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# Journal of Theoretical Biology



journal homepage: www.elsevier.com/locate/jtb

# Stochastic cellular automata model of tumorous neurosphere growth: Roles of developmental maturity and cell death



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#### ARTICLE INFO

Article history: Received 10 July 2018 Revised 13 December 2018 Accepted 19 January 2019 Available online 29 January 2019

Keywords: Neural stem/progenitor cells Brain tumor stem cells Neurosphere assay Tumor growth Theoretical modeling

## ABSTRACT

The neurosphere assay is a powerful in vitro system for studying stem/progenitor-cell-driven tissue growth. By employing a stochastic cellular automata model, we simulated the development of tumorous neurospheres in response to transformation of a randomly selected progenitor cell into a brain tumor stem cell. Simulated tumorous neurospheres were distinguished from normal neurospheres by their size, which exceeded that of normal neurospheres typically manifold. A decisive factor that determined whether brain tumor stem cells gave rise to tumorous neurospheres was their ability to escape encapsulation by neighboring cells, which suppressed mitotic activity through contact inhibition. In our simulations, the likelihood of tumorigenesis was strongly negatively correlated with the developmental maturity of the neurospheres in which the transformation of a progenitor cell into a brain tumor stem cell was induced. This likelihood was furthermore modulated by the probability of the progeny of dividing cells to undergo cell death. In developmentally immature neurospheres, the number of normal neurospheres, relative to the number of tumorous neurospheres, increased with increasing cell death probability. Markedly, in developmentally mature neurospheres the opposite effect was observed. This dichotomous effect of cell death on simulated tumor progression provides theoretical support for the seemingly paradoxical finding made by other authors in experimental studies that anti-cancer therapies based on induction of apoptosis may both promote and suppress tumor growth.

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## 1. Introduction

Brain tumors are relatively rare, yet they include some of the most lethal tumor types. Glioblastoma multiforme, an extremely aggressive malignant primary brain tumor, has an incidence rate of approximately 3 per 100,000 persons per year (Tamimi and Juweid, 2017), thus accounting for less than 1% of all new cases of cancer projected to occur in the United States in 2018 (Siegel et al., 2018). Despite considerable research and clinical treatment trials over the past few decades, prognosis of this glioma has remained poor. In untreated patients, the median survival time after diagnosis is just 3–4 months (Omuro and DeAngelis, 2013). When treated with a combination of surgery, radiotherapy, and chemotherapy, median survival is still only 15 months (Stupp et al., 2005).

The cellular origin of gliomas, including glioblastoma, remains a matter of controversy. The currently prevalent hypothesis proposes that gliomas originate from adult neural stem cells and/or their progenitor cells (NSPCs), harbored in the subventricular zone

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https://doi.org/10.1016/j.jtbi.2019.01.028 0022-5193/© 2019 Elsevier Ltd. All rights reserved. (Sanai et al., 2005; Vescovi et al., 2006), an anatomical structure comprising the lining of the lateral ventricles. The NSPCs in the subventricular zone are a subpopulation of the astrocytes in this region (Doetsch et al., 1999). These stem cells generate transiently dividing progenitor cells, which, unlike their mother cells, have a limited proliferative potential in vivo (Ponti et al., 2013). Furthermore, the progenitors produce neurons *or* glia, but not both, like done by adult neural stem cells.

Sanai and co-authors have argued that the adult neural stem cells and their progenitors are particularly susceptible to tumor transformation because they possess already the activated cellular and molecular machinery required for mitotic division (Sanai et al., 2005). They might, therefore, require less mutations than the 4–7 thought to be needed for malignant transformation of a differentiated cell into a tumor cell (Hanahan and Weinberg, 2000).

One of the most valuable tools for studying NSPCs and their progeny has been the neurosphere assay (Reynolds and Weiss, 1992). The clonal clusters of cells formed in this in-vitro system under serum-free conditions are referred to as neuro-spheres. By growth factor withdrawal and addition of serum to the culture medium, they can be induced to differentiate into the

three major types of cells of the central nervous system – neurons, astrocytes, and oligodendrocytes.

The neurosphere assay has been instrumental in obtaining evidence for the existence in glioblastoma multiforme of cells that exhibit the critical properties of stem cells (Galli et al., 2004; Ignatova et al., 2002). Cells from patients diagnosed with glioblastoma form clones under the growth conditions of this in-vitro system. While they give rise only to astroglia in vivo, they have revealed multilineage potential under cell-culture conditions by demonstrating their capability of producing neurons, astroglial, and oligodendroglia. Most importantly, when transplanted into the striatum of adult immunodeficient mice, they can induce tumors closely resembling glioblastoma multiforme in humans (Galli et al., 2004). Based on this evidence, these cells have been termed brain tumor stem cells (BTSCs).

Since the initial identification of BTSCs, a wealth of experimental data has been accumulated, particularly on the signaling pathways involved in initiation by these stem cells of malignant brain tumors and their propagation. Selective targeting of these pathways, with the aim to eradicate the BTSCs, has led to the development of promising novel therapies (Schonberg et al., 2014; Zhou et al., 2009). Nevertheless, there is a significant lack of theoretical understanding of the cellular dynamics underlying BTSC-driven tumor initiation and growth.

Recently, we developed a stochastic cellular automata (CA) model to examine the role of various cellular phenomena, including proliferative potential, contact inhibition, cell death, and phagocytosis of dead cells, on growth rate, size, and composition of neurospheres (Sipahi and Zupanc, 2018). Based on this model of normal neurosphere growth, we present, in the current study, a modified CA model that incorporates transformation of progenitor cells into BTSCs, and subsequent propagation of the transformed cells. Using this approach, we have specifically examined how developmental maturity of the tissue, and cell death occurring at different stages of maturity, affect tumorigenesis.

We have chosen CA modeling over other approaches because it is particularly well suited to adequately represent, through its array of cells, the discrete nature of individual cells in biological tissue; and, through if-conditions and probabilities, rule-driven biological mechanisms that govern cellular events, such as cell proliferation, cell migration, and cellular differentiation (for reviews, see Jones and Chapman, 2012; Newgreen et al., 2013). Taking advantage of these features, this approach has been used successfully in previous studies of tumor growth (e.g., Alemani et al., 2012; Escobar Ospina and Perdomo, 2013; Monteagudo and Santos, 2015; Oduola and Li, 2018; Sabzpoushan and Pourhasanzade, 2018; Santos and Monteagudo, 2015; Shrestha et al., 2013).

## 2. Model development

#### 2.1. General structure of the CA model

The CA model used in the present study is a modified version of the model developed previously to simulate the growth of neurospheres based on the proliferation of NSPCs (Sipahi and Zupanc, 2018). As mentioned in the Introduction, the major difference of the current model, compared to the previous version, is that it incorporates the possibility of a simulated progenitor cell to transform into a BTSC (Fig. 1; Table 1). Model rules were programmed in MATLAB ver. 2013a. In the following, we will describe the core features of the current model.

The model was constructed on the (x, y) plane, with dimensions of  $L_x \times L_y$ , where  $L_x = L_y = 200$  (Table 2). The resulting plane was divided into squared lattices of unit edge length, yielding a



Fig. 1. Flow chart of algorithm used to initiate and regulate simulated neurosphere growth. Solid lines are associated with generation, development, and death of cells, as well as clearance of dead corpses. Dashed lines indicate influences on decisions. The colors used for the different cell types is identical to the color coding applied to snapshots of neurospheres in Figs. 2–5. *Red*, stem cell; *orange*, progenitor cell; *green*, brain tumor stem cell; *blue*, differentiated cell; *black*, dead cell. The encircled numbers indicate critical steps in the processes (see text for details).

#### Table 1

Biological properties and model implementation of cell types used to study normal and tumorous neurosphere growth. Listed are only those properties of each cell type that are relevant for model implementation. Some of the properties are hypothetical and do not necessarily cover all developmental scenarios.

Cell types	Simulation color coding	Biological properties	Model implementation
Adult neural stem cell	Red	Unlimited self-renewal capacity; predominantly asymmetric cell divisions, thereby cloning itself and producing one progenitor cell; regulation of proliferative activity through contact inhibition; relative resistance to apoptotic cell death	Divides as long as at least one of four neighboring lattices is vacant ( $N < 4$ ) and probability for mitosis $P_{mit}$ is satisfied; division yields two daughter cells, one carrying stem cell characteristics and the other those of a progenitor; not subject to cell death probability $P_{death}$
Progenitor cell	Orange	No self-renewal capacity; limited proliferative ability; amplification through symmetric cell divisions; possibility of apoptosis soon after asymmetric cell division of one of the daughter cells; regulation of apoptosis through contact inhibition; potential for generation of fate-restricted cells	Divides only if at least one of four neighboring lattices is empty ( $N < 4$ ) and probability for mitosis $P_{mit}$ is satisfied; can divide only up to a maximum number of times $d_{max}$ ; before reaching $d_{max}$ , each division produces two progenitor cells; one of the daughter cells always survives, while the other is subject to cell death probability $P_{death}$ ; when $d_{max}$ is reached, division produces two differentiated daughter cells
Brain tumor stem cell (BTSC)	Green	Unlimited self-renewal capacity; origin from adult neural stem/progenitor cell; self-renewal through symmetric divisions; possibility of apoptosis in daughter cells; regulation of apoptosis through contact inhibition	Divides as long as at least one of four neighboring lattices is vacant ( $N < 4$ ) and probability of mitosis $P_{mit}$ is satisfied; each division produces two BTSC daughter cells; one of the daughter cells always survives, while the other is subject to cell death probability $P_{death}$
Differentiated cell	Blue	No self-renewal capacity; no proliferative ability; after terminal differentiation, no migratory activity; after terminal differentiation, relative resistance to apoptosis	Does not divide, move, or die
Dead cell	Black	Cellular debris caused by dead cell in tissue microenvironment; clearance of cellular debris through phagocytosis	Whenever death probability $P_{death}$ is satisfied after mitosis, a cell transforms into a dead cell; a dead cell occupies the lattice for $t_{clear}$ number of time steps; after $t_{clear}$ , dead cell is removed from the lattice, emptying the lattice space

### Table 2

Parameter definitions

Simulation input	Definition	Numerical settings
	Definition	Numerical settings
$L_x$ , $L_y$	Dimensions of the x-y plane on which the simulation is performed	$200 \times 200$ lattices
t <sub>final</sub>	Simulation time	100 time steps
d <sub>max</sub>	Maximum number of time steps a progenitor cell can perform symmetric divisions	6 time steps
Ν	Number of lattices occupied around a cell	$N \le 4$
P <sub>mit</sub>	Probability at which a mother cell performs a mitotic division; this probability is inversely proportional to N	$N = 0, P_{mit} = 1.0$
		$N = 1, P_{mit} = 0.833$
		$N = 2, P_{mit} = 0.667$
		$N = 3, P_{mit} = 0.5$
		$N = 4$ , $P_{mit} = 0.0$
P <sub>death</sub>	Probability that a daughter cell dies after a division of the mother cell; this probability is proportional to $N$	$N = 0, P_{death} = 0.05$
		$N = 1$ , $P_{death} = 0.20$
		$N = 2$ , $P_{death} = 0.35$
		$N = 3$ , $P_{death} = 0.50$
t <sub>clear</sub>	Number of time steps that a dead cell remains dead and occupies a lattice before it is cleared from this lattice	10 time steps
t <sub>dm</sub>	Time step at which a randomly selected progenitor transforms into a BTSC, thus reflecting the developmental maturity of the simulated neurosphere	$t_{dm} = [2, 3,, 20]$
n <sub>sim</sub>	Total number of neurospheres simulated	500
Simulation outputs		

(x,y) Position recordings of all cell types

Counts of cell types

 $n_{live}$  Number of live cells (adult neural stem cell + progenitor cells + BTSCs + differentiated cells) counted at  $t_{final}$ 

total of 40,000 lattices. The simulation is initialized by placing a single adult neural stem cell into the center of the plane (Fig. 1, Step 1). Each lattice could be either empty or occupied by one cell only (Fig. 1, Step 2). To allow each lattice location (x, y) to have four orthogonally adjacent lattices at (x + 1, y), (x - 1, y), (x, y + 1), (x, y - 1), thereby forming a von Neumann neighborhood, spatial limits where cell growth could take place were given by  $2 \le x \le L_x - 1$  and  $2 \le y \le L_y - 1$ . The states associated with the lattice sites were updated after each discrete time step t, until  $t = t_{final} =$  100. The different types of neurosphere cells were represented by different agents in the model's lattice. The cellular processes considered to contribute to the normal and tumorous growth of the neurospheres were governed by specific rules motivated by cell bi-

ological observations and expressed accordingly using if-conditions and probabilities, as detailed in Fig. 1 and the following sections.

# 2.2. Cell proliferation and control of cell cycle progression by contact inhibition

In all simulations, we assumed that the cells within a neurosphere are the progeny of a *bona fide* neural stem cell, with unlimited capacity for self-renewal. In line with the notion that during adulthood a stem cell undergoes predominantly asymmetric divisions (for review, see Morrison and Kimble, 2006), thereby cloning itself and producing one progenitor cell, we implemented this mode of cell division in our model (Fig. 1, Step 6). Each progenitor cell had a limited potential for amplification through symmetric divisions, up to a maximum number,  $d_{max} = 6$  (Fig. 1, Steps 7, 9). This number is in line with quantitative analysis of the proliferation dynamics of neural progenitor cells in the adult vertebrate central nervous system (Ponti et al., 2013; Sîrbulescu et al., 2017, 2015). In our model, the  $d_{max}$  value was compared with the actual number of divisions of each progenitor stored in an array and updated at the end of each time step. The final (symmetric) division of the progenitor cell produced two differentiated cells, which, subsequently, neither divided nor migrated (Fig. 1, Step 9).

Tumorous growth was induced by transforming a randomly selected progenitor cell into a BTSC at a specific time step  $t = t_{dm}$ , denoting the developmental maturity of the neurosphere tissue. BTSCs had an unlimited ability for self-renewal by performing symmetric amplifying divisions (Fig. 1, Step 8).

The decision of the bona fide stem cell, and any progenitor cell or BTSC regarding cell cycle progression is made at the G<sub>1</sub>-S transition of the cell cycle. This time point separates the G<sub>1</sub>-phase (during which metabolic changes prepare the cell for division) and S-phase (during which DNA is replicated) of the interphase. In agreement with experimental evidence (Streichan et al., 2014), we assumed that during the G1-S transition a mechanosensitive cell cycle checkpoint senses the local cell density in the immediate neighborhood (Fig. 1, Steps 3, 4). This information, in turn, determined, at the beginning of each iteration cycle, the probability of the simulated cell to undergo mitosis (Fig. 1, Step 5). As supported by cell biological observations ((Eagle and Levine, 1967; Ingber et al., 1994; Streichan et al., 2014); (for review, see Moeendarbary and Harris, 2014)), we furthermore assumed that this probability is inversely proportional to an extrinsic mechanical force indicating the cell density in the neighborhood. Contributors to the cell density were any 'live' or 'dead' cells occupying the four neighbor lattice sites. The inversely proportional relationship between local cell density, defined by the number of neighbors, N (0  $\leq N \leq 4$ ), and the probability to undergo mitosis,  $P_{mit}$ , during each iteration was incorporated into our model by applying the following rules: If N = 0,  $P_{mit} = 1.0$ ; if N = 1,  $P_{mit} = 0.833$ ; if N = 2,  $P_{mit} = 0.667$ ; if N = 3,  $P_{mit} = 0.5$ ; if N = 4,  $P_{mit} = 0$ .

In biological tissue, the mother cell divides into two daughter cells upon completion of the M-phase of mitosis. In our model, we implemented the following rule that governs the subsequent behavior of the two daughter cells: If the mother cell initially occupied position (x, y) in the lattice, then one of the two daughter cells occupies (x, y), whereas the other occupies, with equal probability, one of the vacant positions at  $(x \pm 1, y)$  or  $(x, y \pm 1)$ . The selection which of the two daughter cells moved to the site of the mother cell or to one of the vacant sites in the surrounding, respectively, was made randomly with equal probability.

Since we showed previously (Sipahi and Zupanc, 2018) that even major differences in contact inhibition have no significant effect on the final size of the neurospheres or on the degree of differentiation of tissue but only regulate the speed of neurosphere growth, we did not attempt to examine in the present study the effect of contact inhibition on tumor growth. Instead, we applied the above moderate contact inhibition to all simulations.

#### 2.3. Cell death and phagocytosis

Cell death occurs spontaneously in neurospheres, and is predominantly apoptotic in nature (Bez et al., 2003; Lobo et al., 2003; Milosevic et al., 2004; Young et al., 2006). Quantitative analysis has revealed significant spatial and temporal overlap of cell proliferation and apoptosis – an observation consistent with the notion that apoptosis of NSPCs occurs shortly after mitotic division in one of the two daughter cells, whereas the other survives and potentially continues to proliferate or differentiates (for reviews, see Kuan et al., 2000; Sommer and Rao, 2002). This differential fate of the two daughter cells appears to be regulated by asymmetric distribution of pro- and anti-apoptotic proteins during the final stages of mitosis (Bieberich et al., 2003). Like in the case of cell proliferation, a major factor that controls cell death appears to be the local cell density (for review, see Eisenhoffer and Rosenblatt, 2013).

To accommodate these observations in our model, the local cell density was used to determine the probability  $P_{death}$  of death of one of the two daughter cells after the decision was made that the mother cells undergoes mitosis (i.e., N < 4).  $P_{death}$  was determined as follows: If N = 0,  $P_{death} = 0.05$ ; if N = 1,  $P_{death} = 0.20$ ; if N = 2,  $P_{death} = 0.35$ ; if N = 3,  $P_{death} = 0.50$ . The following three scenarios were distinguished:

- (1) If the mother cell was the *bona fide* stem cell, the cloned stem cell was always destined to survive, whereas the progenitor cell was subject to  $P_{death}$  dictated by the number of cells *N* in the immediate neighborhood (Fig. 1, Step 6)
- (2) If a progenitor cell was the mother cell, one of the two daughter cells was always destined to survive, whereas the other (excluding the two differentiated cells in case of a terminal division of a progenitor cell) was subject to the same rules as any progenitor cell arising from the mitotic division of a *bona fide* stem cell (Fig. 1, Steps 7, 9)
- (3) If a BTSC was the mother cell, one of the two BTSC clones was always destined to survive, whereas the other clone was subject to the same rules as a progenitor cell arising from the asymmetric mitotic division of a *bona fide* stem cell, or a progenitor cell resulting from a symmetric division of a mother progenitor (Fig. 1, Step 8)

The implementation into our neurosphere model of rules that make newly generated cells subject to the possibility of apoptosis is in agreement with the observation of spontaneous apoptotic cell death among the progeny of both NSPCs (for review, see Ryu et al., 2016) and BTSCs (Kordek et al., 1996; Kuriyama et al., 2002; Migheli et al., 1994; Patsouris et al., 1996) in the brain.

We have, furthermore, incorporated into our model the removal of dead cells by phagocytosis, as found in neurospheres (Bez et al., 2003; Lobo et al., 2003) (Fig. 1, Steps 10, 11, 12). In the simulations, this process leads to clearance of the lattice space previously occupied by the dead cell. Since phagocytic clearance in the central nervous system is slow (for reviews, see Neumann et al., 2009; Vargas and Barres, 2007), we applied a relatively large clearance time of  $t = t_{clear} = 10$  to all simulations. Assuming that 1 cell cycle (represented by 1 time-step in the model) is approximately 1 day long, this value places the clearance time in the range of experimentally determined values associated with phagocytosis of cellular debris after apoptosis in brain tissue (Zupanc et al., 2003).

### 3. Results

#### 3.1. Normal neurospheres

Simulation of the transformation of progenitor cells into BTSCs led to tumorous growth of neurospheres in some but not all instances. To characterize the structural difference between normal and tumorous neurospheres, we first simulated the growth of normal neurospheres, without inducing the transformation of a progenitor cell into a BTSC. We used a single stem cell as a founder cell, which gave rise to progenitor cells endowed with  $d_{max} = 6$ . We also assumed that in both the stem cell and any of the progenitor cells the probability to undergo mitosis,  $P_{mit}$ , was determined by the number of neighbors, *N*, according to the rules detailed in section 2.2, 'Cell proliferation and control of cell cycle progression by contact inhibition.' After the decision had been made that a stem cell or a progenitor cell divided, and the



**Fig. 2.** Normal neurospheres. Growth of neurospheres was simulated without inducing the transformation of a progenitor cell into a brain tumor stem cell (for details, see text). (a) Cellular structure of a neurosphere at  $t_{final} = 100$ . For an explanation of the color codes of the different cell types, see Fig. 1. (b) Frequency distribution of the size of the 500 simulated neurospheres. The size was assessed by using  $n_{live}$  as a quantitative indicator.

probability test had been passed, local cell density was used to determine the probability  $P_{death}$  that a progenitor cell or one of the two BTSCs would die, according to the rule detailed in section 2.3, 'Cell death and phagocytosis.' The size of the neurospheres was assessed at  $t_{final} = 100$ , based on the total number of 'live' cells,  $n_{live}$  [= 1 stem cell + number of progenitor cells + number of BTSCs ('0' in this simulation) + number of differentiated cells] counted at  $t_{final}$ . The other parameters were  $t_{clear} = 10$  and total number of neurospheres simulated,  $n_{sim} = 500$ .

The simulations carried out under these conditions yielded neurospheres with a two-dimensional cellular structure similar to the one shown in Fig. 2a. Among the 500 simulated neurospheres,  $n_{live}$  ranged between 31 and 122, with a mean of 60 (Fig. 2b). Based on these results, we defined for all of the following simulations any neurosphere with  $n_{live} \le 122$  as normal, whereas neurospheres with  $n_{live} > 122$  were considered to be tumorous.

#### 3.2. Tumorous neurospheres

In the second set of simulations, we transformed a randomly selected progenitor cell into a BTSC at  $t_{dm} = 10$ . All other parameters and rules were identical to the ones applied to the first set of simulations.

Out of the  $n_{sim} = 500$  simulated neurospheres, 343 had grown into tumors, as defined above. Fig. 3a depicts the cellular structure of such a tumorous neurosphere at  $t_{final} = 100$ . Fig. 3b shows the core region of this sphere, with the *bona fide* stem cell, the progenitor cells, and the differentiated cells, at higher magnification. The size, as defined by  $n_{live}$ , of these 343 tumorous neurospheres ranged from 128 to 4431 (mean = 2554), whereas  $n_{live}$  of the 157 normal neurospheres ranged from 33 to 113 (mean = 63) (Fig. 3c).

#### 3.3. Encapsulation of BTSCs

Analysis of the size distribution of the simulated neurospheres showed that normal neurospheres and tumorous neurospheres form, at least when run under the conditions used in the second set of simulations, two distinct populations, separated based on  $n_{live}$  (Fig. 3c). What are the factors that decide whether a neurosphere will develop normally or become tumorous – despite the fact that in each case a randomly selected progenitor cell was transformed into a BTSC?

Closer inspection of normal neurospheres revealed that tumor growth was abolished either immediately after transformation of a progenitor cell into a BTSC, or after a very few mitotic divisions of the BTSCs. In each of the cases examined, the BTSCs stopped dividing because of lack of vacant grid position(s) in their immediate neighborhood (Fig. 4). On the other hand, in neurospheres that developed into tumors the dividing BTSC managed to escape encapsulation by neighboring cells, thereby enabling itself to undergo mitotic divisions in the space available (Fig. 5). Typically, this resulted in a rapid growth of the BTSCs at one pole of the neurospheres. During later iteration cycles the proliferation of the simulated BTSCs extended to vacant grid positions in the periphery of other non-tumorous parts of the neurospheres. Frequently, this growth pattern led to complete encapsulation of the non-tumorous portion of the sphere by BTSCs, thereby ultimately blocking any further proliferation of the stem cell and the progenitor cells – despite their proliferative potential.

# 3.4. Tumor transformation as a function of developmental maturity of neurospheres

Since the last part of our study had demonstrated that the local cellular environment is an important factor in determining whether a neurosphere becomes tumorous, in this part we tested the hypothesis that the likelihood of tumorous growth decreases with increasing developmental maturity of the neurospheres. To test this hypothesis in the simulations, a randomly selected progenitor cell was transformed into a BTSC, as described above. However, now this transformation was induced at different developmental stages of the simulated neurospheres. As a quantitative indication of developmental maturity,  $t_{dm}$ , ranging from 2 (most immature neurosphere) to 20 (most mature neurosphere), was used. All other parameters and rules were identical to the ones applied to the second set of simulations.

The simulations run under these conditions showed that with increasing  $t_{dm}$  the proportion of tumorous neurospheres among the total neurosphere population (= tumorous neurospheres + normal neurospheres) exhibited a strong tendency to decrease (Fig. 6). The Spearman rank correlation coefficient *rho* between  $t_{dm}$  and the relative number of tumorous neurospheres was -0.995 (p < 0.001, 2-tailed). This trend was paralleled by a decrease in  $n_{live}$  with increasing  $t_{dm}$  (rho = -0.998, p < 0.001, 2-tailed) (Fig. 7). On the other hand,  $n_{live}$  of the normal neurospheres did not change significantly with  $t_{dm}$  (rho = -0.351, p = 0.140, 2-tailed).

# 3.5. Regulation of development of normal and tumorous neurospheres by cell death

Previous CA modeling of neurosphere growth indicated that cell death of newly generated cells not only results, as one would ex-



**Fig. 3.** Tumorous neurospheres. Transformation of a randomly selected progenitor cell into a brain tumor stem cell was induced at  $t_{dm} = 10$ . All other parameters and rules were identical to the ones applied to generate the data shown in Fig. 2. (a) Cellular structure of a neurosphere at  $t_{final} = 100$ . (b) Area of adult neural stem cell and neighboring cells at higher magnification. For an explanation of the color codes of the different cell types, see Fig. 1. (c) Frequency distribution of the size of the 500 simulated neurospheres.

pect, in a reduction of growth rate and final size of neurospheres, but also leads to an increase in the degree of differentiation of neurosphere cells (Sipahi and Zupanc, 2018). In our model, differentiated cells have a high potential to suppress mitotic activity of neighboring cells through contact inhibition because they do not vacate their grid positions through cell death (and subsequent clearance through phagocytosis) or migration to an adjacent

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Cell death conditions used to study the effect of cell death on development of normal and tumorous neurospheres.

Cell death	Number of neighbors	Cell death probability of
condition	surrounding mitotic cell (N)	newly generated cell $(P_{death})$
1	N = 0	$P_{death} = 0.00$
	N = 1	$P_{death} = 0.00$
	N = 2	$P_{death} = 0.00$
	N=3	$P_{death} = 0.00$
2	N = 0	$P_{death} = 0.05$
	N = 1	$P_{death} = 0.00$
	N=2	$P_{death} = 0.00$
	N=3	$P_{death} = 0.00$
3	N = 0	$P_{death} = 0.20$
	N = 1	$P_{death} = 0.05$
	N=2	$P_{death} = 0.00$
	N=3	$P_{death} = 0.00$
4	N = 0	$P_{death} = 0.35$
	N = 1	$P_{death} = 0.20$
	N=2	$P_{death} = 0.05$
	N=3	$P_{death} = 0.00$
5	N = 0	$P_{death} = 0.50$
	N = 1	$P_{death} = 0.35$
	N=2	$P_{death} = 0.20$
	N=3	$P_{death} = 0.05$
6	N = 0	$P_{death} = 0.65$
	N = 1	$P_{death} = 0.50$
	N=2	$P_{death} = 0.35$
	N=3	$P_{death} = 0.20$
7	N = 0	$P_{death} = 0.80$
	N = 1	$P_{death} = 0.65$
	N=2	$P_{death} = 0.50$
_	N=3	$P_{death} = 0.35$
8	N = 0	$P_{death} = 0.95$
	N = 1	$P_{death} = 0.80$
	N=2	$P_{death} = 0.65$
	N=3	$P_{death} = 0.50$

position. We, therefore, hypothesized that cell death also plays a role in tumorigenesis both by reducing the number of BTSCs, and by suppressing their mitotic activity indirectly through increased contact inhibition exerted by neighboring differentiated cells.

To test this hypothesis, we induced tumor transformation in neurospheres at two developmental stages – an immature stage defined by  $t_{dm} = 2$ , and a mature stage defined by  $t_{dm} = 20$ . At each of the two developmental maturity stages, the probability of cell death of newly generated cells arising from the mitotic activity of stem cells, progenitor cells, and BTSCs was systematically altered from low to high, resulting in 8 different cell-death conditions. Table 3 summarizes these probabilities as a function of the number of vacant positions *N* in the immediate neighborhood. The other parameters employed in these simulations were identical to those described in section 3.1, 'Normal neurospheres.'

At  $t_{dm} = 2$ , transformation of a randomly selected progenitor cell into a BTSC, combined with absence of cell death (Cell Death Condition 1), resulted in each of the 500 simulations in the formation of large tumorous neurospheres (Fig. 8a). As long as  $P_{death}$  remained low (Cell Death Conditions 2– 4), only tumorous neurospheres were generated (not shown). However, when the probability of cell death reached a relatively high level (Cell Death Condition 5), some normal neurospheres appeared, in addition to the many tumorous spheres (Fig. 8a'). The relative portion of normal neurospheres increased further with increasing cell death probability (Cell Death Conditions 6–8). The frequency distribution corresponding to Cell Death Condition 8 is shown in Fig. 8a".

The mean size of tumorous neurospheres showed a perfect negative correlation with cell death probability defined by the 8 cell-death conditions (rho = -1.00, p < 0.001, 2-tailed). Since normal neurospheres were generated only under four of the eight



**Fig. 4.** Non-tumorous growth of a neurosphere after transformation of a progenitor cell into a brain tumor stem cell. The growth of this neurosphere was simulated on a 200 × 200 lattice using a single *bona fide* stem cell as a founder cell. This stem cell performed asymmetric divisions. The progenitor cells generated as a result of these divisions were endorsed with a  $d_{max}$  of 6. Cell death occurred in a probabilistic manner as detailed in the text. A randomly selected progenitor cell was transformed into a BTSC at  $t_{dm}$  = 20. The cellular structure of the growing simulated neurosphere is shown at t = 10 (a), 20 (b), 30 (c), 40 (d), 50 (e), and 60 (f). For an explanation of the color codes of the different cell types, see Fig. 1 and Table 1.

cell-death conditions simulated, it was not possible to calculate a statistically significant correlation of sphere size and cell death probability.

At  $t_{dm} = 20$ , transformation of a randomly selected progenitor cell into a BTSC, combined with various cell death probabilities, yielded a remarkably different outcome. Absence or low levels of cell death probability led predominantly to the generation of normal neurospheres (Fig. 8b). A marked number of tumorous neurospheres within the sphere population emerged only at relatively high cell death probabilities (Fig. 8b'). At very high cell death probabilities, became confluent (Fig. 8b''). The size of both normal and tumorous neurospheres decreased with increasing cell death probability (rho = -1.00, p < 0.001, 2-tailed).

#### 4. Discussion

# 4.1. Phenotypic similarity of BTSCs during the early stage of tumor progression and development of intratumoral heterogeneity

The present study focuses on the early stages of tumor propagation in tissue, after transformation of a single progenitor cell into a BTSC. The critical importance of these early stages for tumorigenesis is underscored by our simulations, which have demonstrated that, following the initial transformation step, the first few iteration cycles decide whether the simulated neurospheres grow normally or become tumorous (for a detailed discussion, see section 4.2, 'Encapsulation of BTSCs as a determinant of normal vs. tumorous neurosphere development,' below).

BTSCs exhibit the cardinal property of self-renewal inherent to any stem cell, both normal or malignant. In our CA model, selfrenewal is achieved by symmetric division of the BTSC. However, by implementing such a mechanism into our model, we do not necessarily imply that tumors form a homogenous clonal population. Rather, in our model the critical outcome of the mitotic divisions of the BTSCs is that they give rise to daughter cells with high proliferative potential, which, phenotypically, cannot necessarily be distinguished from the proliferative potential of their mother BTSCs. Hence, we have termed the two daughter cells 'BTSCs,' only to indicate that each of them shares with the other, during the developmental stage studied here (i.e., within the limits set by simulation time  $t_{final} = 100$ ), the ability to divide. Irrespective of the shared proliferative potential, these cells may be diverse in terms of other properties not considered here, or develop phenotypic differences at later stages (t > 100) of tumor progression.

The development of heterogeneity is thought to be caused by a variety of mechanisms, including acquisition of additional genetic mutations subject to Darwinian evolution (Cairns, 1975; Nowell, 1976); elevated genetic instability operating particularly at later stages of tumor progression (Hanahan and Weinberg, 2011); and epigenetic alterations layered on top of genetic mutations (Shackleton et al., 2009). These modifications will result in clonal heterogeneity of cells and may, as frequently observed, include non-tumorigenic cancer cells. Together with normal cells (like the stem cell, progenitors, and differentiated cells in the simulated tumorous neurospheres), these diverse cell types form the tumor microenvironment. Such an intratumoral cellular heterogeneity is characteristic of many tumors at later stages of tumor progression and has shown to be of critical importance for the maintenance



**Fig. 5.** Tumorous growth of a neurosphere after transformation of a progenitor cell into a BTSC. Growth of this neurosphere and tumor transformation were simulated under the same conditions as used for the simulation shown in Fig. 4. The cellular structure of the growing neurosphere is shown at t = 10 (a), 20 (b), 30 (c), 40 (d), 70 (e), and 100 (f). For an explanation of the color codes of the different cell types, see Fig. 1. Note difference in scaling between subfigures (a-c) and (d-f).

and possible further progression of tumors (Kreso and Dick, 2014). Clearly, extensions of our current CA model to include simulations of later stages of tumor development will have to take intratumoral cellular heterogeneity into account.



**Fig. 6.** Likelihood of tumorous growth of neurospheres as a function of their developmental maturity. The growth of neurospheres was simulated on a 200 × 200 lattice using a single *bona fide* stem cell as a founder cell. This stem cell performed asymmetric divisions. The progenitor cells arising from these mitotic divisions were endorsed with  $d_{max} = 6$ . Cell death occurred in a probabilistic manner (for details, see text). A randomly selected progenitor cell was transformed into a BTSC at  $t_{dm}$  ranging from 2 to 20. The size of the neurospheres was determined at  $t_{final} = 100$ , using  $t_{live}$  as a quantitative indicator. Tumorous neurospheres were defined as spheres with  $n_{live} > 122$ , whereas spheres with  $n_{live} \le 122$  were considered to be normal. For each time step at which tumor transformation was induced, the number of tumorous neurospheres relative to the total number of simulated neurospheres ( $n_{sim} = 500$ ) is shown.

4.2. Encapsulation of BTSCs as a determinant of normal vs. tumorous neurosphere development

The simulations based on our CA model have produced, among others, two remarkable outcomes. First, transformation of a randomly selected progenitor cell into a BTSC does not necessarily result in development of a tumorous neurosphere. Second, the decision whether a neurosphere develops into a neurosphere of normal size, without including more than a very few BTSCs, or into a tumorous neurosphere much larger than a normal neurosphere



**Fig. 7.** Neurosphere size as a function of developmental timing of tumor transformation. The data shown in the graph are derived from the simulated neurospheres on which Fig. 6 is based. Tumor transformation of a randomly selected progenitor cell was induced at  $t_{dm}$  ranging from 2 to 20. The mean of  $n_{live}$  as an indicator of average neurosphere size is shown separately for tumorous neurospheres (*red*) normal neurospheres (*blue*).



**Fig. 8.** Effect of cell death on normal and tumorous neurosphere development. Tumor transformation in neurospheres was induced at  $t_{dm} = 2$  (a-a") or  $t_{dm} = 20$  (b-b"). At each of these two developmental maturity stages of the neurospheres, the probability that newly generated cells undergo cell death was changed systematically from zero ('Cell Death Condition 1') to high ('Cell Death Condition 8'). For details, see Table 3. The graphs depict, at  $t_{final} = 100$ , the frequency distribution of  $n_{live}$  of 500 neurospheres simulated each under Cell Death Conditions 1 (*a*, *b*), 5 (a', b'), and 8 (a", b"). Note differences in scaling of the *x* axes.

and dominated by BTSCs in terms of its cellular composition, is made during the first few rounds of mitotic divisions of BTSCs. This prompts the question of what factors determine the course of normal versus tumorous development.

Closer inspection of the growing neurospheres has indicated that in each of the cases when tumor growth was abolished, the BTSCs stopped dividing because they were completely encapsulated by cells occupying all four grid positions in the immediate neighborhood, thereby suppressing any mitotic activity. We showed previously that this encapsulation of stem and progenitor cells is the major determinant that restricts growth of non-tumorous neurospheres (Sipahi and Zupanc, 2018). Here, we demonstrate that the same mechanism prevents BTSCs from giving rise to tumors.

Although reduced contact inhibition, mediated by transmembrane cell surface receptors coupled to intracellular signaling pathways, has for long been considered one of the hallmarks of cancer (Hanahan and Weinberg, 2000), recent research has indicated that cancer cells still sense contact with neighboring cells and alter their invasive behavior both in vitro and in vivo, depending on the cell density at which they were grown previously (Sharif and Wellstein, 2015; Sharif et al., 2015). Quite unexpectedly, even some of the most aggressive cancer cell lines may switch to a noninvasive phenotype when sensing a high growth density in culture (Sharif and Wellstein, 2015). In our model, we have implemented such a mechanism, which, upon sensing high cell density, forces the cells out of the proliferative cycle into the quiescent ( $G_0$ ) state. This was achieved by prohibiting any cell, including BTSCs, to undergo mitosis if all four neighboring grid positions were occupied.

On the other hand, if BTSCs succeeded in evading encapsulation by neighboring cells, their proliferation led inevitably to the development of tumorous neurospheres. The evasion of encapsulation was facilitated if the progenitor cell selected for tumor transformation was located near the periphery of the neurosphere. In such cases, the outgrowing tumor, carried by the proliferating BTSCs, frequently encapsulated the non-tumorous cells and blocked their further growth. It is important to note that the infinite open space available to cells in the periphery of the modeled neurospheres simulates well the morphology of epithelial tissues, which form not only the external surface of the body but also line the lumina of most internal organs, such as those of the intestinal tract or of glands. It has been estimated that as many as 90% of all human carcinomas arise from epithelial tissue (Hinck and Näthke, 2014). The present study provides a plausible mechanistic explanation for the prevalence of epithelial cancers: the open space faced by the epithelial cells greatly facilitates the evasion of encapsulation by neighboring cells. This spatial configuration helps the epithelial cells to avoid contact inhibition, and thus ultimately promotes growth of cancerous tissue.

### 4.3. The effect of maturity of tissue on tumor development

As discussed in the last section, simulations employing our CA model have suggested that encapsulation of stem and progenitor cells by other cells is a powerful mechanism to restrict growth of normal neurospheres, and a major determinant of the fate of neurospheres — to grow either normally or develop into a tumor. Among the simulated cell types considered here (stem cells, progenitor cell, differentiated cells, and dead cells), differentiated cells are the most effective ones for blocking mitotic activity of neighboring cells. According to the rules implemented into our model, only the differentiated cells cannot vacate a grid position, either by moving, or by dying followed by removal of the dead cell through phagocytosis. Furthermore, the probability that a progenitor cell is neighbored by a differentiated cell increases with increasing time t. We, therefore, hypothesized that the probability of a neurosphere to become tumorous will decrease with increasing t.

We have tested this hypothesis by simulating tumor transformation of progenitors at different time points of neurosphere development, reflected by  $t_{dm}$  ranging from 2 to 20. As predicted by our hypothesis, the simulations demonstrated a strong trend of neurospheres to become tumorous with decreasing time  $t_{dm}$ at which tumor transformation took place (Fig. 6). This trend was paralleled by an increase in the final size of tumorous - but not normal – neurospheres at  $t_{final} = 100$ . The latter difference can be explained by our previous observation that normal neurospheres approximate their final size most typically within less than 60 iteration cycles (Sipahi and Zupanc, 2018), whereas tumorous neurospheres, once on the path of tumor development, have the potential to grow indefinitely (cf. Fig. 5). Thus, a progenitor cell that was transformed into a BTSC at  $t_{dm} = 20$  has 18 iteration cycles less to add newly generated BTSCs to the neurosphere than a progenitor transformed at  $t_{dm} = 2$ . Furthermore, it is more likely that the progenitor cell that is transformed at  $t_{dm} = 20$  is surrounded by other cells, thereby preventing a rapid spread of tumor cells. We, therefore, conclude that the developmental stage of the tissue in which a progenitor cell is transformed into a BTSC has a major effect on the probability that this tissue progresses

into a tumor, whereas the growth rate of tumors is little, if at all, affected by differences in developmental maturity.

#### 4.4. The effect of cell death on tumor progression

Spontaneous cell death is a common feature of cancers, including brain tumors (Kordek et al., 1996; Kuriyama et al., 2002; Migheli et al., 1994; Patsouris et al., 1996). Cell death, induced through radiation or cytotoxic agents, is used as a major mechanism of anti-cancer therapies. However, as straightforward as such a therapeutic approach for the elimination of cancer cells seems to be, recent studies have indicated that the actual situation is more complex. In both lymphoma and prostate cancer, loss of part of the tumor cells through induction of apoptosis can promote tumor growth (Ford et al., 2015; Roca et al., 2018).

It has been hypothesized that tumor cell repopulation is mediated by compensatory proliferation (Zimmerman et al., 2013). This process is usually activated after loss of tissue during regeneration, most markedly in anamniote vertebrates, and involves a precise temporal orchestration of apoptosis of injured cells, removal of cellular debris through phagocytosis by macrophages, and mitosis of stem and progenitor cells (Sîrbulescu and Zupanc, 2011; Sîrbulescu and Zupanc, 2013; Zupanc and Sîrbulescu, 2013). Tumors might have usurped the evolutionary conserved mechanism of compensatory proliferation to offset the loss of cells caused by apoptosis-inducing anti-cancer therapies.

Despite the obvious implications for such therapies, the analysis of the interactions between cell death and tumor repopulation from a dynamical systems point of view is still in its infancy. In the present investigation, we have examined a specific aspect of these interactions, namely the effect of apoptotic cell death on the growth of tumorous neurospheres following transformation of a progenitor cell into a BTSC. The simulations yielded a notable result, which is reminiscent of the dichotomous effect that may occur after application of apoptosis-inducing anti-cancer therapies: Cell death can both promote and suppress tumor growth. Most significantly, our simulations suggest that the specific effect depends on the developmental maturity of the tissue in which the transformation of a progenitor cell into a BTSC is induced. As a corollary, this hypothesis predicts that anti-cancer therapies based on induction of apoptosis are most effective in tissues with a low degree of cellular differentiation, whereas in tissues with a high degree of differentiation such treatment, although initially reducing the number of tumor cells, will ultimately facilitate tumor growth. Efforts to optimize the efficacy of such therapies might benefit from the biological testing of this hypothesis.

#### Acknowledgments

The work was supported by the National Science Foundation, Grant no. 1538505 (G.K.H.Z., R.S.).

G.K.H.Z. designed and coordinated the research. R.S. and G.K.H.Z. developed the CA model. R.S. implemented the model. F.B.Z. and G.K.H.Z. conducted the simulations. G.K.H.Z. analyzed the data. G.K.H.Z. wrote the manuscript. R.S., F.B.Z., and G.K.H.Z. prepared the figures. All authors revised and approved the manuscript.

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