Rapid Reprogramming of Primary Human Astrocytes into Potent Tumor-Initiating Cells with Defined Genetic Factors

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Abstract

Cancer stem-like cells (CSC) are thought to drive brain cancer, but their cellular and molecular origins remain uncertain. Here, we report the successful generation of induced CSC (iCSC) from primary human astrocytes through the expression of defined genetic factors. Combined transduction of four factors, Myc, Oct-4, p53DD, and Ras, induced efficient transformation of primary human astrocytes into malignant cells with powerful tumor-initiating capabilities. Notably, transplantation of 100 transduced cells into nude mice was sufficient for tumor forma-

tion. The cells showed unlimited self-renewal ability with robust telomerase activities. In addition, they expressed typical glioma stem-like cell markers, such as CD133, CD15, and CD90. Moreover, these cells could form spheres in culture and differentiate into neuron-like, astrocyte-like, and oligodendrocyte-like cells. Finally, they also displayed resistance to the widely used brain cancer drug temozolomide. These iCSCs could provide important tools for studies of glioma biology and therapeutics development.

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Introduction

Cancer stem cells (CSC) or tumor-initiating cells are tumor cells with characteristics of normal stem cells and strong ability to form tumors in mice (1–3). They have been shown to play key roles in carcinogenesis (4, 5), metastasis (6, 7), and tumor response to treatment (5, 8, 9). CSCs have been isolated from a wide variety of cancer types. Examples of those include: leukemia (1), glioma (3), and breast cancer (2), etc. However, there are still many confu-
sions and controversies concerning CSCs (10, 11). For example, in many instances, there is no consensus on the most appropriate molecular markers to identify CSCs, in contrast to normal tissue stem cells or embryonic stem cells (11). Despite tremendous growth in CSC research in recent years, the molecular mechanisms involved in the development and maintenance of CSCs are poorly understood.

We are particularly interested in brain CSCs because they are one of the first to be isolated, and they have been implicated in tumor therapeutic responses (5, 8, 9). An important question in brain cancer is whether differentiated brain cells, such as astro-

ocytes, can dedifferentiate and become CSCs. One approach to answer this question is to carry out oncogene-mediated in vitro transformation of astrocytes and determine whether brain CSC-like cells could be obtained. Previously, successful in vitro transformation of primary human astrocytes has been achieved by serial transduction of oncogenes, such as SV40T(t) antigen, HPV E6, E7 genes, H-ras, and hTERT genes (12, 13). The transformed cells can form colonies in soft agar and are tumorigenic in nude mice. However, they were significantly less tumorigenic when compared with patient-derived human brain CSCs in their tumorigenic abilities, and there was no evidence that the transformed cells possess CSC properties.

In this study, we attempted to derive CSCs from cultured primary human astrocytes, which are the cells of origin for several types of brain tumors, including glioblastoma multiforme, a devastating brain malignancy with very poor prognosis. Drawing lessons from the success in making induced pluripotent stem cells (iPSC) from differentiated human cells, we were able to generate brain CSC-like cells from primary human astrocytes with only 4 defined genetic factors.

Materials and Methods

Tissue culture conditions

Normal human primary astrocyte cells purchased from Lonza (cat# CC-2565) in 2012 were used in the experiments. Cells arrived as passage 3 and were cultured in AGM BulletKit Astrocyte Growth Medium (Lonza cat# CC-3186). U87MG cells (ATCC) were obtained in 2012 from the Tissue Culture Facility of the Duke Cancer Institute (Durham, NC), which validated the cell lines by the use of microsatellite analysis. U87MG and the transformed astrocytes were cultured in DMEM plus 10% FBS. In some experiments, the transformed cells were also cultured in neurosphere growth medium. A patient-derived glioma stem cell line ALPS1459, which was derived from a patient at Duke University Medical Center (Durham, NC), was obtained in 2012. It was
cultured in neural stem cell growth medium: DMEM/F12 supplemented with nonessential amino acid, glutamine, B-27 supplement without vitamin A, 0.2% heparin, 20 ng/mL EGF, and 25 ng/mL b-FGF.

**Soft agar assay**

Soft agar assay were carried out according to established protocols (14). Growth of cells in soft agar was determined by plating 0.5–5 × 10^4 cells in triplicate in 0.3% Noble agar in 6-well tissue culture plates. Three weeks after plating, soft agar plates were stained with 0.05% crystal violet. Colonies were then photographed and counted with the aid of a microscope.

**Neurosphere formation**

Transformed cells were cultured at low density (1–2 cells/mm^2) on uncoated plates in neurosphere growth medium (DMEM/F12 supplemented with nonessential amino acid, glutamine, B-27 supplement without vitamin A, 0.2% heparin, 20 ng/mL EGF, and 25 ng/mL b-FGF). Cells were cultured up to 10 days, during which time they were monitored for neurosphere formation. To generate secondary neurospheres, primary neurospheres harvested dissociated with 0.05% trypsin/EDTA and replated under identical neurosphere growth conditions for an additional 10 days.

**Tumor growth in vivo**

For tumor xenograft experiments, athymic nude mice (6–8 weeks old, The Jackson Laboratory) were used. For subcutaneous tumor growth, 100 of the transformed cells grown in neurosphere medium were injected subcutaneously into the hind leg of a mouse. Intracranial tumor cell injection was carried out using a published protocol (15) in commercially obtained nude mice. Briefly, transformed astrocytes were labeled with an EGFP and luciferase fusion reporter introduced through lentivirus infection. About 500 cells in 3 μL of DMEM/F12 medium were injected into the brains of mice (4–5 weeks old of immunodeficient athymic nude mice, The Jackson Laboratory). Tumor growth was monitored for intracranial bioluminescence by use of an IVIS Kinetic imager (PerkinElmer) available through the Optical Molecular Imaging and Analysis Core of Duke Cancer Institute. Tumor samples from mouse brain were fixed in 10% formalin. The samples were embedded with paraffin, and sections were made for immunohistochemical or hematoxylin and eosin (H&E) analysis. All animal procedures described above have been reviewed and approved by the Institutional Animal Use and Care Committee at the Duke University Medical Center.

Additional information on methods used in this study is provided in the Supplementary Information section.

**Results**

We decided to include factors involved in making iPSCs (16) in our transformation protocol because we reasoned that there should be a significant amount of epigenetic reprogramming if successful transformation of differentiated astrocytes into CSCs were to happen. We therefore tested iPSC-inducing factors, Oct4, Nanog, Klf4, Sox2, and Lin28, in addition to established oncogenic factors, H-Ras(G12V), Myc(T58A), p53DD (a dominant negative form of p53), cyclin D1, CDK4(RC24), and hTERT. The latter factors have been shown to induce oncogenic transformation in primary human mammary epithelial and muscle cells independent of oncogenes (17). All factors were cloned into recombinant lentiviral vectors. The vectors were then used to infect primary human astrocytes following a scheme depicted in Supplementary Fig. S1A. We evaluated each of the five iPSC factors in combination with a cocktail of the six oncogenic factors for their ability to transform the astrocytes. Transduced astrocytes showed a generally similar pattern of behavior: initial cell death, survival of a small fraction of cells, and finally, growth and expansion from the surviving cells (Fig. 1A). Transduced cells that survived and proliferated showed a very different morphology when compared with parental astrocytes (Fig. 1A). After 2 to 3 weeks, colonies were visible in the Petri dish after staining with crystal violet, indicating cellular survival and clonal expansion (Fig. 1B). It is interesting to note that the timing of the emergence of the transformed astrocyte colonies was very similar to the induction of iPSCs. After the transformed cells grew to sufficient numbers, we evaluated their ability for anchorage-independent growth in soft agar, which is a well-recognized characteristic of tumorigenic cells. Our results show that cells transduced with Oct4 or Klf4 in combination with the 6-gene oncogenic cocktail (6G in short) could grow in soft agar and form large colonies after 3 weeks (Fig. 1C). Cells derived from other gene transductions did not form any soft agar colonies. In further experiments, we show that cellular transformation and growth in soft agar could be achieved using only 4 factors: Oct4, Myc(T58A), H-ras(G12V), and p53DD (OMRP or Oct4 + 3G; Fig. 1D). These 4 factors could not be reduced any further. Among OMRP-transduced cells, about 0.45% (~45 of 10,000 seeded) of the cells could form soft agar colonies. In contrast, in the Oct4 + 6G–transformed cells, about 105 colonies formed out of 10,000 cells seeded (Fig. 1D). Supplementary Fig. S1B shows the expression of the various exogenous factors in Oct4 + 6G– and Oct4 + 3G–transformed astrocytes. Please see Supplementary Table S1 for information on the antibodies used in this study.

Microarray analysis indicated that both the 7-gene and 4–gene–transformed cells shared similar gene expression profiles (Supplementary Fig. S2A; ArrayExpress accession #E-MTAB-4771). The profiles from both cells are also similar to that of ALPS1459, a patient-derived glioma stem cell line. Less but still significant similarities also exist between the transformed cells and U87G, a well-established glioma multiforme cell line. Principal component analysis confirmed the similarities between the transformed cells and with patient-derived glioma stem cells (Supplementary Fig. S2B). An analysis of genes also showed the major biological pathways related to differentially expressed genes in transformed versus parental astrocytes (>2 fold difference; Supplementary Fig. S2C).

We also conducted flow cytometry analysis of the cell-cycle distribution of the transformed and parental astrocytes. Compared with the parental astrocytes, the transformed cells have very different cell-cycle distribution profiles (Fig. 2A). In the transformed cells (Oct4 + 3G or Oct4 + 6G), the fractions of cells in G1 are much less than those in the control cells. Oct4 + 3G–transformed cells had a similar fraction of S-phase cells as the controls, while Oct4 + 6G–transformed cells had a significantly increased fraction of S-phase cells. In both Oct4 + 3G– and Oct4 + 6G–transformed astrocytes, the fraction of cells in the S or G2–M phases was significantly higher. A careful examination indicates that in Oct4 + 6G transduced astrocytes, many cells had >4N DNA content. Chromosome number analysis indicates that more than 60% of transformed cells had chromosome aneuploidy, with most possessing more than 46 chromosomes, indicating wide-
spread chromosomal instability (Supplementary Fig. S3A and S3B).

Our analysis also showed that compared with the parental astrocytes, both the 4-factor (Oct4 + 3G) and the 7-factor (Oct4 + 6G)–transformed cells showed significantly faster growth rate (Fig. 2B). The doubling times for transformed cells are 16.7 and 25.1 hours for Oct4 + 3G- or Oct4 + 6G–transformed cells. In contrast, it is longer than 43.6 hours for the parental astrocytes. Both Oct4 + 6G- and OMRP-transduced cells showed unlimited growth potential, exhibiting robust proliferative ability even after 12 months of continuous cell culture (data not shown). In comparison, the parental human primary astrocytes could only undergo 4 to 5 population doublings before losing their ability to proliferate. Indeed, TRAP (telomeric repeat amplification protocol) assay showed that OMRP-transformed cells had strong telomerase activities, which indicated activation of the endogenous hTERT genes (Fig. 2C). Further evidence of hTERT gene activation was demonstrated when semiquantitative RT-PCR was carried out to examine hTERT mRNA transcripts in parental and Oct4 + 3G or Oct4 + 6G–transformed astrocytes. Our data showed that hTERT transcript increased from barely any expression to a robust level comparable with established cancer lines (Supplementary Fig. S3C). The activation of the hTERT gene indicated that OMRP-transformed cells gained immortality after OMRP gene transduction. Activation of the endogenous hTERT gene in the 4-factor–transformed astrocytes is reminiscent of iPSC induction procedures, in which cells gained immortality through endogenous rather than exogenous hTERT gene activation. In comparison, most previously published oncogenic protocols called for the use of an exogenous copy of the hTERT gene (18, 19).

OMRP-transformed astrocytes showed clear sphere-forming ability in stem cell media, an important characteristic of CSCs. Furthermore, when the spheres were disaggregated into single cells and seeded into 96-well plates, the cells showed strong ability to form spheres again, with most individual cells able to form spheres. When cells grown as spheres in 96-well plates were taken out and plated as individual cells in wells of new 96-well plates, they formed secondary spheres at a frequency similar to parental cells. Cells from the secondary spheres, in turn, formed tertiary spheres also at similar frequencies when plated again in 96-well dishes, indicating undiminished ability to form spheres in serially diluted cultures. Furthermore, limited dilution assays (Fig. 2D; refs. 20, 21) indicated that the average number of OMRP-transformed cells it took to form a sphere was around 1 in 2.1, a

Figure 1.
Rapid induction of transformation in primary human astrocytes. A, changes in astrocyte cellular morphology during the course of induced transformation. Shown are examples from Oct + 6G-transformed group. Scale bars, 200 μm. B, colony outgrowth in astrocytes transformed with a variety of gene combinations; n = 3. C, soft agar colony growth from cells transduced with a variety of gene combinations; n = 3. D, soft agar colony growth from astrocytes transduced with either the Oct4 + 3G or the Oct4 + 6G combinations; n = 3. B–D, error bars, SEM.
number that compared very favorably with published frequency of CSCs isolated from patient-derived tumor tissues (3, 22).

We next examined the OMRP-transduced cells for expression of various CSC markers established by previous studies. Flow cytometry analyses showed that CD133, one of the most commonly used glioma stem cell markers (3), was expressed by OMRP-transformed astrocytes at robust levels (Fig. 3A, left). In addition, CD15, an established neural stem cell and brain CSC marker (23), was also detected by flow cytometry in the cells (Fig. 3A, middle). Furthermore, CD90, another well-recognized glioma stem cell marker (24), was expressed at very high levels in OMRP-transformed astrocytes (Fig. 3A, right). In addition, the transformed cells also showed positive staining of Sox2 and nestin expression (Fig. 3B). Western blot analysis confirmed the presence of CD133, Sox2, and nestin in both Oct4 + 3G- and Oct4 + 6G-transduced cells, similar to patient-derived glioma stem cells (Fig. 3C).

In subsequent experiments, we show that when spheres of OMRP-transduced cells cultured in stem cell medium were disaggregated and placed into neuron-specific media, they could differentiate into cells with neuron-like morphology that stained positive for Tuj1, a neuron-specific marker (Fig. 3D, left). We also show that sphere-derived cells could be differentiated into oligodendrocyte-like cells that stained positive for galactocerebroside (Fig. 3D, middle) or astrocyte-like cells that stained positive for glial fibrillary acidic protein (GFAP; Fig. 3D, right). Therefore, the OMRP-transformed astrocyte cells grown in stem cell media can form spheres and behave like brain tumor stem cells, which can differentiate into different cell types in a manner similar to neural stem cells (22).

Thus, OMRP-transformed astrocytes behaved similarly to patient-derived glioma stem cells in several aspects as described above. However, the "gold standard" of human CSCs is the ability to form tumors in immunodeficient mice. We therefore evaluated the capacity of OMRP-transformed cells to grow in nude mice, which is a more stringent host than either the NOD/SCID or the NOD/SCID/gamma mice that many previous studies used when testing human CSCs. Injection of only 100 OMRP-transformed astrocytes subcutaneously into nude mice gave rise to tumor growth in 8 of 10 injected mice (Supplementary Fig. S4A) during
8 weeks of observation, indicating powerful tumor-initiating ability of the cells. Because primary tumors do not grow outside of the CNS in human patients, we examined the tumor-forming abilities of the OMRP-transformed cells to form orthotopic tumors by injecting them intracranially into nude mice. Intracranial injections of about 500 of firefly luciferase–EGFP–labeled OMRP-transduced astrocytes into nude mice led to robust tumor growth in 5 of 5 mice when measured through bioluminescence imaging (Fig. 4A). On the basis of luciferase signals, it is estimated that during the course of 4 weeks (from week 2 to week 6), tumor cellular numbers increased exponentially almost 1,000-fold (Fig. 4B), with a doubling time of 64.6 hours. These numbers demonstrated the extraordinary potent tumor-forming abilities of the OMRP-transduced cells orthotopically. When mice with the
intracranial tumors were sacrificed, their brains showed signs of invasive tumor growth (Fig. 4C). In addition, immunohistologic staining showed diffuse tumor growth in large areas of the brain (as shown by GFP staining, Fig. 4D, left). They also show infiltration of astrocytes (as revealed by GFAP staining) in the tumors (Supplementary Fig. S4D, middle), which has been associated with invasive brain tumors (25). H&E staining shows areas of high cellularity and vascularity mixed with necrotic areas (Fig. 4D, right). Further immunofluorescence staining indicates expression of the stem cell marker CD133 (Supplementary Fig. S4B). Expression of the original exogenous factors was confirmed by immunofluorescence (Oct4, Supplementary Fig. S4C) and immunohistochemical (Myc, p53, and Ras; Supplementary Fig. S4D–S4F) staining.

Another reported characteristic of CSCs is their elevated resistance to chemotherapy. To determine whether the OMRP-transduced cells exhibit similar properties, we measured the resistance of the cells to temozolomide, an alkylating agent used commonly for advanced stage brain cancer patients. OMRP-transduced cells are significantly more resistant to temozolomide than U87MG (Fig. 5A), with IC50 at least more than 10× higher in the former (Fig. 5B).

Figure 4.
Tumor initiation in vivo from OMRP-transduced astrocytes. A, tumor formation in nude mice from 500 firefly luciferase–labeled, OMRP-transformed astrocytes injected intracranially. Bioluminescence imaging was used to follow tumor growth noninvasively at different time points. B, exponential increase in intracranial bioluminescence signals from the OMRP-transformed cells during weeks 2 to 6. Error bars, SEM; n = 5. Luc., luciferase. C, brains of noninjected mice (left) and those injected with OMRP-transformed cells (middle and right). Notice smaller, deformed shapes of the brains and abnormal tissue growth with greenish tint (from EGFP-labeled tumor cells) in the latter group. D, immunohistochemical (left and middle) and H&E (right) staining of brain sections from the tumor-bearing mice. Scale bars, 50 µm.

Figure 5.
Drug resistance of OMRP-transduced astrocytes. A, crystal violet staining of OMRP-transformed astrocytes (Astr) and U87MG cells after treatment with various concentrations of temozolomide. B, quantitative estimate of the relative survival of OMRP-transduced astrocytes and U87MG (UB7) cells after temozolomide treatment. Error bars, SD (n = 3).
Discussion

Thus, we have successfully generated CSC-like cells from primary human astrocytes with a relatively simple cocktail of defined genetic factors: Oct4, Myc, Ras, and p53DD. Most remarkably, the cells possess strong tumor-initiating abilities and express various molecular markers of glioma stem cells, which distinguish them from previously successful transformation of human astrocytes (12, 13). The critical importance of Oct4 implicates a key role for epigenetic reprogramming in facilitating the transformation process.

Our study should contribute to the cell-of-origin studies in brain tumor. Mouse genetic studies conducted so far have indicated that glioma can arise from neural stem cells (26, 27) and oligodendrocyte progenitor cells (28). Attempts to transform mature astrocytes were less successful (29). However, a more recent study indicated that lentivirus-mediated transduction of shNF1-shp53 or H-RasV12-shp53 could cause dedifferentiation and transformation of mature astrocytes in mice (30). Our results therefore provide evidence that mature human astrocytes could be dedifferentiated and transformed into glioma-like cells, similar to mouse cells.

Of the four factors used for carrying out oncogenic transformation of astrocytes, Oct4 and myc have been shown to be overexpressed in brain tumors (31), while p53 (32) has been shown to be frequently mutated. Therefore, all three are quite relevant for human glioma development. On the other hand, H-Ras, one of the four factors used in our transformation of primary astrocytes, is not frequently mutated in brain cancers. Despite the fact that Ras has been shown to play some important roles in glioma (33), we believe our model could be made more relevant by replacing Ras with other more relevant oncogenic factors, such as EGFR or EGFRvIII (34).

We propose to name the transformed cells induced CSCs (iCSC) or induced tumor-initiating cells (iTIC). We believe it should be possible to establish iCSCs from other cell lineages using similar procedures. The iCSCs or iTICs should provide genetically tractable models to study cancer stem cell biology or develop novel cancer therapeutics.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: F. Li, J.H. Sampson, C.-Y. Li
Development of methodology: F. Li
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): F. Li, X. Liu, J.H. Sampson, D.D. Bigner
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F. Li, J.H. Sampson, C.-Y. Li
Writing, review, and/or revision of the manuscript: F. Li, J.H. Sampson
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): F. Li, J.H. Sampson, D.D. Bigner
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