Title: Glioblastoma-initiating cell heterogeneity generated by the cell-of-origin, genetic/epigenetic mutation and microenvironment

Toru Kondo

Division of Stem Cell Biology, Institute for Genetic Medicine, Hokkaido University, Sapporo, 060-0815, Japan

Correspondence: Toru Kondo
Division of Stem Cell Biology, Institute for Genetic Medicine, Hokkaido University
Kita-15, Nishi-7, Kita-ku, Sapporo 060-0815, Japan

Tel: +81-11-706-6082, Fax: +81-11-706-7870
E-mail: tkondo@igm.hokudai.ac.jp

Abstract

Glioblastoma (GBM) and other malignant tumours consist of heterogeneous cancer cells, including GBM-initiating cells (GICs). This heterogeneity is likely to arise from the following: different sets of genetic mutations and epigenetic modifications, which GICs gain in the transformation process; differences in cells of origin, such as stem cells, precursor cells or differentiated cells; and the cancer microenvironment, in which GICs communicate with neural cells, endothelial cells and immune cells. Furthermore, considering that various types of GICs can be generated at different time points of the transformation process, GBM very likely consists of heterogeneous GICs and their progeny. Because cancer cell heterogeneity is responsible for therapy resistance, it is crucial to develop methods of reducing such heterogeneity. Here, I summarize how GIC heterogeneity is generated in the transformation process and present how cell heterogeneity in cancer can be addressed based on recent findings.

Keywords: Glioblastoma (GBM) , GBM-initiating cells (GICs), heterogeneity, Temozolomide (TMZ), dihydroorotate dehydrogenase (DHODH)

1. Introduction

Glioblastoma (GBM) is a malignant glioma with a median survival of approximately 15 months [1,2]. Despite the development of multimodal treatments involving surgery, chemotherapy and radiotherapy, the overall survival rate of patients with GBM has not improved over the past few decades. The discovery of GBM-initiating cells (GICs) has strongly impacted the direction of GBM research, as these cells have been shown to possess strong tumourigenic ability and to be resistant to irradiation and anticancer drugs such as temozolomide (TMZ) [3-7]. It is therefore crucial to characterize GICs and to identify new methods and compounds that specifically eliminate GICs. Although researchers have elucidated essential factors and mechanisms associated with the characteristics and maintenance of GICs, the development of new therapeutic methods and the identification of anti-GIC compounds through screening remain problematic for the following reasons. First, it is uncertain whether all kinds of GICs from patients can be maintained using current culture methods. Second, there is little information about the heterogeneity of cultured GICs. Third, there is no method for maintaining GICs as a homogenous population in culture. In this review, I discuss these hurdles and present recent findings as possible approaches to overcome such challenges and treat GBM.

2. Tumour heterogeneity

2.1. Cell of origin in GBM

Cancers arise from cells that acquire genetic mutations, epigenetic regulation or both. To characterize cancer appropriately, it is also essential to determine the cell of origin as well as genetic/epigenetic alterations acquired. Globus and Kuhlenbeck have suggested that malignant brain tumours arise from cells residing in the ventricular/subventricular zone (VZ/SVZ), where neural stem cells (NSCs) exist [8]. Hopewell and Wright demonstrated that in the brains of mice, tumours were frequently induced from the VZ/SVZ when tumour promoters, such as 3,4-benzpyrene and 20-methylcholanthrene, were transplanted into various regions [9]. Furthermore, Holland et al. succeeded in inducing GBM-like tumours in transgenic mice that express the avian leukaemia virus receptor; expression was controlled by the promoter of either the NSC marker Nestin or the astrocyte marker Glial fibrillary acidic protein via virus infection with the *Ras* oncogene [10]. In addition, DePinho and colleagues induced malignant glioma in mice by transplanting NSCs that express the constitutively active form of *epidermal growth factor receptor* (*EGFR*) [11]. Kondo and colleagues showed that the combination of oncogenic *Ras* overexpression and p53 inhibition induced NSCs and oligodendrocyte precursor cells (OPCs) to transform into GICs, which formed brain tumours with pathological features of human GBM and that the same mutations did not transform the differentiated mature oligodendrocytes but did astrocytes to form non-transplantable lower grade tumours, suggesting that these cells need additional mutation(s) to transform into GICs [12-14]. These authors also revealed that OPCs lost their original characteristics and acquired NSC characteristics (reversion) during the transformation process [15,16]. Using *p53* and *type 1 neurofibroma* (*NF1*) mutant–mosaic analysis

with a double-marker (MADM) mouse model, Liu et al. found significant aberrant growth prior to malignancy only in OPCs but not in any other NSC-derived lineages or NSCs themselves [17]. Overall, these findings suggest that NSCs, OPCs and astrocytes are possible cells of origin for GBM and that non-NSCs acquire NSC characteristics in their transformation process, contributing to tumour heterogeneity, including GICs and differentiation marker-positive cancer cells (Fig. 1A).

2.2. Genetic/epigenetic mutations in GIC development

Among genes involved in GBM development, both the Cancer Genome Atlas Research Network and Parsons et al. have independently revealed that genes mutated in GBM are involved in three signalling pathways: p53 (approximately 90%), retinoblastoma (Rb) (approximately 80%) and receptor tyrosine kinase (RTK) (approximately 90%) pathways [18,19].

Loss of p53 function is essential to promote accelerated cell proliferation and malignant transformation [20]. Indeed, over 65% of human gliomas have been shown to contain *TP53* gene deletions and mutations [21]. Moreover, additional evidence indicates that other p53 signalling factors, including *Murin-double-minute 2* (*MDM2*), which binds to, destabilizes, and inactivates p53, and *chromodomain helicase DNA binding domain 5* (*Chd5*), which regulates cell proliferation, cellular senescence, apoptosis, and tumourigenesis, are mutated in malignant glioma [22,23]. Although the effector molecule of the p53 pathway is the p21 cyclin-dependent kinase (cdk) inhibitor, which regulates the progression of cells through the G1 cell cycle phase, it has not been demonstrated that the *p21* gene itself is an oncogenic target in human cancers.

Mutations in the Rb signalling pathway, including *cdk4* amplification and *p16/Ink4a* deletion, are frequently identified in many types of malignant tumours [24]. The hypophosphorylated form of Rb sequesters the E2F transcription factor and arrests cells at the G1 checkpoint. After Rb is hyperphosphorylated by cyclin D and the cdk4/6 complex, phosphorylated Rb releases E2F, which induces expression of cell cycle regulators, and the cells enter S phase. In contrast, the p16/Ink4a cdk inhibitor binds to cdk4/6, prevents complex formation by cdk4/6 and cyclin D, and maintains Rb hypophosphorylation [25].

Signalling pathways (Ras/Raf/MAPK and phosphatase tensin homologue (PTEN)/AKT pathways) of RTKs, including *EGFR*, *platelet-derived growth factor* (*PDGF*) *receptor α* and *transforming growth factor-beta* (*TGFβ*) *receptor*, many of which participate in the maintenance of GICs and amplifying precursors, are also frequently mutated in tumours [26]. For example, *EGFR* is overexpressed in up to 60% of gliomas [21]. It has also been shown that the small GTP protein *Ras*, an essential oncogene, and its negative regulator *NF1* are mutated in many types of human cancers, including glioma; moreover, *PTEN*, which inhibits the function of Akt-activating phosphoinositol tri-phosphate kinase (PI3K), is frequently inactivated in malignant glioma [27]. In addition, TGFβ has been shown to induce the proliferation of GICs through stimulation of the PDGFβ and LIF signalling pathways [28,29]. Furthermore, TGFβ activates the Sox4-Sox2 axis and polycomb complex protein Bmi1 (B lymphoma Mo-MLV insertion region 1 homologue), both of which are essential regulators in GIC, to maintain tumourigenesis [30,31]. Considering that TGFβ is an inducer of regulatory T cells, which inhibit immune system activation, these results suggest that TGFβ is a central player in gliomagenesis. On the basis of these findings, many researchers have

revealed that various combinations of oncogene activation and tumour suppressor gene inhibition cause NSCs, OPCs or astrocytes to become GICs, apparently contributing to heterogeneity in progressive cancer.

Similar to genetic mutations, epigenetic modifications have been shown to be involved in GBM development. Abdouh et al. found that both polycomb repressive complex1 (PRC1) and 2 (PRC2) are involved in the self-renewal of GICs and their tumourigenesis: both oncogenes Bmi1 and EZH2, the main components of PRC1 and PRC2, respectively, are expressed in CD133-positive GICs and GBM. *Bmi1* knockdown prevents GIC proliferation and tumourigenesis by inducing apoptosis and differentiation, while inhibition of EZH2 impairs GBM growth [32].

In another study, Gallo et al. showed that expression of the histone 3 variant H3.3 is negatively regulated by the epigenetic repressor mixed lineage leukaemia 5 (MLL5), which controls self-renewal and tumourigenesis by orchestrating reorganization of the chromatin structure. Given that H3.3 prevents self-renewal of GBM cells and promotes their differentiation, the authors concluded that MLL5/H3.3-associated epigenetic status maintains self-renewal hierarchies in GBM [33].

Gene expression profiles of GICs as well as bulk tumours have revealed that GICs were classified into four molecular subtypes, classical, pro-neural (PN), mesenchymal (MES) and neural [34]. MES-GICs display more aggressive phenotypes than the others and are resistant to chemoradiotherapies, resulting worse prognosis. It has also been shown that GICs are derived from PN-GICs and MES-GIC but not from classical-type GBM. Wang et al. addressed whether PN-GICs and/or MES-GICs are sufficient to generate GBM heterogeneity [35]. Using single-cell/nucleus RNA sequencing of 28 gliomas and single-cell assays for transposase-accessible chromatin

sequencing, these authors elegantly showed that GICs reside on a single axis of variation ranging from PN to MES. *In silico* lineage tracing using transcriptome and genetics analyses has also revealed that MES-GICs are progenitor cells of PN-GICs. Correctively, these results elucidate the lineage relationship between glioma cell types further generate GBM heterogeneity.

It is likely that cell of origin, genetic/epigenetic mutations, GIC microenvironment or these combination generate the molecular subtypes in GBM. According to Bhat et al., expression of *transcriptional coactivator with PDZ-binding motif* (*TAZ*, also known as *WW domain-containing transcription regulator 1*, *WWTR1*) is lower in PN-GBMs and lower-grade gliomas than in MES-GBMs due to hypermethylation of the *TAZ* promoter [36]. The researchers demonstrated that knockdown of *TAZ* in MES GICs prevents proliferation, invasion and tumourigenesis but that its overexpression in PN GICs and NSCs induces expression of MES markers and their transdifferentiation into osteoblasts and chondrocytes. These findings indicate that *TAZ* promoter methylation status determines MES phenotypes in GBMs. Taken together, combined genetic/epigenetic mutations cause cell of origin to transform GICs.

Recent progress of genome editing technology make it possible to manipulate specific genetic/epigenetic changes in GICs [37]. We can normalize genetic mutations or delete oncogenes using a combination of CRISPR-associated 9 nuclease (Cas9) and specific signal guide RNA (sgRNA). In addition, the finding of a catalytically inactive Cas9 (dCas9) enabled to manipulate gene expression, positively and negatively. Using dCas9 fused with VP64 transcriptional activation domain (dCas9-VP64) and a specific single guide RNA (sgRNA), Maeder et al. have successfully shown to activate expression of VEGFA and NTF3 in human cells [38]. Gao et al. have also succeeded to

induce mouse embryonic fibroblast to become induced pluripotent stem cells using dCas9-VP64 and sgRNA targeting Oct4 and Nanog [39]. Gilbert et al. demonstrated that combination of dCas9 fused with Krüppel-associated box (KRAB) domain of Kox1 (dCas9-KRAB) and sgRNA repressed expression of endogenous target genes, trasnferrin receptor and chemokine receptor type 4, in HeLa cells [40]. Taken together, these findings suggested that we can modify expression of target genes positively and negatively in GICs, once we establish its delivery system into GICs.

2.3. Oncogene or tumour suppressor gene, the first hit in GIC development

In the beginning of tumorigenesis, the cell of origin of cancer undergoes either genetic mutation or epigenetic alteration (first hit). The mutated cells (pre-transformed cells) proliferate and then acquire further different mutations (second hit, third hit, further hits) and eventually transform into cancer cells (Fig. 1B). After the first hit in tissue-specific stem cells, they generate pre-transformed stem cells and differentiating (precursor) cells via asymmetric division. Pre-transformed stem/precursor cells are thought to readily become GICs through second or third hit acquisition, whereas non-proliferating/differentiated pre-transformed cells may not transform into GICs as these cells do not easily acquire genetic mutations, suggesting that the first hit may occur in tumour suppressor genes and increase the tumorigenicity by accelerating proliferation.

On the other hand, there are many evidences that oncogenes are the first targets in tumorigenesis, even though oncogene activation induces cellular senescence, a state of permanent cell-cycle arrest [41]. For instance, the premalignant tumours, including Nevi and colon adenomas, have been shown to increase the expression of senescence associated beta-gal and INK4A, definitive markers of cellular senescence [42,43].

Nonetheless, when premalignant cells lose senescence mediators, such as p53 and the histone methyltransferase of lysine 9 in histone 3 that is involved in the heterochromatin formation, the cells bypass senescence states and transform into malignant tumours, raising a question of how premalignant tumour cells lose the expression of senescence mediators in their progression. Feng et al have recently uncovered that proto-oncoprotein c-Myc, one of downstream effectors of EGFR, not only drives cell proliferation but also inactivates p53 by inducing the Long noncoding RNA Inactivating p53 (MILIP) [44]. Ye et al have shown that Zeranol, produced from a natural carcinogenic product Zearalenone, induced heterochromatin formation in p53 promoter by the DNA methyltransferase 1-dependent manner, preventing the p53 expression [45]. Ladds et al have also found that inhibitors of dihydroorotate dehydrogenase (DHODH), a key enzyme in de novo pyrimidine synthesis pathway, killed melanoma cells by increasing p53 synthesis [46]. These findings suggest that various types of pre-transformed cells obtain different mutations/modifications at different time points in their transformation process, generating heterogeneity in cancer, although it remains to elucidate how and when genetic/epigenetic mutations happen.

2.4. GIC microenvironment

GICs as well as other CICs are likely retained in a special microenvironment, the GIC niche, formed by various types of cells, including endothelial cells (ECs), mesenchymal stem cells (MSCs), glial cells, neurons and immune cells (Fig. 1C). These niche cells express many factors, such as Notch ligands, TGFβ, interleukin-6 (IL-6), neuroligin-3 (NLGN3) and scavenger receptors, to maintain the stemness, proliferation and motility of GICs [47]. For example, Li and Neaves showed that ECs express the Notch ligands

Dll4 and *Jagged 1*, which activate Notch1 and 2 on GICs, and that GICs secrete VEGF, which promotes the migration and proliferation of both ECs and MSCs [48].

Zhou et al. demonstrated that periostin generated by GICs polarizes macrophages/microglia into the M2 phenotype (tumour-associated macrophages/microglia, TAMs) and induces GIC proliferation through integrin αvβ3 [49]. In fact, there is much evidence that TGFβ from TAMs induces expression of matrix metalloproteases (MMPs) in GICs and promotes their invasion and that TGFβ from GICs also polarizes macrophages/microglia towards the TAM phenotype [50-53].

Two teams have shown that oesophageal cancer-related gene 4 (Ecrg4, also known as Chromosome 2 open reading frame 40 (c2orf40) and Augurin), a hormone-like peptide, acts as a novel type of tumour suppressor [54-57]. Using mouse GIC models, Moriguchi et al. found that *Ecrg4*-deficient GICs form tumours with characteristics resembling those of human GBM, including necrosis, haemorrhage and massive angiogenesis, in the brains of immunocompetent mice; in contrast, Ecrg4-expressing GICs tended to be eliminated by the immune system [58]. By using retrovirus-mediated expression cloning, the authors also identified scavenger receptors, including lectin-like oxidized LDL receptor-1, CD36 and Scarf1, as receptors of the Ecrg4 (71-132 amino acid) fragment. Furthermore, they reported that Ecrg4 (71-132) induces microglial secretion of inflammatory factors, such as IL-6 and tumour necrosis factor α, via the NF-κB signalling pathway [59]. Independently, Lee et al. discovered that the carboxy terminal fragment of Ecrg4 (133-148) is able to recruit microglia/macrophages and activated them, resulting in tumour regression [60]. The team also found that Ecrg4 (133-148) binds to Toll-like receptor 4, becomes internalized and modulates inflammation via non-canonical NF-κB signalling [61,62].

Collectively, these finding indicate that Ecrg4 is an essential intercellular communication factor in the GIC niche.

In addition, much evidence indicates that cross-talk between reactive astrocytes and GICs promotes tumour progression. Physical contact between astrocytes and glioma cells through gap junctions reduces the cytotoxicity of chemotherapeutic drugs in glioma cells [63]. IL-6 produced by astrocytes induces expression of cytomembrane *MMP14* in glioma cells and increases their migration and invasion via activation of MMP2 [64]. Astrocytes also supply L-glutamine, which is essential for GBM growth [65]. Furthermore, when GICs are transplanted into the brain parenchyma, they tend to migrate into the VZ/SVZ, move along the zone, and form disseminated tumours, suggesting that the VZ/SVZ cells secrete chemoattractants, such as Cxcl12 (also known as the stromal cell-derived factor, SDF1), for GICs and foster them as well as NSCs [66,67, Kondo et al., unpublished observation]. Thus, GICs are exposed to many factors in the niche, raising the question of whether all GICs can be maintained and expanded in GIC culture medium composed of serum-free NSC medium supplemented with basic FGF and EGF.

Neurons have been also shown to communicate with glioma cells by paracrine signaling and synapse formation. Venkatesh et al have shown that a soluble form of synaptic protein NLGN3, which is secreted from active neurons, increases glioma proliferation by inducing the expression of FOS and NLGN3 [68,69]. Yu et al revealed that glioblastoma cells keep proliferate by increasing the glypican 3-dependent synapse formation with tumour-surrounding neurons, while two groups demonstrated that neurons promote glioma progression through AMPA receptor-dependent neuron-glioma synaptic communication [70]. Although it is important to examine whether GICs also

communicate with neurons using same mechanism, these findings suggest that neuronal activity apparently contributes to gliomagenesis.

There is increasing evidence that extracellular microvesicles (EVs), such as exosomes, contain a portion of the mRNA, microRNA and proteins of the cells secreting them and are delivered to target cells that are nearby or even at a long distance and affect their characteristics [71-74]. Therefore, EVs have been extensively investigated as important intercellular communication factors. Skog et al. discovered that exosomes from GBM contain 6 angiogenic proteins, IL-6, IL-8, tissue inhibitor of metalloprotease (TIMP)1, TIMP2 and angiogenin, and activate angiogenesis. The authors also detected *EGFR variant III* (*EGFRvIII*) mRNA in the exosomes. Given that exosomes can be purified from body fluids, such as the blood and cerebrospinal fluid, exosomes may be employed to analyse the EGFRvIII status of GBM patients [75]. In addition, Gabrusiewicz et al. have shown that GIC-derived exosomes promote the immune-suppressive M2 phenotype in monocytes, which involves expression of programmed death-ligand 1, by transferring components of the STAT3 signalling pathway [76]. Mirzaei et al. have also reported that GIC-secreted exosomes contain tenascin-C, which suppresses T cell activity through interaction with α5β1 and αvβ6 integrins on T cells [77]. However, Figueroa et al. found that glioma-associated MSCs release exosomes that induce proliferation of GICs and increase their clonogenicity by *miR-1587*-dependent downregulation of the nuclear receptor corepressor 1 [78].

GICs may be present both in the vascular niche, which continuously supplies nutrients and oxygen to GICs, and in the hypoxic area, where both nutrients and oxygen are relatively low. Therefore, it is likely that the gene expression profile of GICs is largely dependent on the niche. Indeed, according to Kathagen et al., there is a

reciprocal metabolic switch between the pentose phosphate pathway (PPP) and glycolysis in GICs [79], whereby expression of glycolytic enzymes is upregulated in hypoxia but downregulated in normoxia. In contrast, PPP enzymes display the inverse expression pattern. Exosomes from GBM cells in hypoxia were shown to contain hypoxia-regulated mRNAs and proteins and induce angiogenesis by activating ECs [80]. Moreover, Bao et al. revealed that GICs express hypoxia-inducible transcription factor (HIF) 1α and HIF2α, which regulate many genes involved in angiogenesis, metabolism, proliferation and survival in hypoxia, even in normoxia, and play essential roles in GIC tumourigenesis [81]. These results indicate that an unknown mechanism maintains expression of HIF1α/2α in GICs in the vascular niche or the hypoxic area.

3. Single-cell analysis for characterising heterogeneous cancer cell populations

To understand tumour heterogeneity properly, knowledge of how many types of GICs exist in GBM is essential. Many researchers have unveiled heterogeneity using two types of single-cell analyses: (1) clonal analysis of GICs and (2) direct analysis of single cells prepared from GBM (Fig. 2).

Hide et al. established single GIC clones from mouse GIC models and revealed that only 2 of 10 clones, all of which were *p53* deficient and expressed oncogenic *Ras*, retained tumourigenicity. Analysis of gene expression profiles has revealed that these tumourigenic clones differed, suggesting that mouse GIC models consist of heterogeneous GICs. By comparing the gene expression profiles of tumourigenic and non-tumourigenic clones, 547 and 402 genes were observed to increase and decrease, respectively, in both tumourigenic clones. Among these genes, the authors focused on Sox11 and Plagl1, as their expression also decreased and increased, respectively, in

human GICs. It was demonstrated that *Sox11* overexpression inhibits GIC proliferation by preventing expression of *plagl1* but that enforced expression of *plagl1* promoted tumourigenesis in non-tumourigenic clones, and the authors concluded that Sox11 and Plagl1 reciprocally regulate GIC tumourigenesis [12].

Meyer et al. established 44 single cell clones, which were directly prepared from human GBM tissues, and analysed their gene expression, proliferation, differentiation, tumourigenicity and drug resistance, observing heterogeneous expression of PTEN, EGFR and EGFRvIII and different sensitivities to TMZ in the tumourigenic clones and revealing the existence of heterogeneous GICs in human GBM [82].

Additionally, Chen et al. performed transcriptome analyses of single cells from primary and relapsed GBM and discovered three independent mutations in Ankyrin Repeat and PH Domain 1 (ASAP1), CD44 and catenin α-like 1 protein (CTNNLA1), all of which are involved in RAS/GEF/GTP signalling regulation, in relapsed GBM. Based on meta-analysis of NIH Genomic Data Commons, they confirmed expression of the three genes to be increased in GBM [83].

Because it examines the tumourigenicity of expanded lines, clonal analysis is the best method for analysing GIC heterogeneity. Nonetheless, in clonal analysis, it is necessary to verify that GICs are maintained as a homogenous population in culture. Additionally, there is concern about whether the direct analysis of single cells from GBM can unveil the characteristics of *bona fide* GICs because it is impossible to examine which single cell retains tumourigenicity. Therefore, novel methods for analysing heterogeneity in cancer still needed.

4. Discovery of chemicals and factors targeting TMZ-resistant GICs

Although radiation and anticancer drugs may kill proliferating GICs as well as cancer cells, some GICs are obviously resistant to these therapies due to enhanced DNA repair ability (e.g., Chk1/2), cytotoxicity neutralization (e.g., ALDH1A3), drug efflux (e.g., ABCG2) or their combination, leading to recurrence [11,84,85]. As GBM appears to contain heterogeneous GICs, each of which differs in sensitivity against cytotoxic compounds, the most effective therapeutic methods are to target factors/mechanisms, such as Notch, Sox2, STAT3 and EGFR, all of which are shared by all GICs. Notably, it should be considered that targeting such factors/mechanisms may cause severe side effects, with homeostasis loss, as tissue-specific stem cells very likely use the same factors/mechanisms for their maintenance and proliferation. An alternative method is to identify factors/mechanisms that specifically act on therapy-resistant GICs but not on therapy-sensitive GICs and normal cells, including tissue-specific stem cells (Fig. 3A).

Wang et al have been demonstrated that a drug combination synergistically targeting PN-GICs and MES-GICs effectively prevented the proliferation of U87 glioma cells *in vitro* [19]. These suggest that the chosen drug combination, which independently eliminates different types of GICs, is useful for GBM therapy with fewer side effects.

TMZ is currently used as a standard medicine for GBM, but its efficacy is limited, indicating the existence of TMZ-resistant tumourigenic GICs. To uncover the molecular mechanism involved in TMZ resistance, several laboratories have established TMZ-resistant GBM cells and have used them for chemical screening, knockdown/lethality screening and genome-wide CRISPR/Cas9 screening (Fig. 3B). Li et al. conducted whole-transcriptome sequencing analysis to screen TMZ-resistant

GBM cells and parental GBM cells to identify the molecular mechanism of TMZ resistance. They selected 55 microRNAs (miRs) with expression that was significantly changed by TMZ treatment. Using Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analyses, the authors noted *miR-1268a* that was decreased by TMZ treatment. They further demonstrated that overexpression of *miR-1268a* inhibits translation of ABCC1, an ABC transporter contributing to drug exclusion, but that its knockdown increased ABCC1 in GBM cells. These findings suggest that *miR-1268a* is involved in the multidrug resistance of TMZ-resistant GBM [86].

Ding et al. used a high-throughput synthetic lethality screen with a pooled short hairpin DNA repair library, in combination with TMZ, to identify targets that enhance TMZ-induced anti-tumour effects. BRCA1, which sensitizes TMZ-induced cell death in p53 wild-type GBM cells, was identified: *BRCA1* knockdown enhanced the cytotoxicity of TMZ in *p53*-wild-type glioma sphere-forming cells (GSCs) but not in *p53*-mutant GSCs. Moreover, *BRCA1* knockdown with TMZ increased DNA damage and cell death in *p53*-wild-type GSCs, and the authors concluded that the combination of BRCA1 inhibition and TMZ can be applied for GBM therapy [87].

In a genome-wide screening with a CRISPR/Cas9 library using TMZ-resistant *EGFRvIII*-expressing U87 cells (TMZ-resistant U87-EGFRvIII) and parental U87 cells, Huang et al. identified CD28, NF-κB and NFAT pathways as the most significantly altered pathways in TMZ-resistant U87-EGFRvIII. Ingenuity pathway analysis further revealed that the EGFRvIII/PI3K/AKT/NF-κB and G protein-coupled receptor (GPCR)/PLA/PKC/NFAT pathways are most responsible for TMZ resistance in U87-EGFRvIII cells. The authors eventually determined E2F6, a target of NF-κB, to be a

TMZ resistance gene in GBM and demonstrated that either knockdown of *E2F6* or pharmacological inhibition of NF-κB/E2F6 sensitizes GBM to TMZ [88].

Teng et al. performed drug screening against TMZ-resistant GBM cells and patient-derived stem-like neurospheres. They eventually found that hydroxyurea sensitized recurrent GBM to TMZ both *in vitro* and *in vivo*, regardless of *MGMT* promoter methylation status, tumour subtype or stem cell characteristics [89].

Tsukamoto et al. succeeded in establishing TMZ-resistant GICs (GICRs) by culturing GICs with 200 μM TMZ, which is 4 times higher than that in the blood of TMZ-treated patients, for 3 weeks. The GICRs as well as GICs proliferated similarly in culture, were positive for NSC markers, and formed tumours when transplanted into the brains of immunodeficient mice, indicating that the GICRs retained the characteristics of GICs [90]. The authors then performed chemical screening to identify new compounds, which killed GICRs but did not affect normal cells including NSCs, and eventually found that 1-(3-C-ethynyl-b-D-ribopentofuranosyl) uracil (EUrd) strongly kills both GICs and GICRs in culture and eradicates GICR tumours *in vivo* by blocking pyrimidine synthesis.

Same team have performed another drug screening by collaborating with the FUJIFILM company and finally identified novel compound 10580 that targets DHODH [91]. The researchers also discovered a new metabolic mechanism that de novo pyrimidine synthesis pathway is crucial for the survival and maintenance of GICs/GICRs: *DHODH* is more highly expressed in GICs/GICRs than in various types of normal cells, including NSCs. *DHODH* knockdown kills GICs. Small amounts of uridine diphosphate, a downstream product of pyrimidine biosynthesis, rescues 10580-induced cytotoxicity in GICs/GICRs, whereas large amounts of uridine, the substrate

for the salvage pathway, are required to achieve the same results. In addition, pyrimidine is mainly synthesized through the salvage pathway in many types of normal cells and tissues, particularly in the brain. Furthermore, the authors demonstrated that 10580 induced nuclear export of SOX2 and that blocked GIC tumorigenesis in vivo. Given that 10580 administration did not show any visible toxicity in mice, they concluded that 10580 is a potential candidate compound for GBM therapy (Fig. 3C).

Wang et al. have also reported a similar result: knockdown of *DHODH* by using specific short hairpin RNA induced cell cycle arrest in GICs and inhibited their tumourigenesis. It was also demonstrated that the combination of the DHODH inhibitor teriflunomide and the PI3K inhibitor BKM120 significantly prevented GIC tumourigenesis *in vivo* [92]. This combination was utilized because the medical efficacy and/or BBB permeability of teriflunomide may not be sufficient for preventing tumourigenesis *in vivo*. Nonetheless, given that pharmaceutical companies are now developing new DHODH inhibitors, such as ASLAN003 (http://aslanpharma.com/drug/aslan003/) and BAY2402234 (https://clinicaltrials.gov/ct2/show/NCT03404726), as promising anticancer compounds for acute myeloid leukaemia, pancreatic cancer, triple-negative breast cancer and melanoma, DHODH inhibitors may be used as promising GBM eradicating drugs in the near future [93-97].

5. Conclusion

To date, it is unknown how many types of GICs exist in GBM, and it is also uncertain whether all types of GICs can be maintained using the current culture methods. Therefore, establishing tailor-made medicines that eradicate individual GBM effectively and completely is challenging. It should be noted that targeting the factors/mechanisms that are shared among all GICs may cause severe side effects, with homeostasis loss, as tissue-specific stem cells very likely use the same factors/mechanisms for their maintenance and proliferation. At the moment, the best strategy is to target factors/mechanisms that are specifically essential for therapy-resistant GICs but not for therapy-sensitive GICs and normal tissue-specific stem cells. Accordingly, researchers have found DHODH to be a promising therapeutic target for GBM and have verified DHODH inhibitors including 10580, as potential anti-GBM drugs. Using similar methods, it is highly expected that innovative therapeutic drugs/methods without side effects will be developed in the near future.

Declaration of competing interest

No potential conflicts of interest were disclosed.

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Figure legends

Fig. 1 Tumour heterogeneity is generated by cell of origin, genetic mutation/epigenetic regulation and tumour environment factors

(A) Neural stem cells (NSCs) self-renew and generate mature neurons, astrocytes and oligodendrocytes via proliferating precursor cells (left). Similarly, glioblastoma-initiating cells (GICs) self-renew and generate differentiation marker-positive cancer cells. NSCs, oligodendrocyte precursor cells (OPCs) and astrocytes, GIC cells of origin, transform into at least 3 types of GICs when these cells acquire genetic mutations/epigenetic changes. It should be noted that OPCs acquire NSC characteristics and lose their specific characteristics in the transformation process (reversion). (B) The first hit (red) occurs in the cell of origin of GIC. During proliferation, the mutant cells acquire further hits (yellow, blue and grey), eventually generating GICs. Various combinations of hits in the p53, Rb and RTK signalling pathways can occur in proliferating mutant cells, generating GIC heterogeneity. The heterogeneous population contains both therapy-sensitive and -resistant cells. (C) Many types of tumour-surrounding cells, such as endothelial cells (EC), mesenchymal stem cells (MSCs), astrocytes, microglia/macrophages and neurons, foster GICs by maintaining stemness/proliferation (e.g., Notch signal, IL6, TGFβ, miR-1587, NLGN3) and supplying energy (e.g., L-Gln). In turn, GICs supply factors, such as TGFβ, periostin, angiogenic factors and STAT3 signalling components, and increase synapse formation through Glypican 3 to maintain their niche cells.

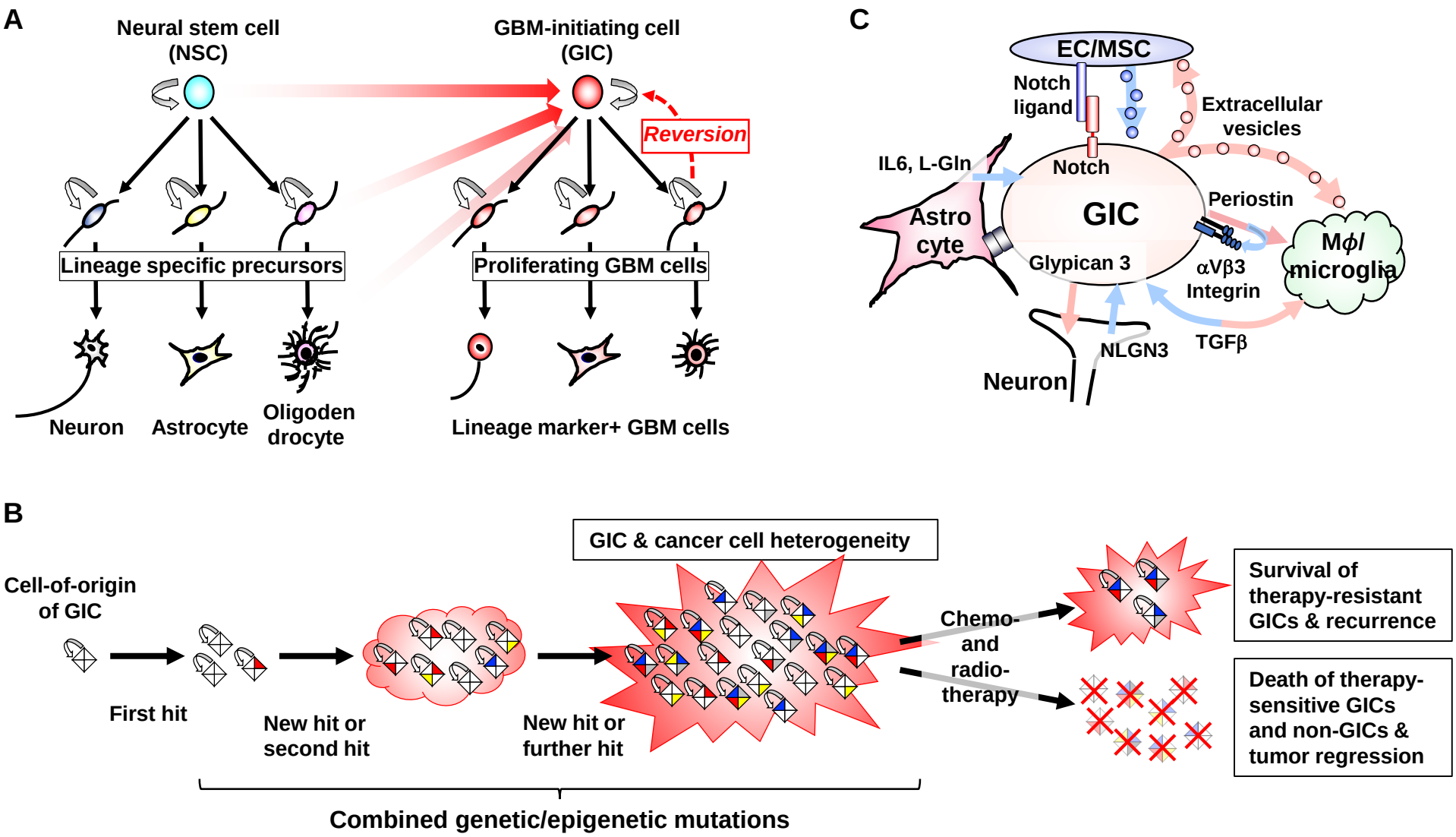
Fig. 2 Single cell analyses of GICs

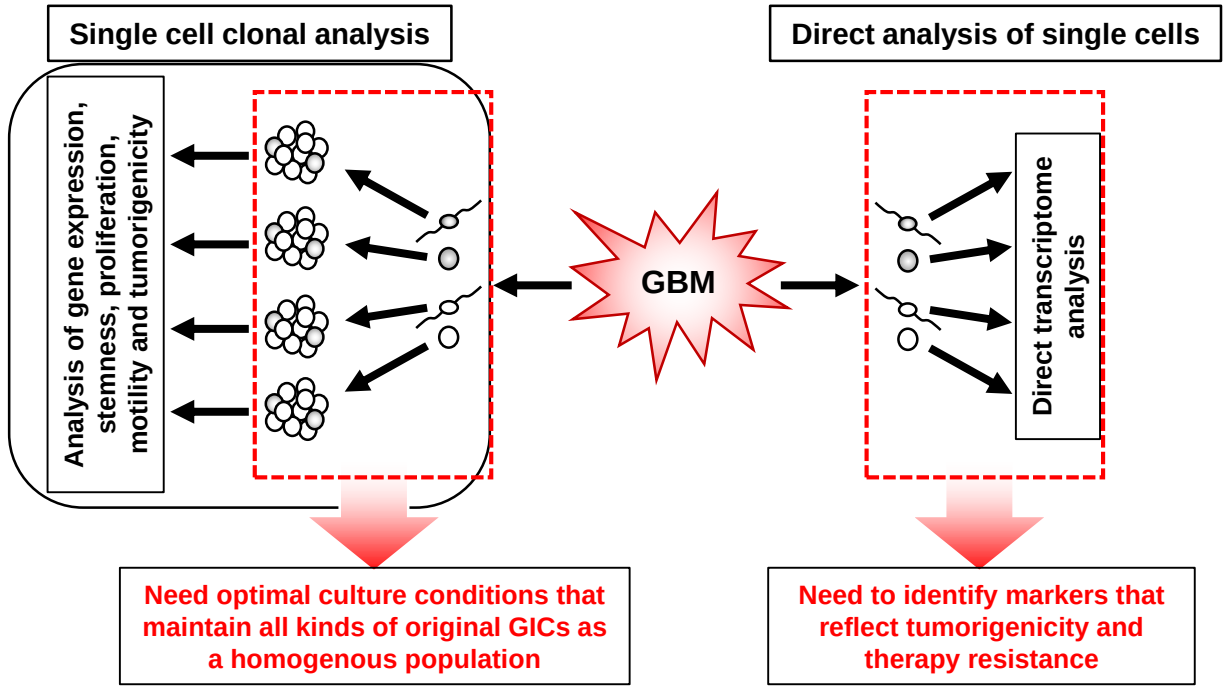
Two single-cell analyses, clonal analysis and direct analysis of single cells, can be applied to characterize heterogeneous GICs. Notably, identification of optimized culture conditions that maintain all types of GICs as a homogenous population is essential for clonal analysis. Additionally, essential markers, which reflect tumourigenicity and therapy resistance, should be determined for direct analysis of GBM-derived single cells.

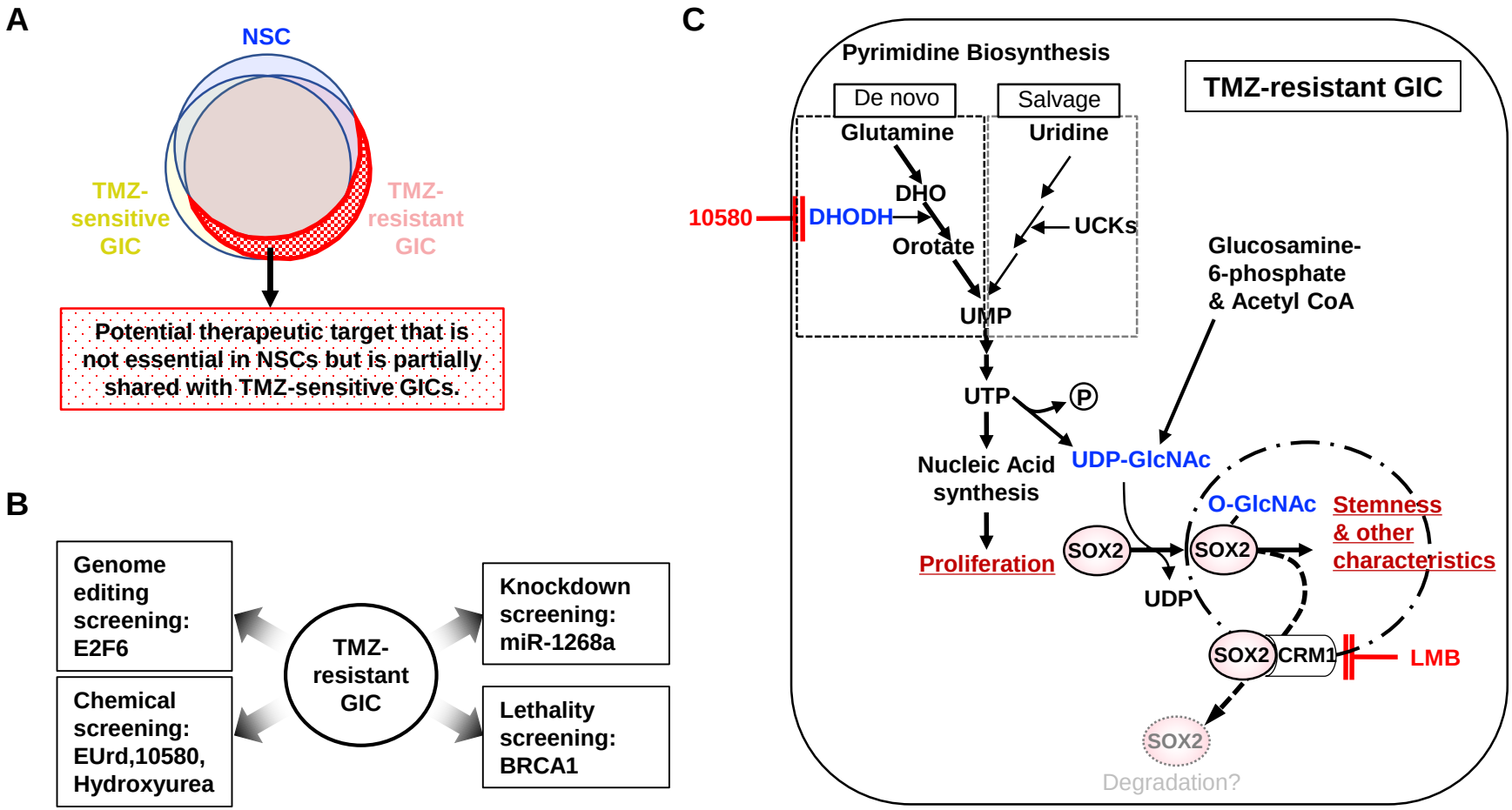
Fig. 3 Discovery of chemicals and factors targeting TMZ-resistant GICs

(A) TMZ-resistant GICs can be used for identifying critical factors/mechanisms and compounds for GBM therapy through comparison with both normal NSCs and TMZ-sensitive GICs. (B) By using TMZ-resistant GICs, various types of screenings have identified novel therapeutic targets, E3F6, *miR-1268a*, BRCA1 and DHODH, and novel anti-GBM compounds, EUrd, hydroxyurea and 10580. (C) Molecular mechanism of 10580-dependent cytotoxicity and stemness deprivation in GICs. 10580-dependent DHODH inhibition causes pyrimidine exhaustion, resulting in cell cycle arrest. Decreased pyrimidine also blocks UDP-GlcNAc production, inducing CRM1-dependent nuclear export of SOX2 in GIC.

Kondo, Figure 1




Single cell clonal analysis
Direct analysis of single cells
Analysis of gene expression, stemness, proliferation, motility and tumorigenicity
GBM
Direct transcriptome analysis
Need optimal culture conditions that maintain all kinds of original GICs as a homogenous population
Need to identify markers that reflect tumorigenicity and therapy resistance


A
NSC
TMZ-sensitive GIC
TMZ-resistant GIC
Potential therapeutic target that is not essential in NSCs but is partially shared with TMZ-sensitive GICs.
B
Genome editing screening: E2F6
Chemical screening: EUrd,10580, Hydroxyurea
TMZ-resistant GIC
Knockdown screening: miR-1268a
Lethality screening: BRCA1
C
Pyrimidine Biosynthesis
TMZ-resistant GIC
De novo
Salvage
Glutamine
Uridine
DHO
10580
DHODH
UCKs
Orotate
UMP
Glucosamine-6-phosphate & Acetyl CoA
UTP
P
Nucleic Acid synthesis
UDP-GlcNAc
O-GlcNAc
Stemness & other characteristics
Proliferation
SOX2
SOX2
UDP
SOX2
CRM1
LMB
SOX2
Degradation?