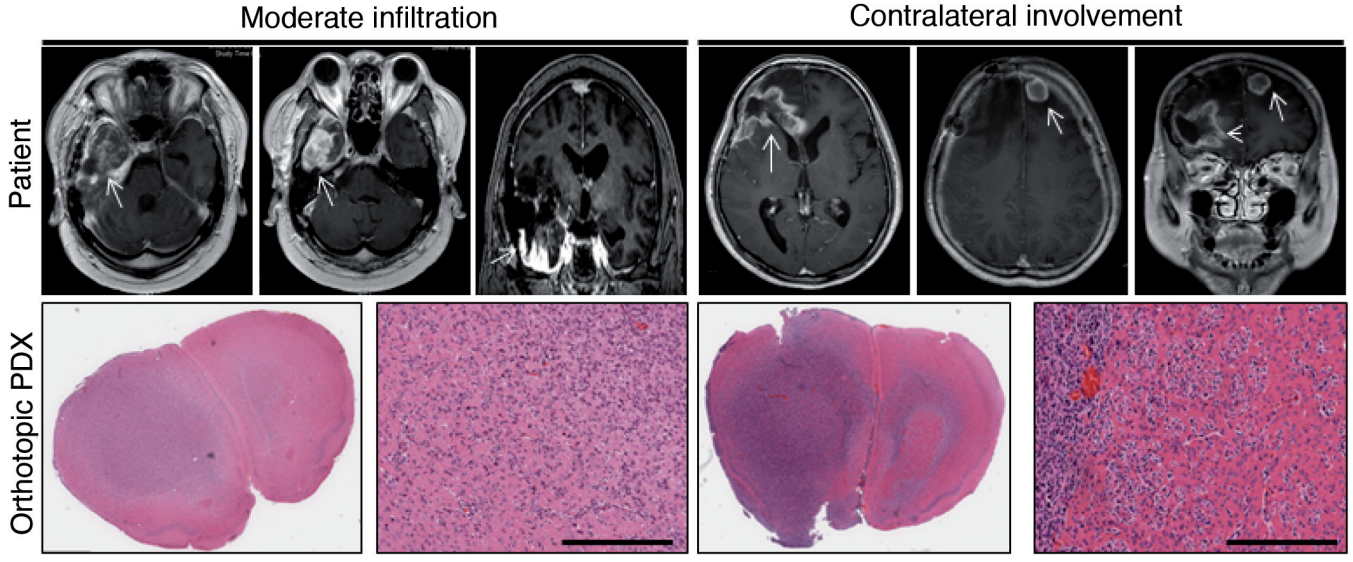

Fig. 1. Patient-mimicking GBM orthotopic PDX models convey unmet clinical needs. The direct orthotopic transplantation of primarily dissociated GBM patient-derived cells can recapitulate the infiltrative and invasive growth pattern observed in GBM patients. White arrow: tumor. Scale bar: 300 μm.

As such, GBM PDXs have the potential to reflect the clinical activity of novel drugs more accurately, and readily identify predictive molecular characteristics as an ideal preclinical test platform (Jimeno et al., 2009).

Pros of GBM orthotopic models

Major hurdles to drug delivery in GBMs

Important delivery considerations for novel therapies for GBMs include effective transport across the BBB, enhanced movement through brain and tumor tissue to achieve distribution in regions with infiltrating tumor cells, and sustained multi-modal actions on tumor cells (Groothuis et al., 2007; Neuwelt et al., 2011). Indeed, the ability of TMZ to penetrate the BBB and reach the diffusely infiltrated tumor cells due to favorable brain pharmaco-kinetic (PK) profiles is a likely reason for its clinical efficacy (Agarwal et al., 2011), while the failure of EGFR TKIs was likely due in part to their poor brain PKs. In addition to size and physico-chemical restrictions, the presence of active efflux pumps and the integrity of the BBB, influence the access of the drug to brain parenchyma and the tumor itself (Groothuis et al., 2007; Neuwelt et al., 2011), causing relatively low drug concentrations in the tumor surroundings (Portnow et al., 2009). BBB break-down is often heterogeneous throughout GBMs and generally remains intact in brain regions with infiltrating cells (Nathanson and Mischel, 2011).

More recently, poor distribution of agents within the brain and/or tumor tissue itself has emerged as a major delivery challenge (Baish et al., 2011). The extracellular space in brain tissue represents the major route for the transport of many signaling molecules and metabolites, as well as therapeutic and diagnostic substances (Sykova and Nicholson, 2008). Importantly, this space may be significantly altered in and around GBMs, further increasing the challenge of movement within the extracellular space (Vargova et al., 2003; Papadopoulos et al., 2005). More closely defining the size limits and surface property characteristics required for movement within the brain extracellular space has greatly aided the establishment of effective drug delivery systems.

Important players in GBM microenvironment

The tumor microenvironment in the brain consists of numerous specialized cell types that include brain-resident and brain-infiltrating cells such as astrocytes and microglia/macrophages. Astrocytes are the most abundant glial cell population comprising approximately 50% of the human brain volume (Charles et al., 2012). Astrocyte activation in GBMs is known as reactive gliosis and shows cellular hypertrophy and up-regulation of Glial Fibrillar Acidic Protein (GFAP) (Zhang and Olsson, 1995). These cells play an important role in GBM progression through a variety of different mechanisms including the promotion of cancer cell proliferation and invasion (Marchetti et al., 2000; Hoelzinger et al., 2007). Interestingly, astrocytes may protect cancer cells from chemotherapy-induced apoptosis by sequestering intracellular calcium via direct contact with GBM cells (Lin et al., 2010; Chen et al., 2015). Finally, astrocytes are also implicated in immunosuppression in the CNS by impairing the antigen-presenting capability of monocytes/microglia to T-cells for promoting T-cell activation (Kostianovsky et al., 2008), and inducing apoptosis in brain-infiltrating T-cells by expressing CD95L (Bechmann et al., 2002).

Microglia/macrophages account for 8-78% of all cells in human gliomas (Morantz et al., 1979) and the microglia/macrophages associated with the brain tumor have been shown to proliferate, which most likely contributes to their increased quantity (Badie et al., 2001). Microglia are primary immune effector cells of the CNS and are capable of generating significant immune responses via at least two different and functionally distinct morphological states, termed as activated and reactive/amoeboid microglia (Yang et al., 2010; Kettenmann et al., 2011). Activated microglia have a hyper-dilated stellate morphology and express only major histocompatibility complex class I (MHCI). Reactive microglia are large cells with amoeboid morphology that express both MHCI and MHCII, and therefore possess increased antigen presenting capability as well as phagocytic activity (Kettenmann et al., 2011).

One of the most commonly detected phenomena in GBM is the abundant macrophage infiltration without apparent phagocytic activity (Hao et al., 2002). The percentage of tumor-associated macrophage (TAM) infiltrating GBM can reach up to 30% of tumor mass (Cretu et al., 2005). Accumulating evidence has suggested that the TAM infiltration can be linked to the poor prognosis in GBM (Abou-Ghazal et al., 2008) and significantly higher numbers of TAMs were detected in adult mesenchymal GBMs compared to non-mesenchymal tumors (Engler et al., 2012). The infiltration of TAMs in GBM can be, at least in part, ascribed to GBM cancer cells. Multiple soluble factors produced by GBM cancer cells, including glial cell-derived neurotrophic factor (GDNF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and transforming growth factor (TGF-β1) are involved in TAM recruitment (Wu et al., 2010; Ku et al., 2013; Sielska et al., 2013). The high percentage of supportive TAM in GBM tumor mass makes it possible to be a good target for GBM treatment (Zhou and Bao, 2014).

Activated microglia and TAMs are different populations with distinct specific surface antigens (Saederup et al., 2010; Mizutani et al., 2012), different functions and distinct distribution within tumors (Roggendorf et al., 1996) in GBM tumor microenvironment. Microglia is different from monocyte-derived TAMs and whether it attenuates or promotes GBM tumor growth is still questionable (Liu et al., 2008). In general, TAMs in GBMs are not likely to be classically activated macrophages that are supposed

to attenuate tumor growth by phagocytosis (Black et al., 1992). TAMs in GBMs manifested strong M2 tumor supportive macrophage characteristics (Tran et al., 1998; Ludwig et al., 2000; Komohara et al., 2008). Several lines of evidence underscore the supportive role of TAMs in GBM tumor progression by promoting proliferation (Wagner et al., 1999; Jenny et al., 2006; Samaras et al., 2007; Fonseca et al., 2012), facilitating neo-vascularization (Hirano et al., 2001; Tanioka et al., 2001; Kanamori et al., 2006), contributing to resistance to radio-chemotherapy (Deininger et al., 2001), enhancing invasiveness of GBM cells (Wesolowska et al., 2008; Coniglio et al., 2012), and interfering with the functions of other immune cells to help GBM tumor progression (Cowan et al., 1991; Morford et al., 1999).

However, it could be extremely difficult to eliminate the TAMs in GBMs. A more attractive hypothesis is to restore the anti-tumor activities in TAMs to destroy the GBM cancer cells. Several preclinical studies have taken this approach. It was demonstrated that ectopic expression of a membrane associated isoform of M-CSF in GBM cells elicited an anti-tumor response along with TAMs infiltration in a rat intracranial model (Graf et al., 1999), suggesting that M-CSF may re-activate TAMs into a tumor suppressive phenotype. Moreover, some preliminary studies in GBM rodent models have proved the potential to treat GBMs by depleting TAMs. Targeting tumor-associated macrophages in C6 glioma xenografts in nude mice with a recombinant immunotoxin to Folate receptor beta (FR-beta) significantly depleted TAMs and reduced tumor growth (Nagai et al., 2009). Propentofylline (PPF), an atypical methylxanthine, significantly decreased tumor growth in a CNS-1 rat model of GBM by targeting TAMs but not tumor cells (Jacobs et al., 2012a,b).

Applications of orthotopic GBM PDX models

To date, the subcutaneous (heterotopic) model remains a popular method for assessing both anti-tumor efficacy and overall tolerability in the early screening of new drugs *in vivo*, due to their reproducibility, easier tumor transfer and precise monitoring of tumor growth (Kim et al., 2009). However, the brain provides a unique environment with paracrine growth factors that differ from most other organs (Zhang et al., 2009). A major limitation of subcutaneous xenografts is that these models fail to account for native GBM micro-environmental influences on tumor pathogenesis and drug response. They have limited pathophysiological relevance and clinical predictability given the absence of critical stromal and micro-environmental interactions with tumor cells (Abate-Shen, 2006; McMillin et al., 2013).

Since the biological function and druggability of mutational targets are likely tissue specific, it is necessary to prove the importance of the drug-targetable gene mutations in the specific clinical context to be investigated. Preclinical data from models that do not accurately reflect tumor histology or its native organ-based microenvironmental interactions are likely to generate misleading results (Becher and Holland, 2006). Recent studies have provided a strong rationale for the clinical investigation of NT113, a novel ErbB inhibitor, and AMG 595, an antibody drug conjugate composed of maytansinoid DM1 attached to a highly selective anti-EGFRvIII antibody, which demonstrate good activity against intracranial GBM xenografts in which wild-type EGFR or EGFRvIII is highly expressed through superior partitioning to intracranial compartments (Yoshida et al., 2014; Hamblett et al., 2015). Therefore, systematic drug efficacy screening in multiple genomically characterized orthotopic GBM PDXs might be useful for prospective identification of sets of potentially responsive tumors. Recently, patient-mimicking GBM orthotopic PDX models generated by implanting GBM cells directly into intra-cranial space of mice, present many opportunities to discover novel effective therapeutics. We established a GBM orthotopic PDX library that can functionally represent tumor heterogeneity and the biology of original GBMs in situ (Joo et al., 2013). The preclinical and clinical implications of our platform were validated by the recapitulation of pathologic characteristics such as proliferation, invasiveness and angiogenesis, the patient-specific response to standard treatments and genomic alterations observed in the parental GBMs. More importantly, our data indicated that the *in vivo* tumorigenic potential and invasion property of primarily cultured GBM cells is associated with clinical aggression in the corresponding patients, suggesting the involvement of 'tumorigenesis' and 'invasion' signatures associated with poor prognosis in GBM patients.

Moreover, the subcutaneous models also do not accurately reflect PK effects because they cannot recapitulate the significant obstacle posed by the BBB and the brain/tumor tissue itself, for the delivery of therapeutics (Nathanson and Mischel, 2011). Since subcutaneous models can overestimate the therapeutic potential of novel agents, their role in prioritizing drugs for clinical investigation should be minimized. For example, palbociclib, a selective inhibitor of the cyclin-dependent kinases CDK4 and CDK6, has failed when tested in orthotopic models due to inability to pass through the drug efflux pumps in the BBB while it shows efficacy in subcutaneous xenografts (Parrish, 2013). MK-1775 that targets Wee1, a regulator of DNA damage checkpoints, was similarly ineffective when combined with TMZ in a GBM orthotopic PDX model due to limited distribution, whereas MK-1775 exhibited both single-agent and combinatorial activity with TMZ in a subcutaneous flank model (Pokorny et al., 2015). Preclinical PK studies in orthotopic GBM models have the potential to predict the clinical failure of targeted agents on the basis of poor PKs if used prior to, or concurrent with, initiation of advanced clinical trials.

Clinical benefits from standard therapies against GBM are limited in part due to the intrinsic treatment resistance of GBM and inefficient targeting of GSCs. On

the bases of the high plasticity of GSCs, modulation of xenograft features depending on the grafting site can be expected because microenvironment factors strongly affect GSC growth and development (Galli et al., 2004). The maintenance of GSC population depends on the presence of the so-called perivascular niche (Calabrese et al., 2007). More importantly, a role for the microenvironment in GBM radio-resistance was validated through the differential response of GSCs and non-GSCs irradiated under *in vitro* and orthotopic conditions (Jamal et al., 2012). Recently, we demonstrated that ubiquitination-specific protease 1 (USP1)-mediated protein stabilization promotes GSC maintenance and radio-resistance using GBM PDCs and orthotopic PDXs, providing a rationale for USP1 targeting as a GBM-specific therapeutic approach (Lee et al., 2015). Another study demonstrated the *in vitro* and *in vivo* efficacy of the on-target Janus kinase 2 (JAK2)/STAT3 pathway using a large set of molecularly diverse GBM-derived brain tumor stem cells (BTSCs) (Stechishin et al., 2013).

The cellular components of the tumor stromal microenvironment as well as soluble cytokines, chemokines, cell matrix components, and adhesion proteins not only influence the natural history of tumor proliferation, angiogenesis, and invasion, but are also specifically capable of altering the response of tumors to therapeutic agents (Loi et al., 2011; McMillin et al., 2013). Therefore, supportive tumor microenvironment in the brain may be a suitable target in anti-GBM therapies, as well as a valuable biomarker for prognostic purposes. For example, one study has identified Tenascin-C (TNC), an extracellular matrix protein overexpressed in GBMs, as a promoter of GSC invasiveness through a mechanism involving disintegrin and metalloproteinase domain-containing protein 9 (ADAM-9) proteolysis (Sarkar et al., 2015). The relevance of ADAM-9 to tumor invasiveness was validated using resected human GBM specimens and orthotopic xenografts where elevation of ADAM-9 and TNC expression was prominent at the invasive front of the tumor. Another study also suggested that targeting Livin, a member of a family of apoptosis inhibitor proteins, using cell-permeable peptides may be an effective therapeutic strategy for tumor microenvironment-induced treatment resistance through a synergistic therapeutic effect with radiation and TMZ in intracerebral GBM-bearing mice (Hsieh et al., 2015).

Finally, *in vivo* RNAi screening in patient-derived GBM models can be a powerful tool to identify and validate potential candidate oncogenic effectors or tumor suppressors in GBM. By implementing combined oncogenomics with *in vivo* RNAi screening using the GBM orthotopic PDX models (Sa et al., 2015), we found that Nemo-Like Kinase (NLK) plays a critical role in tumor restriction through regulation of Wnt/β-catenin pathway and mesenchymal activity in GBM.

Clinical relevance of GBM orthotopic PDX platform

Here we briefly describe our research experience in utilizing GBM orthotopic PDX platform to support their essential roles in personalized medicine for GBM. Establishment of PDXs offers the potential of representing an expanded genetic diversity as well as studying tumor evolution during recurrence if longitudinal samples that failed to respond to conventional therapies are available (pre-/post-treatment). Isocitrate dehydrogenase 1 (IDH1) mutations are predominant in secondary GBM (>75-80%) and are rare in primary GBM (5%) (Ohgaki et al., 2014). It has been found that IDH1 is a marker of secondary GBM and that those primary GBMs diagnosed with IDH1 mutations may have been secondary gliomas that rapidly progressed to GBM with no early low-grade clinical symptoms experienced by patients. Clinically, IDH1 mutation is a favorable prognostic marker, because patients with this mutation had significantly better overall survival (Parsons et al., 2008). In case the PDX model is not established such as in tumors with IDH1 mutation, recurrent tumor samples can be implanted into mice for use in drug validation. For example, we acquired surgically resected treatment naïve secondary GBM and matched the recurrent tumor from a 36-year-old male patient with IDH1 R132H (arginine to histidine) mutation, which is the most common mutation (Fig. 2A) (Balss et al., 2008; Nobusawa et al., 2009; Watanabe et al., 2009; Yan et al., 2009). Following

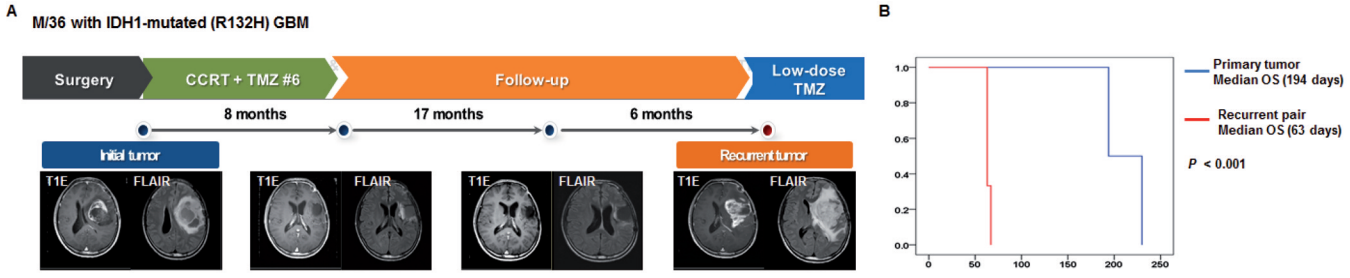

Fig. 2. Establishment of clinical relevant GBM orthotopic PDXs via intra-cranial implantation of primary tumor and matched recurrent tumor. **A.** Clinical course of a 36-year-old male with secondary GBM harboring IDH1 R132H mutation patient. **B.** Kaplan-Meier survival curves for orthotopic PDXs generated from initial tumor and paired recurrent tumor after conventional therapy.

standard concurrent RT/TMZ followed by 6 cycles adjuvant monthly TMZ, a cranial magnetic resonance imaging (MRI) examination demonstrated increased size of the contrast-enhancing lesion and significantly increased surrounding T2/fluid-attenuated inversion recovery (FLAIR) consistent with progressive disease after a disease free period of 30 months. The GBM orthotopic PDXs generated from recurred GBM demonstrated a more aggressive phenotype than those from initial tumor (Fig. 2B). Unfortunately, he presented with rapid progression resulting in the patient's death due to acquired resistance to low-dose TMZ.

IDH mutations are thought to arise early in gliomagenesis and persist during progression to secondary GBM. Of great interest is that the IDH1 status never changed between primary and recurrent GBMs, which suggests that these tumors are initiated by the clonal expansion of cells with IDH1 mutant function (Johnson et al., 2014). Furthermore, an array-based comparative genomic hybridization (CGH) analysis revealed Met gene amplification in paired recurrent tumor (Fig. 3A). The GBM orthotopic PDXs generated from recurred GBM demonstrated up-regulated MET and mutated IDH1 (R132H) compared with those from

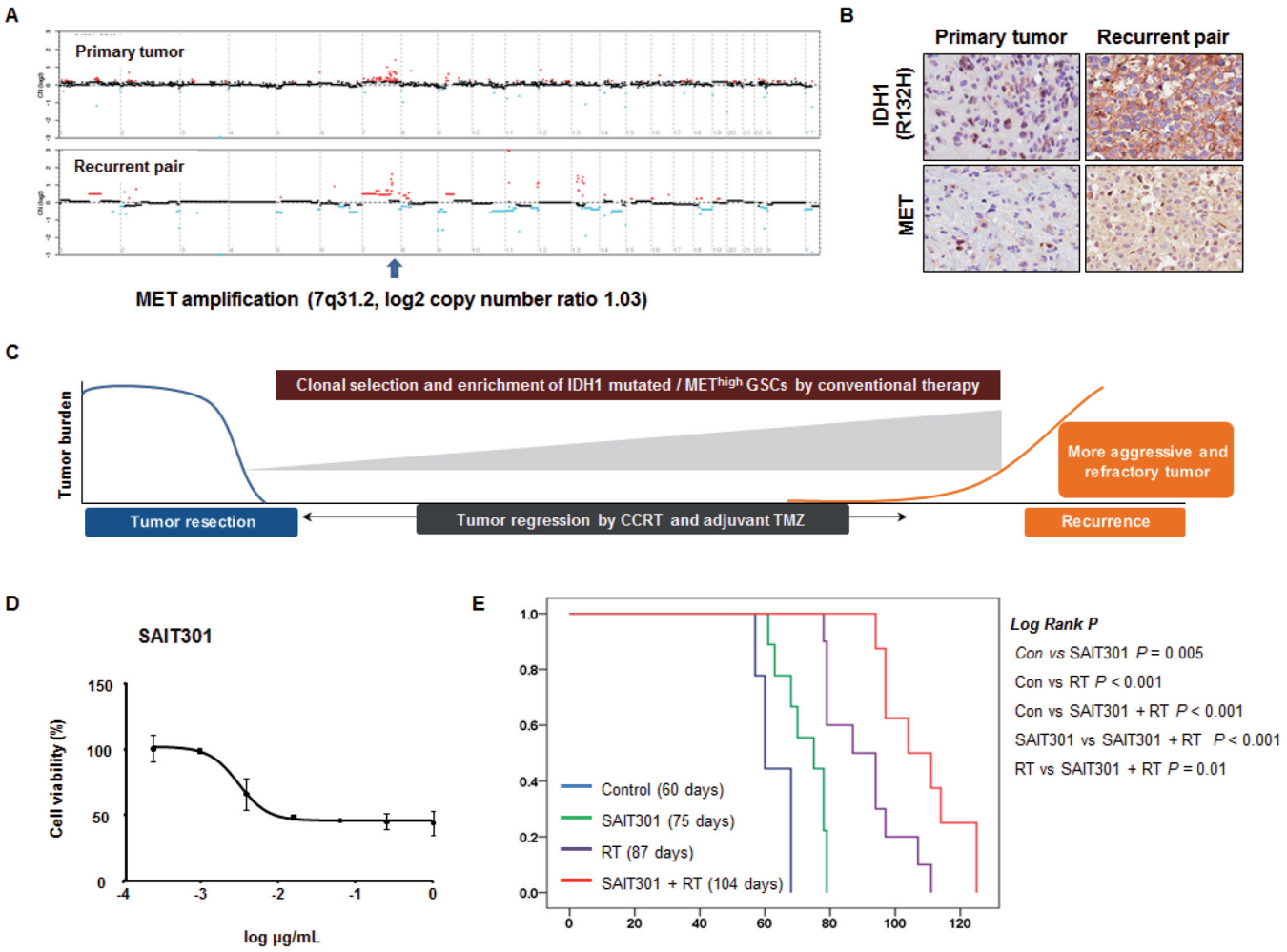

Fig. 3. Validation of anti-tumor efficacy of targeting MET in GBMs harboring MET gene amplification using orthotopic PDX platform. **A.** Array CGH analysis showing MET gene amplification in recurrent tumor tissue. **B.** Relative up-regulation of MET and mutated IDH1 (R132H) in immunohistochemical staining of orthotopic PDXs from recurrent tumor compared with those from initial tumor. **C.** Lethal tumor recurrence induced by clonal selection of IDH1 mutated and MET-high GSC population after standard therapies. **D.** GSCs from recurrent tumor-derived orthotopic xenografts were sensitive to SAIT 301 treatment alone *in vitro*. The relative cell viability (%) represents percent growth compared to the control group (no antibody treatment) and was measured after treating with various concentrations of SAIT301 for 6 days. **E.** Kaplan-Meier survival analysis of BALB/c nu/nu mice transplanted intracranially with GSCs isolated from recurrent tumor-derived orthotopic xenografts treated with SAIT301 or RT or their combination. Differences between survival curves were compared using a log-rank test. All experiments repeated in triplicate with >6 mice per arm. SAIT301 targeting MET enhanced the antitumor response to γ-radiation in pre-established intracranial GBM xenografts. P<0.05 between RT + SAIT301 antibody arm and all other arms.

the primary tumor (Fig. 3B), suggesting aggressive growth of recurrent tumor through clonal selection and enrichment of IDH1 mutated MET^{high} GSC population after treatment (Fig. 3C). A high level of MET amplification is found in ~4% of GBM tumors (Cancer Genome Atlas Research, 2008; Verhaak et al., 2010). In contrast to bulk tumor cells, GSCs survive irradiation and chemotherapy treatment better and therefore are thought to contribute to therapeutic resistance and tumor recurrence (Bao et al., 2006; Liu et al., 2006; Salmaggi et al., 2006; Kang and Kang, 2007). MET activation is a functional requisite for GSC activity and thus represents a promising therapeutic target (Jun et al., 2014). Studies on the genomic heterogeneity of GBMs at the single cell level revealed that a small fraction of GBM cells within a tumor contain focal amplification of MET that is independent of other RTKs (Snuderl et al., 2011; Szerlip et al., 2012). Taken together, MET plays a central role in maintaining GSC populations in human GBMs, suggesting a link between MET signaling and GSCs (Li et al., 2011; De Bacco et al., 2012; Joo et al., 2012; Kim et al., 2013). Kong et al. (2009) and Liu et al. (2011) reported that up-regulation of MET is associated with poor survival outcomes and poor treatment responses in GBM. Therefore, inhibiting the MET pathway by the anti-HGF neutralizing monoclonal antibody L2G7 (Rath et al., 2013) or crizotinib (Zou et al., 2007) potently decreased tumor growth and the expression of stem cell markers in a pre-established GBM xenograft model (Joo et al., 2012).

It is believed that one of the mechanisms by which the therapeutic anti-MET antibodies induce anti-tumor effects in Met overexpressing tumors is via internalization and subsequent degradation of MET from the cell surface. Recently, a potent and selective bivalent Met-targeting antibody (SAIT301) that promotes a Castias B-lineage lymphoma (Cbl) E3 ligase-independent, LRIG1-mediated MET degradation pathway was developed (Lee et al., 2014a). MET degradation by SAIT301 does not require MET activation, and SAIT301 dramatically inhibits growth of tumors with low or no Cbl expression (Lee et al., 2014a; Oh et al., 2014). In addition, MET inhibition by SAIT301 resulted in highly significant inhibition of cell migration and invasion induced by early growth response protein (EGR-1) (Lee et al., 2014b). Consistent with previous reports, treatment with SAIT301 led to a dose-dependent growth inhibition of GBM PDX cells derived from recurrent pair (Fig. 3D), and a strong anti-tumor efficacy in orthotopic PDXs as a monotherapy (10 mg/kg i.v. 3 times per week, Fig. 3E). Importantly, SAIT301 enhances the radiosensitivity of recurred tunmor-derived GSCs under orthotopic *in vivo* conditions (Fig. 3E), consistent with a recent report showing that Targeting the SF/HGF/c-Met pathway markedly potentiates the anti-GBM response to γ-radiation (Lal et al., 2005). Improved survival was demonstrated with combination SAIT301 plus RT compared with either modality alone: median survival was 60 days in the control arm, 75 days in the SAIT301 antibody arm, 87 days in the RT arm, and 104 days in the RT plus SAIT301 therapy arm (all pair-wise combinations $P<0.05$ by log-rank Mantle-Cox). These findings provide strong preclinical evidence to support combining strategies that target MET pathway using antibody with γ-radiation in the treatment of GBMs with high MET expression by MET gene amplification, validating the role of MET in these clinically relevant GBM models.

Tumor angiogenesis has emerged as a primary target of drug development for GBMs over the past decade. It is controlled through a complex balance of angiogenic factors, including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGFR), hypoxia-inducible factor (HIF) 1α, and others (Batchelor et al., 2007; Wick et al., 2010). Due to the importance of VEGF in angiogenesis, targeted efforts to block VEGF pathways have been ongoing for several years, focusing on monoclonal antibodies and tyrosine kinase inhibitors. Bevacizumab, a recombinant humanized monoclonal antibody against VEGF-A, received accelerated approval in 2009 for recurrent GBM in the United States and many other countries based on radiographic response rates (Friedman et al., 2009; Kreisl et al., 2009). Thereafter, attempts to augment the benefit of single-agent bevacizumab included studies evaluating bevacizumab combined with chemotherapeutics (Francesconi et al., 2010; Desjardins et al., 2012), targeted therapies (Drappatz et al., 2012; Galanis et al., 2013) and re-irradiation (Cabrera et al., 2012; Cuneo et al., 2012). Unfortunately, all of these combinatorial regimens failed to improve outcome beyond that of bevacizumab monotherapy. In addition, recent large-scale clinical trials in patients with newly diagnosed GBM failed to deliver a significant prolongation of overall survival by persistent therapeutic effects of bevacizumab (Norden et al., 2008; Chinot, 2012; Gil-Gil et al., 2013; Arrillaga-Romany et al., 2014).

Resistance to bevacizumab inevitably develops, and such patients typically die rapidly due to ineffective therapy (Kreisl et al., 2009; Reardon et al., 2011, 2012). Adaptive resistance after bevacizumab treatment has been characterized by a transition to a mesenchymal and more invasive and infiltrative phenotype (Bergers and Hanahan, 2008; Norden et al., 2008; Chinot, 2012; Lu et al., 2012; Piao et al., 2013). Previously, we found that bevacizumab treatment did not improve overall survival in several GBM orthotopic PDX models and made xenograft tumors more invasive (Joo et al., 2013), which suggests that xenograft tumors derived from GBM surgical samples would efficiently predict the results of clinical trial, while U-87MG glioma cell line generates orthotopic xenograft tumors responding to bevacizumab therapy. Understanding the molecular mechanisms of resistance to anti-angiogenic therapy is a critical unmet need to improve patient outcome. The tumor and its microenvironment release alternative proangiogenic factors (DeLay et al., 2012; Lu and Bergers, 2013) and

recruit pro-angiogenic myeloid cells (de Groot et al., 2011, 2012) to promote VEGF-independent angiogenesis. We recently identified the cytoskeleton protein Talin1 (TLN1) as a key regulator of bevacizumab-resistance by utilizing patient-derived GBM xenografts and serial orthotopic transplantation of *in vivo* bevacizumab-treated GBM cells (accepted by Oncotarget). Bevacizumab resistant clones established by serial orthotopic transplantation were highly enriched with stem-like features and invasive growth pattern. Through comparative transcriptome analysis between untreated and bevacizumab-treated groups, TLN1 was obtained as a novel therapeutic target for GBM to overcome resistance to anti-angiogenic therapies. More importantly, we validated the role of TLN1 in clinically relevant GBM orthotopic PDX models by confirming that TLN1 targeting not only attenuated malignant characteristics of GBM cells but also reversed the resistance to the bevacizumab treatment. Unfortunately, none of the promising neuroimaging, histologic, and circulating markers associated with clinical benefit from VEGF inhibitors among patients with GBM have been validated yet. With these translational research platforms, more studies focusing on predictive biomarkers and escape mechanisms are feasible.

Conclusions

GBM orthotopic PDX models are not widely used in spite of many inherent advantages including clinical relevance and response predictability, because these models are technically challenging due to increased animal morbidity upon orthotopic surgical implantation, latency periods, and varying tumorigenic potential, and require specialized *in vivo* imaging facilities to monitor the growth of tumor xenografts (Bibby, 2004). Additionally, PDX models may be problematic for evaluating effective immunotherapy in GBM as they are derived from highly immune-compromised mice strains (Alvarnas et al., 2001; Shiow et al., 2008; Wang et al., 2008; Ishizawa et al., 2010).

However, patient-mimicking GBM orthotopic PDXs can present many opportunities for discovery of novel effective therapeutics bridging the gap between genomic alteration profiles and drug efficacy *in vivo*. They enable the assessment of the effects of targeted or cytotoxic agents on primary tumor growth in the appropriate tumor microenvironment, as well as impact on tumor invasiveness and the emergence of acquired therapeutic resistance, indicating that they are highly useful resources for individually tailored treatment strategies for patients with GBM. In order to evaluate the predictive accuracy of these models and maximize their utility for biomarker discovery and development, preclinical studies in PDX models should be performed earlier in the drug development process, ideally prior to initiating clinical trials or concurrently as was done in the 'PreCision Neuro-Oncology (PCNO) trials.' In theory, tumor tissue from GBM patient surgery can be implanted into immunocompromised mouse to establish the orthotopic PDX model. During the PDX growth, genomic analysis of this tumor can be performed and candidate drugs can be selected using accumulated associations between genome alterations and drug efficacy data generated by high-throughput screening of patient-derived GSCs. Once the orthotopic PDX model is ready, candidate drugs can be validated *in vivo*. In case the PDX model is not established, surgical or biopsy samples derived from more aggressive recurrent GBM can be implanted into mice for use in drug validation. Generation of relatively large number of GBM orthotopic PDX models with different genomic background panels may thus represent a useful experimental setting to investigate patient-tailored approaches by high-throughput techniques. A deeper scientific understanding of basic biologic principles of drug delivery to the CNS, immune surveillance in the CNS, predictive imaging technologies for brain tumors, and novel clinical trial designs will also be necessary.

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