INDUCTION OF GLIOMA STEM CELLS BY INTERLEUKIN-1BETA AND TRANSFORMING GROWTH FACTOR-BETA

by

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Abstract

Transforming growth factor beta (TGF-β) and interleukin-1β (IL-1β) are both up-regulated in high grade gliomas and their elevated activities have been associated with prognosis in glioma patients. It is known that TGF-β is involved in proliferation and maintenance of glioma stem cells. In this study, I evaluated whether IL-1β also plays an important role in glioma stem cell development. Glioma stem cells are usually identified by using a sphere assay where glioma stem cells proliferate as neurospheres in serum free medium (SFM) in the presence of two growth factors: EGF and bFGF. However, LN229, a human glioblastoma cell line does not form neurospheres in SFM, suggesting that LN229 cells contain very few stem cells. I found that combination of IL-1β and TGF-β, but not IL-1β or TGF-β alone induced LN229 cells to grow as neurospheres in SFM. Furthermore, quantitative RT-PCR analyses show that the expression of stem cell markers (Nestin, Bmi1, Notch2, and LIF), cytokines (IL-1β, IL-6 and IL-8) and invasive genes (SIP1, β-integrin and N-Cadherin) are significantly enhanced in IL-1β /TGF-β induced spheres compared to the control. Using an invasion assay, drug resistance test, and colony assay, I found that LN229 sphere cells induced by IL-1β and TGF-β are more invasive, have increased drug resistant ability, and are more oncogenic in comparison to the control. Together, these results suggest that IL-1β cooperates with TGF-β to induce glioma stem-like cell phenotype.

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Dedication

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Chapter 1- Introduction

1.1 Glioblastoma multiforme

Glioblastoma multiforme is comprised of a collection of morphologically diverse tumor cells within the tumor mass. It is the most common and most aggressive type of primary brain astrocytic tumor in humans (Martuza, Seizinger et al. 1988). In 2000, the World Health Organization (WHO) classified glioblastoma multiforme as a grade IV glioma, which has the highest risk of malignancy in gliomas, by their histological criteria, with grades increasing with more severe malignancy (Black 1991). Unfortunately, in the case of glioblastoma multiforme, prognosis is very poor and the median survival even when radiotherapy and chemotherapy are combined is only 14.6 months (Stupp, Mason et al. 2005). In the past decades, there has been no significant increase in the overall survival of patients with this disease. Additionally, tumor relapse is universal, almost all patients experiencing tumor relapse or progression.

1.2 Inflammation and cancer

Recent data show that the tumor microenvironment, which refers to unique properties conferred by abnormal interactions between tumor and host cells (Sacchi, Ferrari et al. 1979), and which contains tumor compartment and recruited vascular, and stromal elements, is an indispensable factor in the neoplastic process (Lyden, Young et al. 1999; Ylikorkala, Rossi et al. 2001; Haga, Yanagisawa et al. 2003). It has been estimated that almost 25% of all cancers are related to chronic infection and inflammation (Hussain and Harris 2007). A number of gene association and molecular studies suggest that chronic inflammation has a tight connection to cancer initiation, progression and metastatic potential (Hold and El-Omar 2008). For the time being, it is still incompletely understood how the inflammation in the tumor microenvironment is triggered by inflammation. Several mechanisms (nuclear factor-kappa B, induction of damage to DNA and tissue repair response) of oncogenesis promoted by inflammatory responses have been demonstrated (Hold and El-Omar 2008). In addition, anti-inflammatory medications, such as: ibuprofen (Harris, Chlebowski et al. 2003) and piroxicam (Calaluce, Earnest et al. 2000), have

been known to dramatically lower the risk of cancer for patients with certain chronic inflammatory conditions (Garcia-Rodriguez and Huerta-Alvarez 2001). All these findings indicate that tumor initiation and progression has an essential relationship with its inflammatory microenvironment.

1.2.1 Transforming growth factor-β

Transforming growth factor-β1 (TGF-β) is a secreted multifunctional polypeptide that plays a key role in cell proliferation and differentiation (Massague, Blain et al. 2000), especially during the oncogenic process (Derynck, Akhurst et al. 2001). TGF-β signals mainly through activation of Smad transcription factors (Dennler, Goumans et al. 2002). When TGF-β binds to its receptor on membrane, the transcription factors Smad has been phosphorylated, and accumulate in nucleus (Massague 1998). TGF-β is highly active in high-grade glioma and the elevated TGF-β activity confers poor prognosis in glioma patients (Bruna, Darken et al. 2007). Interestingly, TGF-β plays a role in both tumor suppressor and oncogenic activities (Derynck, Akhurst et al. 2001). It is known that TGF-β can inhibit tumor growth at an early stage but it can also promote advanced tumor cell invasiveness and metastasis at a later stage (Stroemer and Rothwell 1998), thus the two faces of transforming growth factor beta in carcinogenesis (Siegel, Shu et al. 2003). In another words, TGF-β can inhibit differentiated cancer cell proliferation, but enhance cancer stem cell proliferation (de Caestecker, Piek et al. 2000).

1.2.2 Interleukin-1β

Interleukin 1 beta (IL-1β) is an important mediator of the inflammatory response (Fleisher, Ferrell et al. 1995). It is involved in cell proliferation and differentiation of multipotent neural precursor cells (Wang, Fu et al. 2007), and plays an essential role in host defense mechanisms and repair (Vonk, Netea et al. 2006). Some data shows that polymorphisms in the promoter region of the gene encoding IL-1β linked to an increased risk of developing cancer (Apte, Dotan et al. 2006). IL-1β has been shown to be produced in the central nervous

system (CNS) in response to the stimulation of traumatic brain injury (Sutcliffe, Smith et al. 2001), acute stress (Ota, Mori et al. 2008), and peripheral administration of lipopolysaccharide (O'Connor and Coogan 1999). IL-1β is thought to mediate tissue damage during inflammation, and it has been found that IL-1β can contribute to hypersensitivity in the central nervous system (Palin, Bluthe et al. 2004). Interestingly, it has been known that low concentrations of IL-1β can exacerbate neuronal damage in cerebral ischaemia (Rothwell and Relton 1993). Long-term production of IL-1β in the brain is associated with activation of glial cells as well as the occurrence of chronic disorders (Tringali, Dello Russo et al. 2000). IL-1β is abundant at tumor sites (Apte, Krelin et al. 2006), where it may affect the process of tumor initiation, growth and invasion. Inhibition of IL-1β reduces tumor invasion and progression in cancer therapy (Apte, Dotan et al. 2006). All these properties of IL-1β indicate to us that IL-1β may play a role in brain oncogensis.

1.3 Glioma stem cells

Normal neural stem cells to self-renew during cell division, give rise to progenitor cells, which in turn differentiate to astrocytes, oligodendrocytes and neurons. Similarly, glioma stem cells are capable of self-renewal while dividing to various progenitor cells, which give rise to tumor (Rich and Eyler 2008). Conventional cancer therapies target differentiated or differentiating cells, which form the tumor and are less resistant to chemotherapy and radiotherapy (Mabon, Svien et al. 1950; Frosina 2009). Tumors easily relapse after a period of treatments (Jordan, Guzman et al. 2006; Frosina 2009). Recently, studies showed that there may be a number of glioma stem-like cells in the glioblastoma mass (Tringali, Dello Russo et al. 2000). The glioblastoma contains a mixture of stem-like cells and differentiated cells, including: astrocytes, oligodendricytes and neurons. The stem-like cells, glioma stem cells (GSCs), play a very important role in glioblastoma multiforme recurrences that occur following current treatments (Larochelle, Vormoor et al. 1996; Lapidot, Goichberg et al. 2007). Therefore, it has become more significant to target and eliminate this population of cancer cells during cancer therapy (Figure.1.1).

It has been known that glioma stem cells are defined to have ability to form neurospheres (Lobo, Alonso et al. 2003) that are free-floating structures when cultured in SFM (Figure.1.2). However, a human glioma cell line, LN229 does not grow as neurospheres in SFM, suggesting that it contains very few stem cells. We use this cell line to study the effects of IL-1β and TGF-β on glioma stem cell development.

1.3.1 Cancer stem cell characteristic: self-renewal

One of the most important characteristics of cancer stem cells is self-renewal ability (Morrison, Prowse et al. 1996; Reya, Morrison et al. 2001; Morrison and Kimble 2006). Self-renewal is a unique cell division process, in which a cell is capable of giving rise to a daughter cell, which has the same properties as the mother cell (O'Brien, Kreso et al. ; Li, Chen et al. 2009). These rare cells reside within normal organs with the ability to self-renew and give rise to all types of cells within the organ to drive organogenesis. Similarly, rare cells within tumors have the ability to self-renew and give rise to the phenotypically diverse tumor cell population to drive tumorigenesis. Usually a balance of cell proliferation and cell death is required in normal tissue, which means a certain number of stem cells in the normal tissue are needed, but they also experience constant regulation (Pardal, Molofsky et al. 2005). However, oncogenesis does not undergo the normal regulation (Carbone and Levine 1990); a subpopulation within the tumor that has the ability to self-renew will constantly expand in the tumor and result in unlimited tumorigenic cancer cells (Al-Hajj and Clarke 2004). In the last thirty years, most cancer therapies have targeted cancer cells, but not cancer stem cells. At the beginning of the treatment, the tumor shrinks; however, the self-renewing cancer stem cells are capable of regenerating the tumor. In contrast, specific cancer stem cell therapy targets these cancer stem cells, which in turn suppresses the regeneration of tumor and eliminates the cancer (Serakinci, Guldberg et al. 2004). It has been known that glioma stem cells can self-renew to form neurospheres in serum free conditions in the presence of certain growth factors, providing a way to select the stem cells in vitro (Galli, Binda et al. 2004; Yuan, Curtin et al. 2004).

In this study, we use both the self-renewal assay, where a low density of cells is cultured in SFM, and the colony assay, where a certain number of cells are cultured in soft agar, to determine whether the cells are self-renewable and oncogenic.

1.3.2 Cancer stem cell characteristic: express stem cell markers

Cancer stem cells share some characteristics with normal stem cells (Al-Hajj, Wicha et al. 2003). For example, both of them have very low proliferation rates, and express some of the same stem cells markers, such as: Bmi-1, LIF, Nestin and Notch. B lymphoma Mo-MLV insertion region 1, Bmi-1, is a member of the polycomb family that functions to repress the transcription of its target genes (Hanson, Hess et al. 1999). It is required for self-renewal and post-natal maintenance of neural stem cells in the central nervous system (Molofsky, Pardal et al. 2003; Molofsky, He et al. 2005). It tends to be turned off as cells differentiate (Lessard and Sauvageau 2003). Leukaemia inhibitory factor, LIF, inhibits the differentiation of embryonic stem cells and promotes the proliferation of stem cells (Smith, Heath et al. 1988; Williams, Hilton et al. 1988). It is necessary and sufficient for the maintenance of stem cell in an undifferentiated state (Gough, Williams et al. 1989; Murray and Edgar 2001). Nestin is a major cytoskeletal protein in neural precursor in the mammalian CNS, it has been found in undifferentiated state and a characteristic neural stem cell marker (Lendahl, Zimmerman et al. 1990). Notch is a cell surface receptor (Artavanis-Tsakonas, Muskavitch et al. 1983) that plays a key role in the development of neurons in the central nervous system (Artavanis-Tsakonas, Muskavitch et al. 1983). It is known that stimulation of the Notch pathway promotes tumor growth, whereas Notch pathway blockade inhibits proliferation and/or survival. In addition, it has been reported that glioma stem cells contain higher Notch activity (Ignatova, Kukekov et al. 2002; Galli, Binda et al. 2004). In normal neural stem cells, Notch regulates their self-renewal and differentiation; in cancer stem cells Notch has a similar function.

1.3.3 Cancer stem cell characteristic: drug-resistance

Tumor relapse is a severe problem under current conventional therapies. Cancer stem cells are responsible for tumor initiation and propagation. Recent data shows that cancer stem cells derived from human glioma surgical specimens display radio-resistance due to increased activation of DNA damage checkpoint and promote tumor angiogenesis (Rich 2007). Similarly, cancer stem cells show stronger resistance to chemo-therapy in comparison to cancer cells. It has been reported that ATP-binding cassette drug transporters have been shown to protect cancer stem cells from those chemo- therapeutic agents (Dean, Fojo et al. 2005). Notch has been demonstrated to play an essential role in regulating the radiation resistance of gliom stem cells (Wang, Wakeman et al.). In mammals, activation of Notch receptor is mediated by binding to transmembrane ligands, Delta-like ligands or Jagged ligands. The ligand bindings were cleavaged, in turn, a γ-secretase complex cleaves Notch and releasing its active signaling portion, the Notch intracellular domain (NICD) (Fan, Matsui et al. 2006). In addition, Knockdown of Notch is sensitizing for glioma stem cells exposed to radiation (Wang, Wakeman et al. ; Zhang, Zheng et al. 2008). It is significant to identify and eliminate cancer stem cells. Therefore, in our study, we used Temozolomide and Carboplatin to investigate drug resistance abilities of the neurosphere cells induced by IL-1β and TGF-β1. Temozolomide is a widely used drug that was licensed by The European Agency for the Evaluation of Medicinal Products in early 1999. It is a novel oral alkylating agent that is efficient in the treatment of primary malignant brain tumors (O'Reilly, Newlands et al. 1993; Newlands, O'Reilly et al. 1996). It efficiently crosses the blood-brain barrier (Patel, McCully et al. 2003). Carboplatin is a common chemotherapy drug used against cancer. It is a cytotoxic platinum complex structurally related to cisplatin, it reacts with nucleophilic sites on DNA, causing intrastrandand interstrand crosslinks and DNA-protein crosslinks (Wheate, Walker et al. ; Kelland 2007).

1.3.4 Cancer stem cell characteristic: stronger invasive ability

The invasive property of glioblastoma is one of the most significant clinical problems (Mikkelsen, R. Bjerkvig et al. 1998). Without chemotreatment and radiotreatment, the mean

survival time of a glioblastoma patient is only 17 weeks (Avgeropoulos and Batchelor 1999). The small populations of glioma stem cells exhibit stronger extensive infiltration to surrounding normal brain separate from the tumor mass. It has been reported that epithelial-mesenchymal transition, EMT, may contribute to stronger invasive ability of cancer stem cells (Karnoub, Dash et al. 2007). When EMT occurs, epithelial cells break down cell-cell extracellular matrix connections and migrate to other locations of body, stem cells associated with mesenchymal cells or epithelial cells possess both stem cell-like properties and mesenchymal phenotype (Pang, Law et al. ; Vernon and LaBonne 2004). EMT plays key role in cancer invasion and metastasis (Vernon and LaBonne 2004). Furthermore, some similar molecules and pathways of normal stem cells and cancer stem cells have been identified to regulate both stem cell migration and cancer metastasis, such as: MET, a proto-oncogene, play an important role of invasive growth. To investigate the strong invasive ability of glioma stem cells, one of the most important characteristics of cancer stem cells, we performed an invasion assay to demonstrate that the neurosphere cells induced by IL-1β and TGF-β1 are capable of migrating compared to control cells. In addition, we perform qRT-PCR of some invasive genes: SIP1, N-cadherin and β-integrin, to investigate their invasive potential. Smad interacting protein 1, SIP 1, is a survival of motor neuron protein interacting protein (Meister, Buhler et al. 2000). It has ability to regulate stem cell differentiatation (Chng, Teo et al.), and controls cell migration during embryonic development (Yang and Weinberg 2008; Sayan, Griffiths et al. 2009) . N-cadherin is a classical cadherin from the cadherin superfamily (Angst, Marcozzi et al. 2001). Its increased activity on the surface of neuron allows the cell to choose the appropriate direction for its migration (Jossin and Cooper). β-integrin is also involved in cell adhesion and recognition in the process of embryogenesis and metastatic diffusion of tumor cells (Blystone, Slater et al. 1999; Arthur, Petch et al. 2000). We chose these invasive genes to examine the invasiveness gene expression in the cells induced by IL-1β and TGF-β.

1.4 Overall hypothesis

It has been known that both IL-1β and TGF-β1 are highly up-regulated in high grade glioma, and their elevated activities have been associated with poor prognosis in glioma patients.

In addition, TGF-β has been demonstrated to enhance cancer stem cell proliferation. Therefore, we hypothesized that IL-1β also plays an important role in glioma stem cell development.

Figure 1.1 Resistance mechanisms in glioma cells (Wen and Kesari 2008). Normal neural stem cells have the ability to self-renewal while division, giving rise to progenitor cells, in turn, differentiate to astrocytes, oligodendrocytes, and neurons. Oligodendrocytes, astrocytes can mutate to transformed stem cells. And the transformed stem cells give rise to tumor. Under traditional therapy, at the beginning, tumor temperarily regressed, after a while, tumor recurrence. However, the stem cell therapy is targeting on stem cells, it lead tumor regressed durably.

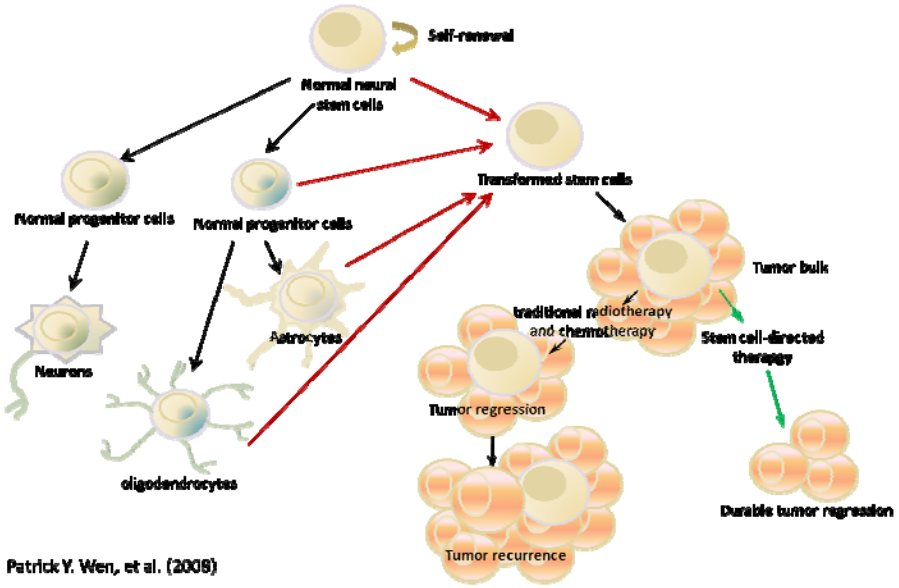
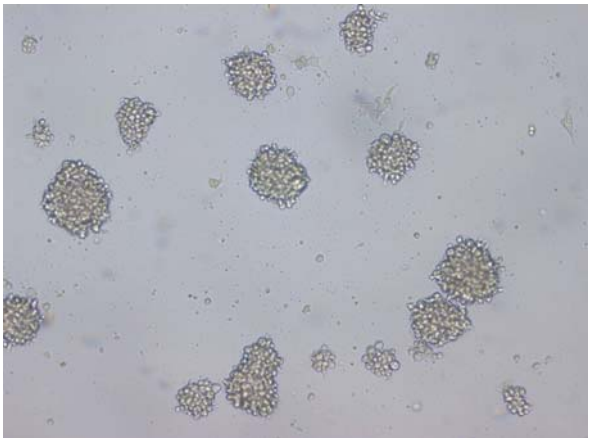


Figure 1.2 U87 cultured in serum free medium formed neurospheres. U87, human glioma cells form round, tight and floating neruospheres in SFM. Image was taken after seven days of culture.



Chapter 2 - Materials & Methods

2.1 Cell line

The LN229 cell line was a gift of Dr. Gilber Cote from Monroe Dunaway Anderson cancer center. LN229 was established in 1979 from cells taken from a patient with right frontal parieto-occipital glioblastoma (Pang, Law et al.). LN229 were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (GIBCO) in a humidified incubator at 37°C with 5% CO_2.

2.2 Sphere assay

LN229 cells grew as monolayers attached to T75 flasks in serum containing medium (SCM). When the cells reached ~70% confluency the culture medium was removed and the cells were rinsed once with 1X PBS. Trypsin-EDTA (0.05%, Cellgro, Inc) was added to the cells for two minutes at 37°C to trypsinize the cells from the T75 flasks. After approximately two minutes, most of the cells had detached from the flasks and 5ml of SCM was added to inhibit the function of Trypsin-EDTA. The cell suspension was centrifuged at 250 g, 24°C, for five minutes. The supernatant was removed and the cells were washed once with 1X PBS to remove any serum around the cells, the cell suspension was centrifuged again as described previously, and the supernatant was removed. The cell pellet were resuspended with serum free medium comprised of neurobasal-A medium (GIBCO/Invitrogen) with supplements 1X B27 (Invitrogen), 20ng/ml of epidermal growth factor (EGF, R&D systems), 20ng/ml of basic fibroblast growth factor (bFGF, R&D systems), 1X GlutaMAX-I supplement (Invitrogen), 50ng/ml heparin (Sigma-Aldrich) and 1% penicillin-streptomycin. Cells were seeded at $1x10^4$ cells/well in 24-well plates that contained 0.5ml SFM with or without Interleukin-1 beta (IL-1β, R&D) and Transforming Growth Factor (TGF-β1, R&D), and grown in the incubator at 37°C and 5% CO_2 for seven days. IL-1β (4ng/ml) and TGF-β (5ng/ml) were added every other day, and the media was changed on the fourth day of culture. After seven days of culture, images were taken and spheres were counted microscopically.

2.3 Cell proliferation assay

LN229 cells were cultured as sphere assay described above and $1x10^3$ cells/well with 100μl SFM was placed in a 96-well plate for seven days. IL-1β (4ng/ml) and TGF-β (5ng/ml) was added every other day, and the media was changed on the fourth day of culture. On the seventh day of culture, the cell proliferation ratio was measured by using the cell proliferation kit (MTT, Roche Applied Science) as follows: 10μl/well of MTT labeling reagent was added, mixed well and incubated in a humidified incubator at 37°C, after four hours solubilization solution was added, mixed well and incubated in the incubator overnight. The next morning, the 96-well microplate was evaluated with a spectrophotometer at 550 nm with a reference wavelength of 690 nm.

2.4 Self-renewal assay

LN229 cells were cultured as sphere assay described above, 100 cells/well with 100μl SFM achieving a low cell density, 1 cell per micro liter SFM, was placed in a 96-well microplate. Plates were incubated for 14 days in the absence or presence IL-1β (4ng/ml) and TGF-β (5ng/ml). Fresh cytokines were added every other day, and the SFM media was changed every four days. After 14 days culture in a humidified incubator at 37°C, images were taken and the neurosphere number was counted under the microscope.

2.5 Drug resistant assay

LN229 cells were cultured as sphere assay described above, $1x10^3$ cells/well with 100μl SFM was placed in a 96-well microplate for five days with or without IL-1β (4ng/ml) and TGF-β (5ng/ml). Fresh cytokines were added every other day, and the media was changed on the fourth day. On the fifth day of culture, when cells in the control group were approximately 60-70% confluent in the wells, 0μM, 250μM, 500μM, or 1000μM of Temozolomide or Carboplatin was mixed in each well. After 48 hours of treatments with drug in the incubator with 37°C and 5%

CO_2, the cells were dissociated, stained with Trypan blue (Amresco Inc.) and counted under the microscope. The viability was determined by the percentage of live cells over the sum of live and dead cells.

2.6 Invasion assay

The invasion assay was performed on 24-well BD Falcon cell culture inserts containing an eight micron pore size membrane with a thin layer of matrigel basement membrane matrix (BD Biosicences). LN229 cells were cultured as sphere assay described above, 8 x 10^5 cells/well with 1.5ml SFM was placed in a 6-well plate for seven days, in the absence or presence of IL-1β (4ng/ml) and TGF-β (5ng/ml). Fresh cytokines were added every other day, and the media was changed on the fourth day. After seven days of culture, cells in each treatment group were dissociated with Trypsin-EDTA as described above. After the cells were washed with 1X PBS, they were resuspended in SFM, and counted under the microscope. The cells were diluted to reach the total number of $5x10^4$cells/ml. The top layer of the rehydrated inserts was seeded with 0.5ml of the cell suspension and 0.75ml of media containing 20% FBS was added in the lower layer as chemoattactant. After 48 hours of incubation in the humidified incubator at 37°C, 5% CO_2, non-invaded cells were removed from the upper surface of the membrane by using moist cotton tipped swabs to "scrub" twice. The invaded cells at the lower surface were fixed by 100% cold methanol for two minutes, and stained by 0.005% crystal violet for ten minutes. The wells were then washed with distilled water three times, using a beaker to remove excess stain, until the color did not come off anymore. Images were taken immediately.

2.7 Colony assay

LN229 cells were cultured as sphere assay described above, 8 x 10^5 cells/well with 1.5ml SFM were placed in a 6-well for seven days, in the absence or presence of IL-1β (4ng/ml) and TGF-β (5ng/ml). Fresh cytokines were added every other day, and the media was changed on the fourth day. After seven days of culture, the monolayer cells in the control treatment and the

sphere cells in the IL-1β/TGF-β treatment were trypsinized as described above and resuspended in 2 X SCM, counted under a microscope, and resuspended to reach the total number of 2 x 10^4 cells/ml. Then 0.5 ml control or pretreated cell suspension was mixed with 0.5 ml of 0.8% 40°C Sea Plague Agar to make the top layer of soft agar in a 6-well plate. The bottom layer contains a mixture of 0.5 ml 1.6% Sea Plague Agar and 0.5 ml 2X SCM (2X DMEM containing 20% FBS and 2% Pen/Strep). Then the plates were placed in the incubator at 37°C and 5% CO_2. Images were taken and the cell number was counted microscopically after two weeks.

2.8 Immunocytochemistry

LN229 cells were cultured as sphere assay described above, 5x 10^3cells/well with 500μl SFM were placed in slide chambers (4-chambers per slide, Fisher Scientific) for seven days with or without IL-1β (4ng/ml) and TGF-β (5ng/ml). Fresh cytokines were replenished every other day, and the media was changed on the fourth day. On the seventh day of culture, the cells were approximately 80% confluent in the slide chambers, and the media was removed and the chamber slides were gently washed twice with 0.5 ml phosphate buffered saline (PBS). The cells were then fixed with 4% paraformaldehyde for 20 minutes in the dark at room temperature, washed three times with PBS, followed by blocking with 5% BSA plus 0.2% Triton X-100 for two hours at room temperature. After washing three times with PBS, mouse anti-nestin (1:50; Santa Cruz, USA) primary antibody was diluted with 2% BSA and 0.2% Triton X-100, added to the cells and incubated overnight at 4°C. The following day the slides were gently washed with PBS three times, five minutes each. Secondary antibody, chicken anti-mouse IgG (H+L) (1:200, Invitrogen), was added for one hour. Slides were then mounted with VECTASHIELD Mounting Medium (1 drop per slide, Vector) and images were taken using confocol microscope.

2.9 ELISA

LN229 cells were cultured as sphere assay described above, 8 x 10^5cells/well with 1.5ml SFM were placed in 6-well plates for seven days with or without IL-1β (4ng/ml) and TGF-β

(5ng/ml). Additional cytokines were added every other day, and the media was changed on the fourth day. On the sixth day of culture, cells had reached approximately 80% confluent in the wells of the control treatments. The media in each well was removed and the cells were washed once with 1X PBS to get rid of the external cytokines, then fresh SFM was added in each well without the cytokines, and cultured in the incubator overnight. These supernatants from the cell culture were used for IL-1β and IL-8 testing by ELISA (kits from R&D) following the kit instructions. The microplates were read at the wavelength of 450nm, with a correction wavelength of 540nm.

2.10 RNA extraction

LN229 cells ($8x10^5$/well) were cultured in SFM with or without IL-1β and TGF-β stimulation for seven days as indicated above for the sphere assay, followed by RNA extraction by TRI reagent. Cells were rinsed with ice cold PBS once and lysed directly in the well by adding 1ml of TRI Reagent (Sigma) per 3.5cm diameter. Cell lysates were passed several times by thorough pipetting and vortexing. Chloroform (Fisher) was added at 0.2ml per 1ml of TRI Reagent, and the samples were vortexed vigorously for 15 seconds and then incubated at room temperature for two to three minutes. The samples were centrifuged at 14,000 rpm for 15 minutes at 4°C. Following centrifugation, the upper aqueous phase was transferred carefully without disturbing the interphase into a fresh tube. The RNA was precipitated by mixing with 0.5ml isopropyl alcohol (Sigma) per 1ml of TRI Reagent. Samples were incubated at 4°C for 30 minutes and centrifuged at 14,000 rpm for 15 minutes. The supernatant was removed completely and the RNA pellet was washed once with 1ml 75% ethanol per ml of TRI Reagent. The RNA was centrifuged at 10,000 rpm for 5 minutes at 4°C, and the supernatant was removed. The RNA pellet was air-dried for 10 minutes and dissolved with DEPC-treated water. In order to avoiding contamination of genomic DNA, RNA samples were digested by a DNAse kit (Ambion, AM1906) as follows: 0.1 volume 10x DNase I buffer and 1μl rDNase I were added to the RNA. The enzymes and RNA were incubated in 37°C for 20 minutes. 0.1 volume of DNase inactivation reagent was added to the RNA and the reaction was incubated at room temperature

for 2 minutes followed by centrifugation at 10,000 x g for 1.5 minutes. After centrifuging, the supernatant which contains the RNA was collected.

2.11 Gene expression

Relative mRNA expression was performed by two-step qRT-PCR. cDNA was reverse transcribed by using the iScript cDNA synthesis kit (Bio-Rad 170-8891). A total volume of 20ul, consisting of 500ng RNA, 4µl 5X iScript reaction mix, 1µl iScript reverse transcriptase and the balance as water were mixed per reaction.

qPCR were performed by using SsoFast Eva Green Supermix kit (Bio-Rad 172-5201). The reaction mix consisted of 10µl Eva Green mix buffer, 0.5µl forward primer, 0.5µl reverse primer, and 0.5µl template, which was obtained from the reverse transcription step as described above. The condition of the qPCR steps was as follows: enzyme was activated by 30 seconds at 95°C, denatured at 95°C, then annealing and extension at 62°C for 30 seconds, and last, melting at 78°C for 20 minutes and increase 0.5°C in 10 seconds per step from 65°C to 85°C. The amplificationof cDNA was performed with the primers (Table 2.1).

2.12 qRT-PCR analysis

Relative mRNA expressions were analyzed by $2^{-\Delta\Delta C}{}_{T}$ method (Winer, Jung et al. 1999). β-actin was used as internal control gene. The C_T values were provided from real time instrumentation (AB system). qPCR was performed on the corresponding cDNA synthesized from each sample. The data was analyzed using the equation: amount of target = $2^{-\Delta\Delta C}{}_{T,}$ where $\Delta\Delta\ C_T = (C_{T.\ target} - C_{T.\ Actin})_{treated} - (C_{T.\ target} - C_{T.\ Actin})_{control}$. The fold change in the target gene, normalized to β-actin and relative to the expression of control, was calculated for each sample.

2.13 Statistic analysis

Statistical analyses were compared by a paired, two-tailed student's t test in excel. A p value of less than 0.05 was considered significant. Analysis of variance was used for comparison of multiple groups.

Table 2.1 Sequences of primers were used in qRT-PCR.

	Forward primer sequence	Reverse primer sequence
Nestin	5’-GAAACAGCCATAGAGGGCAAA-3’	5’-TGGTTTTCCAGAGTCTTCAGTGA-3’
LIF	5’- CCCATCACCCCTGTCAACG-3’	5’- GGGCCACATAGCTTGTCCA-3’
Bmi1	5’-TCATCCTTCTGCTGATGCTG-3’	5’-GAACTTGCCGGAACTGAGAAC-3’
Notch2	5’- TATTGATGACTGCCCTAACCACA-3’	5’- ATAGCCTCCATTGCGGTTGG-3’
SIP1	5’-CGCTTGACATCACTGAAGGA-3’	5’-CTTGCCACACTCTGTGCATT-3’
N-cadherin	5’- CAACTTGCCAGAAAACTCCAGG-3’	5’- ATGAAACCGGGCTATCTGCTC-3’;
β-integrin	5’-GTTACACGGCTGCTGGTGTT-3’	5’-CTACTGCTGACTTAGGGATC-3’
β-actin	5’-CCTGGGCATGGAGTCCTGTGG-3’	5’- CTGTGTTGGCGTACAGGTCTT-3’
IL-1β	5’-AAGCTGAGGAAGATGCTG-3’	5’-ATCTACACTCTCCAGCTG-3’
IL-6	5’-AACCTGAACCTTCCAAAGATGG-3’	5’-TCTGGCTTGTTCCTCACTACT-3’
IL-8	5’-TAGCAAAATTGAGGCCAAGG-3’	5’-AGCAGACTAGGGTTGCCAGA-3’

Chapter 3 - Result and Discussion

3.1 Combination of IL-1β/TGF-β induces sphere formation of LN229 cells in SFM

The glioma cell line we used is LN229. The cells were grown as monolayers attached to the bottom of flasks in the presence of serum. We hypothesized that LN229 induced by IL-1β and TGF-β would form neurospheres in SFM. To test the hypothesis, we performed LN229 with or without IL-1β and TGF-β as sphere assay based on the ability of GSCs to generate neurospheres in SFM (Reynolds and Weiss, 1996; Seaberg and van der Kooy, 2002). In sphere assays, LN229 cells were switched from SCM to SFM, which is known to be permissive for stem cell proliferation and cultured for seven days in the absence or presence of IL-1β and TGF-β. From the sphere assay, our images show that LN229 cells do not form neurospheres in SFM, suggesting that these cells contain very few stem cells. In addition, we found some morphological changes of treated cells: with single IL-1β or TGF-β treatment, cells formed only a small number of neurospheres in SFM. Surprisingly, the combination of IL-1β and TGF-β induced significantly more tight, round and floating neurospheres in SFM (Figure 3.1A-D). The total neurosphere numbers were counted (Figure 3.1 E). Treatment with the combination of IL-1β and TGF-β formed one thousand more neurospheres as compared to control treatment and IL-1β or TGF-β alone. This result indicates that IL-1β cooperates with TGF-β to induce neurosphere formation.

3.2 IL-1β/TGF-β induces sphere formation in SFM at a very low cell density

The morphological results of our sphere assay suggested that the combination of IL-1β and TGF-β induced neurosphere formation. Additionally, stem cells usually form spheres. One of the most important characteristics of glioma stem cells is self-renewal (Pilkington 2005; Vescovi, Galli et al. 2006). To test whether the neurosphere cells induced by IL-1β and TGF-β

were stem cells, we performed a self-renewal assay. Self-renewal is one of the most important characteristics of GSCs (Reya, Morrison et al. 2001). We cultured LN229 cells at a very low cell density, 1 cell per 1μl SFM. After 14 days of culture in SFM with or without IL-1β and TGF-β stimulation, neurospheres were only formed in the presence of the cytokines (Figure 3.2A, B, C). This result suggests that IL-1β and TGF-β induce self-renewal of LN229 cells.

3.3 IL-1β/TGF-β slows down LN229 cell proliferation ratio

To further determine the effect of IL-1β and TGF-β on cell proliferation, proliferation assays on LN229 cells were performed. LN229 cells were cultured in SFM similar to the sphere assay with or without IL-1β and TGF-β stimulation for seven days, followed measurement of the proliferation ratio by MTT proliferation assay. The proliferation ratio of the sphere cells in the presence of IL-1β and TGF-β was 50% lower as compared to the cells in the absence of the cytokines (Figure 3.2 D). It has been known that cancer stem cells proliferate slower than tumor cells (Moore and Lyle). These results suggest that neurospheres induced by IL-1β and TGF-β enriched stem cells.

3.4 IL-1β/TGF-β-induced sphere cells form more and larger colonies than control cells

To investigate the tumorigenic potential of sphere cells is induced by IL-1β and TGF-β, we performed colony-forming assays. LN229 cells were cultured in the soft agar for 14 days. The semi-solid agar reduces cell movement and allows individually transformed cells, which lose anchorage-dependence, to develop into colonies. Normal cells do not possess transformed properties to grow as colonies in the soft agar, however, cancer stem cells, which are more tumorigenic, can self-renew and grow on top of each other to form colonies in the soft agar. The number of colonies was counted under the microscope and the size of colonies was measured by using Image J. The sphere cells induced by IL-1β and TGF-β formed significantly more and

larger colonies compared to control cells (Figure 3.3 A-C). These results suggest that the cytokine-induced sphere cells have stronger tumorigenic potential.

3.5 IL-1β/TGF-β-stimulated cells express stem cell markers

To study whether LN229 cells in the presence of IL-1β and TGF-β express more stem cell markers (Nestin, Bmi-1, LIF and Notch2) compared to the cells in the absence of the cytokines. We performed immunocytochemistry assays of Nestin, which is an extensively used as CNS stem cell marker (Uchida, N. et al. 2005) (Frederiksen, Jat et al. 1988). LN229 cells were cultured in SFM in the absence or presence of IL-1β and TGF-β for seven days. The cells on chamber slides were fixed and stained with Nestin antibody. Images were taken using confocol microscopy. LN229 cells stimulated with IL-1β and TGF-β expressed more Nestin compared to the control cells (Figure 3.4 A). This result suggests that the sphere cells induced by IL-1β and TGF-β contain more stem cells compared to the control cells. In addition, we tested gene expression of other stem cell markers, including Bmi-1, LIF and Notch. Notch has been known to be involved both in the maintenance of neural progenitors and in the generation of glial during development of the brain (Gaiano and Fishell 2002). Bmi-1 has been demonstrated to be essential for the self-renewal of neuronal stem cells. LIF is necessary to maintain the stem cells in an undifferentiated state (Matsuda, Nakamura et al. 1999). Our qRT-PCR results show that all these stem cell markers were expressed more in IL-1β and TGF-β-stimulated cells compared to control cells (Figure 3.4 B-E). These data suggests that the combination of IL-1β and TGF-β increases the pool of glioma cells expressing cancer stem cell markers.

3.6 IL-1β /TGF-β-induced sphere cells developed drug resistance

Temozolomide is the most commonly used chemotherapeutic agent in the therapy of glioblastoma (O'Reilly, Newlands et al. 1993). Carboplatin is another common drug used against cancer (Wheate, Walker et al.). To analyze the drug resistance ability, we cultured LN229 cells in SFM in the absence or presence of IL-1β and TGF-β for five days, followed by different concentrations of Temozolomide or Carboplatin for 48 hours. When we measured viability of the

cells after exposure to Temozolomide for 48 hours, we found that the cytokine-induced spheres had significantly increased resistance to the drug at 250μM, 500μM, and 1000μM concentration of temozolomide. Similarly, the viabilities of the sphere cells when exposed to 1000μM concentration of carboplatin showed significantly greater resistance compared to control cells (Figure 3.5 A, B). It has been reported that one of the most critical characteristics of cancer stem cells is stronger drug resistance ability compared to cancer cells (Dean, Fojo et al. 2005; Kang and Kang 2007). These results suggest that there were more stem cells in spheres induced by IL-1β and TGF-β.

3.7 IL-1β /TGF-β-induced sphere cells have increased invasion and invasive gene expression

It has been known that one of the most important characteristics of stem cells is stronger invasive ability compared to normal cells (Schichor, Birnbaum et al. 2006; Mani, Guo et al. 2008). To test the invasive ability of LN229 cells in the presence of IL-1β and TGF-β, we performed invasion assays. LN229 cells were seeded in the top of insert in serum free media, while medium with 20% FBS was added to the lower chamber as chemoattractant. After 48h incubation, non-invaded cells were removed from the top chamber, invaded cells migrated through pores of membrane to the lower surface of the chamber and were fixed and stained with 0.005% crystal violet. Our data shows that IL-1β and TGF-β pretreated sphere cells have significantly more cells migrated and stained compared to the control cells (Figure 3.6 A-C). In addition, the expression of invasive genes SIP1, β-integrin, and N-Cadherin was higher in the IL-1β and TGF-β treated sphere cells than control cells (Figure 3.6 D). Smad interacting protein 1, SIP1, a multi-zinc finger transcriptional repressor, has been demonstrated that it associates breast cancer cells migration and invasion (Bindels, Mestdagt et al. 2006). Knockdown of SIP1 inhibited glioma cells migration and invasion (Xia, Hu et al.). Beta1-integrin play an important role of glioma cell migration (Huang, Tian et al. 2006) and promotes an invasive phenotype. Some data has been reported that N-Cadherin levels were significantly higher in gliomas than in low-grade astrocytomas or normal brain (Shinoura, Paradies et al. 1995). N-Cadherin plays an

essential role to promote migration and invasion in glioma cells (Shinoura, Paradies et al. 1995). Therefore, these results suggest that the sphere cells induced by IL-1β and TGF-β contain more stem cells compared to the cells in the absence of the cytokines.

3.8 IL-1β/TGF-β induces inflammatory cytokines secretion

It has been demonstrated that IL-1β, IL-6 and IL-8 are involved in the innate immune response (Fleisher, Ferrell et al. 1995; Birnbaum, Roider et al. 2007; Naugler and Karin 2008). It is also known that IL-6 can induce cancer stem cell proliferation (Ernst, Gearing et al. 1994), while IL-8's role is not well understood. Many recent reports show a relationship between inflammation and oncogenesis (Grivennikov, Greten et al. ; Balkwill 2004; Kim and Joh 2006; Allavena, Garlanda et al. 2008). To investigate protein and gene expression of inflammatory cytokines, we performed qRT-PCR and ELISA. Our data from qRT-PCR show more than 80 fold increases of IL-8, more than 20 fold increases of IL-1β and more than 30 fold increase of IL-6 gene transcripts were expressed in cells treated with IL-1β and TGF-β (Figure 3.7 A). To confirm the gene expression of IL-1β and IL-8, we also performed ELISA by using culture media to determine the protein expression of IL-1β and IL-8. Our results demonstrate a 400 fold increase in IL-1β protein and 1000 fold increase in IL-8 protein was measured in the media of cells treated with IL-1β/TGF-β compared to control cells (Figure 3.7 B). The results of increased expression of gene and protein in the treated cells indicate that IL-1β/TGF-β induces IL-1β and IL-8. In addition, to test whether IL-8 mediates IL-1β/TGF-β-induced sphere formation, we performed sphere assays in the presence and absence of IL-8 and TGF-β. Our representative images were taken after seven days of culture (Figure 3.7 C-E). This result shows that only monolayer cells were observed in the control and IL-8 treatment, while the cells treated with combination of IL-8/TGF-β formed more spheres. These representative images suggest that IL-8 is also capable of synergizing with TGF-β to induce neurosphere formation.

3.9 Spheres cells induced by IL-1β/TGF-β were still able to form cell clusters of LN229 cells in SFM without IL-1β/TGF-β stimulation

To further characterize properties of cells induced by IL-1β and TGF-β, we performed a second passage of sphere cells. That is LN229 cells cultured in SFM in the absence or presence of IL-1β and TGF-β for seven days [Passage 1, (P1)]. After the seven days of culture, control cells or sphere cells were trypsinized and 1,000 dissociated cells of each type were plated in SFM in the absence of IL-1β and TGF-β for another seven days [Passage 2, (P2)]. Surprisingly, without IL-1β and TGF-β stimulation, the dissociated sphere cells were still able to form significantly more cells clusters (Fig. 3.8 A-C). To further investigate the characteristics of P2 cells, we performed qRT-PCR to examine the gene expression of stem cell markers Notch, Bmi-1, LIF, and Nestin. There was significant decrease of Nestin, LIF and Notch gene expression in P2 compared to P1 (Fig. 3.8 D-G). In contrast, Bmi-1 in P2 still maintained as highly expressed as that in P1 cells. This key role of Bmi-1 in self-renewal and maintaining neural stem cell proliferation (Bruggeman, Hulsman et al. 2007; Bruggeman, Hulsman et al. 2009) may explain the cluster formation in P2 cells. To further investigate the oncogenic potential of P2 cells, I performed the colony-forming assays for P1 and P2 cells. Cells were cultured in the soft agar for 21 days and the number of colonies was counted under the microscope and the size of colonies was measured using Image J. Interestingly, the P2 cells induced by IL-1β and TGF-β formed significantly more colonies compared to control cells without cytokine pretreatment (Figure 3.8 H-I). Consistent with the results of gene expressions, sizes of P2 colonies are significantly larger than control cells, but significantly smaller than to P1 colonies. These results suggest that the P2 cells have stronger tumorigenic potential compared to control cells.

3.10 Discussion

Studies on leukemia (Lapidot, Sirard et al. 1994) and breast carcinoma (Al-Hajj, Wicha et al. 2003) have demonstrated that a small group of cells within the tumor mass is responsible for tumor formation and maintenance. Glioma stem cells, the small population of glioma cells, play a key role in brain tumor recurrences after current treatments. There is no question that GSCs

will open a new approach of cancer therapies. It is becoming more and more important to target and eliminate this small population of glioma cells.

TGF-β, an oncogenic factor (Bruna et al., 2007), confers a poor prognosis in human glioma patients (Bruna, Darken et al. 2007). Recent evidence indicates that TGF-β increases self-renewal capacity, induces proliferation of glioma stem cells and prevents their differentiation (Penuelas, Anido et al. 2009; Watabe and Miyazono 2009). In addition, silencing of TGF-β expression by small interfering RNA (siRNA) technology has been reported to abrogate glioma cells tumorigenicity in vivo (Golestaneh and Mishra 2005).

The application of principles used for studying the chemokines/cytokines and GSC to cancer biology indicates a link between inflammation and brain tumorigenesis. Does IL-1β, a pro-inflammatory cytokine, have significant effects on GSCs development? Our data show that LN229 sphere cells induced by IL-1β and TGF-β in serum free condition contains a small fraction of cells that are capable of displaying stem cell characteristics: self-renewal, invasion, anti-cancer drug resistance and expression of stem cell markers. This indicates that IL-1β cooperates with TGF-β to induced GSC-like cells, which may explain the association of IL-1β and TGF-β with poor prognosis of gliomas in human patients.

Notch has been reported to play a critical role in the regulation of radio-resistance of glioma stem cells (Wang, Wakeman et al.). Our qRT-PCR result shows that there is significantly increased expression of Notch in IL-1β/TGF-β-induced sphere cells, which might explain the increase of drug resistance ability induced by IL-1β and TGF-β.

We have observed that IL-1β synergizes TGF-β to induce neurospheres formation of LN229 cells and increase their capacity of self-renewal. Bmi-1, B-cell-specific Moloney murine leukemia virus integration site 1, is required for the self-renewal of stem cells in central nervous systems (Lessard and Sauvageau 2003; Molofsky, Pardal et al. 2003). Knockdown of Bmi-1 in human brain cancer cell lines causes inhibition of proliferation in vitro, and suppression of tumor formation in vivo (Cui, Zhao et al. ; Bruggeman, Hulsman et al. 2009). An interesting finding of our results shows that expression of Bmi-1 is not only significantly increased in P1 sphere cells induced by IL-1β and TGF-β, but also maintained highly expressed in P2 cluster cells, which are the sphere cells followed by 7 days culture without IL-1β and TGF-β stimulation in serum free condition. Moreover, the P2 cells show almost similar oncogenic potential as P1 in colony assay. These results suggest that Bmi-1 may be a major factor for increased oncogenic potential of P1

and P2 cells. However, the molecular mechanism underlying the regulation of human stem cells by Bmi-1 remains unknown. Some people reported that Bmi-1 regulates by the repression of p16INK4a and p19ARF (Molofsky, He et al. 2005); and some people suggest that Bmi-1 may play a role in the regulation of stem cells because of the stabilization of telomeres (Silvestre, Pineda Marti et al.). An understanding of the exact mechanisms implicated in the regulation of GSCs will provide successful therapeutic strategies. Determination of the molecular mechanisms of our present work demands further investigation.

Figure 3.1

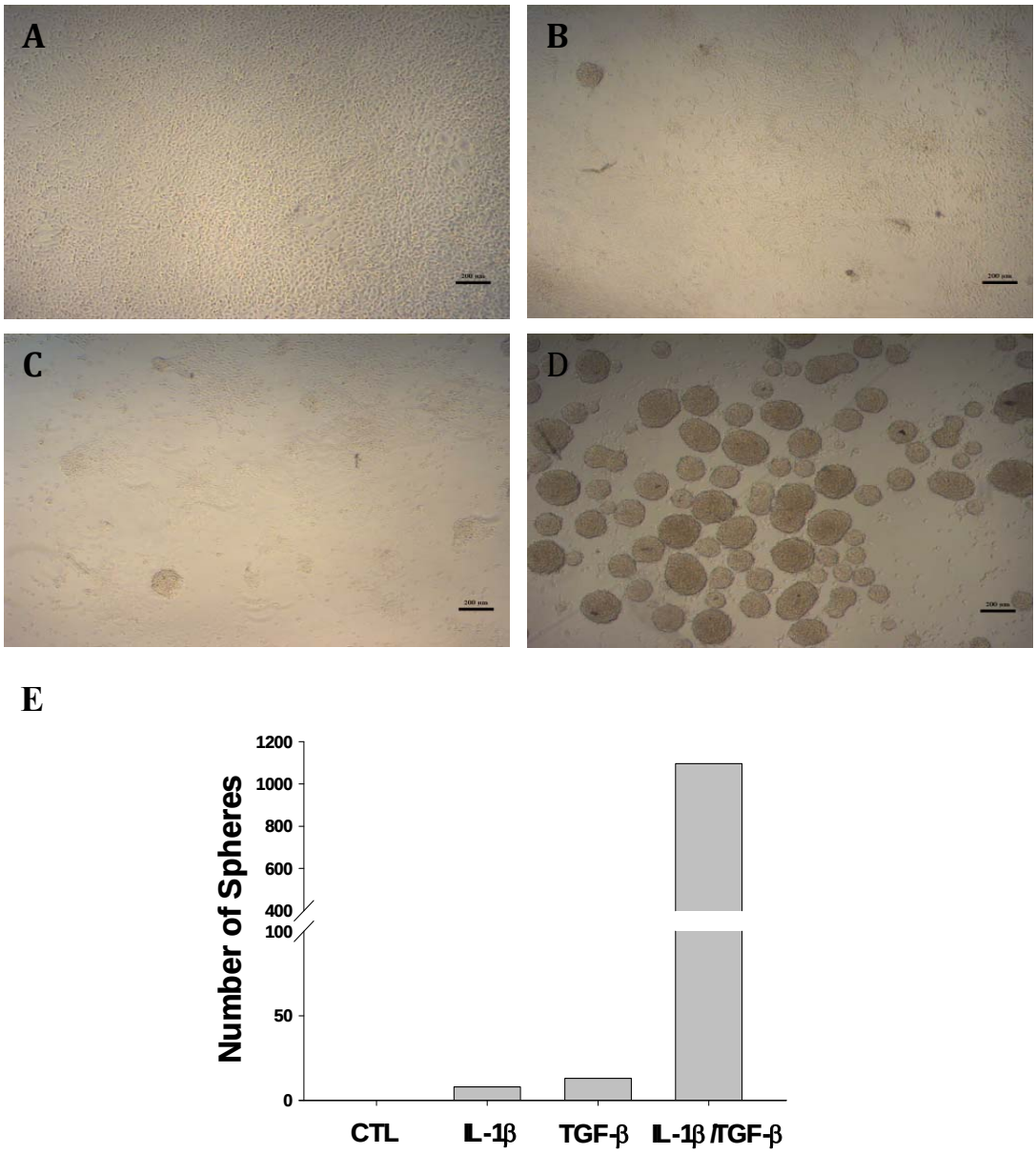


Figure 3.1 Combination of IL-1β/TGF-β induces sphere formation of LN229 cells in serum-free medium (SFM). LN229 cells (10,000 cells/well) were cultured in SFM in the absence or presence of IL-1β and TGF-β for seven days. Spheres were counted under microscope. **A**. Control, cells are found as monolayer cells. **B**. With IL-1β treatment, cells form several cell clusters. **C**. With TGF-β treatment, cells form several cells clusters. **D**. With IL-1β+TGF-β treatment, many very tight neurospheres are found. **E**. Quantitative number of the neurosphere forming cells shows a significant increase when cells are treated with IL-1β and TGF-β. Scale bar = 200μm. Figures are representative of three independent experiments.

Figure 3.2

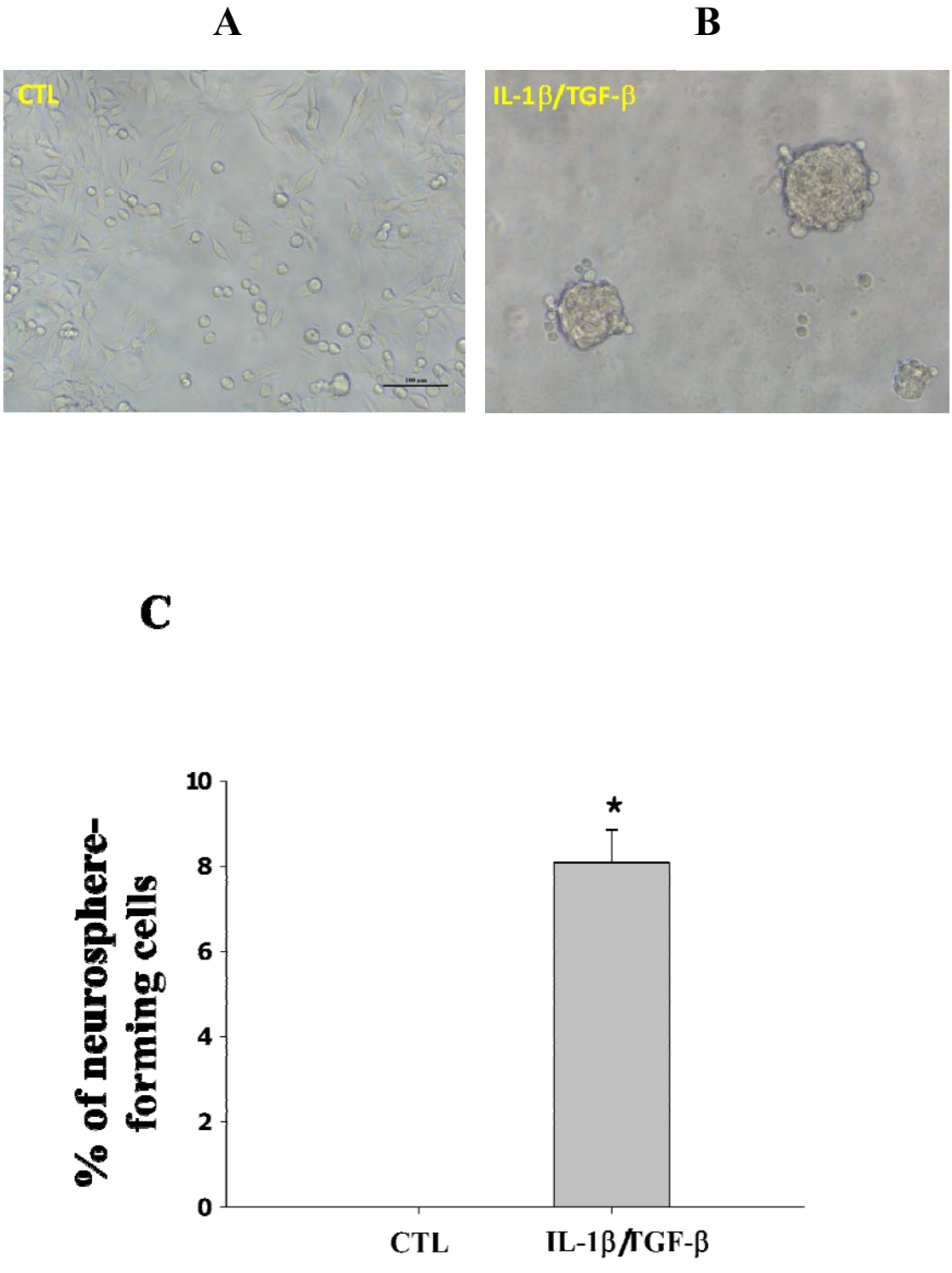

A
B
CTL
IL-1β/TGF-β
C
% of neurosphere-forming cells
10
8
6
4
2
0
*
CTL
IL-1β/TGF-β

Figure 3.2

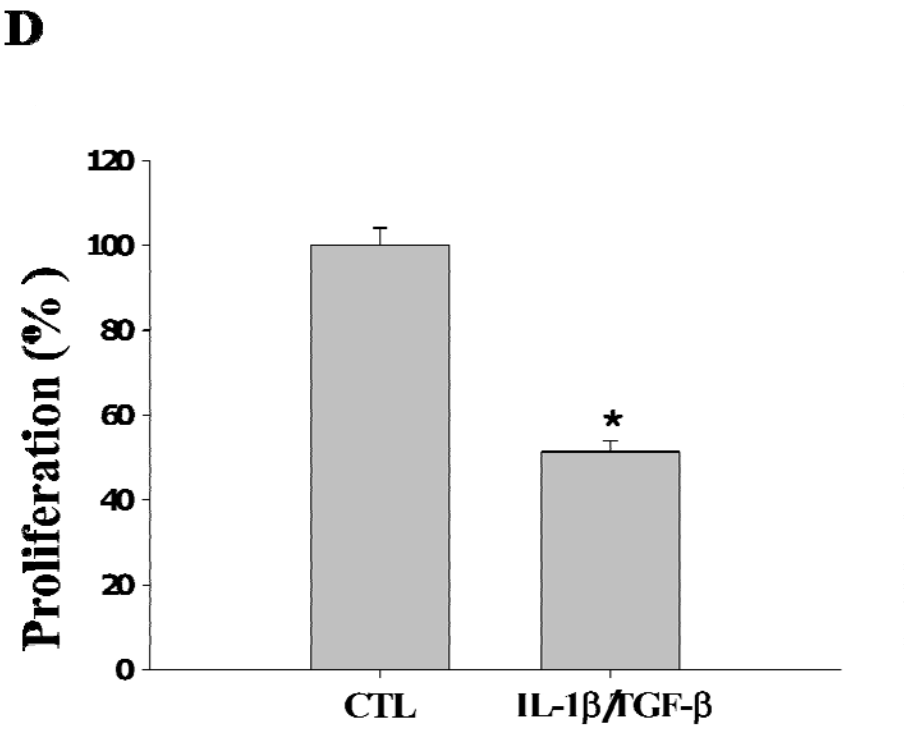


Figure 3.2 IL-1β/TGF-β induces sphere formation in SFM at a very low cell density. A., B., and C. Self-renewal assay. LN229 cells (1 cell/μl) were cultured in SFM in the absence or presence of IL-1β and TGF-β for 14 days. Spheres were counted microscopically. **A.** Control cells, cells are found as monolayer cells. **B.** With IL-1β and TGF-β treatment, cells form tight neurospheres. **C.** Quantitative number of neurosphere forming cells shows a significant increase when cells are treated with IL-1β and TGF-β. **D**. Proliferation Assay. LN229 cells (500 cells/well) were cultured in SFM in the absence or presence of IL-1β and TGF-β for seven days. Proliferation ratio was measured by MTT assay. Scale bar = 200μm. * $P < 0.05$. Figures are representative of three independent experiments.

Figure 3.3

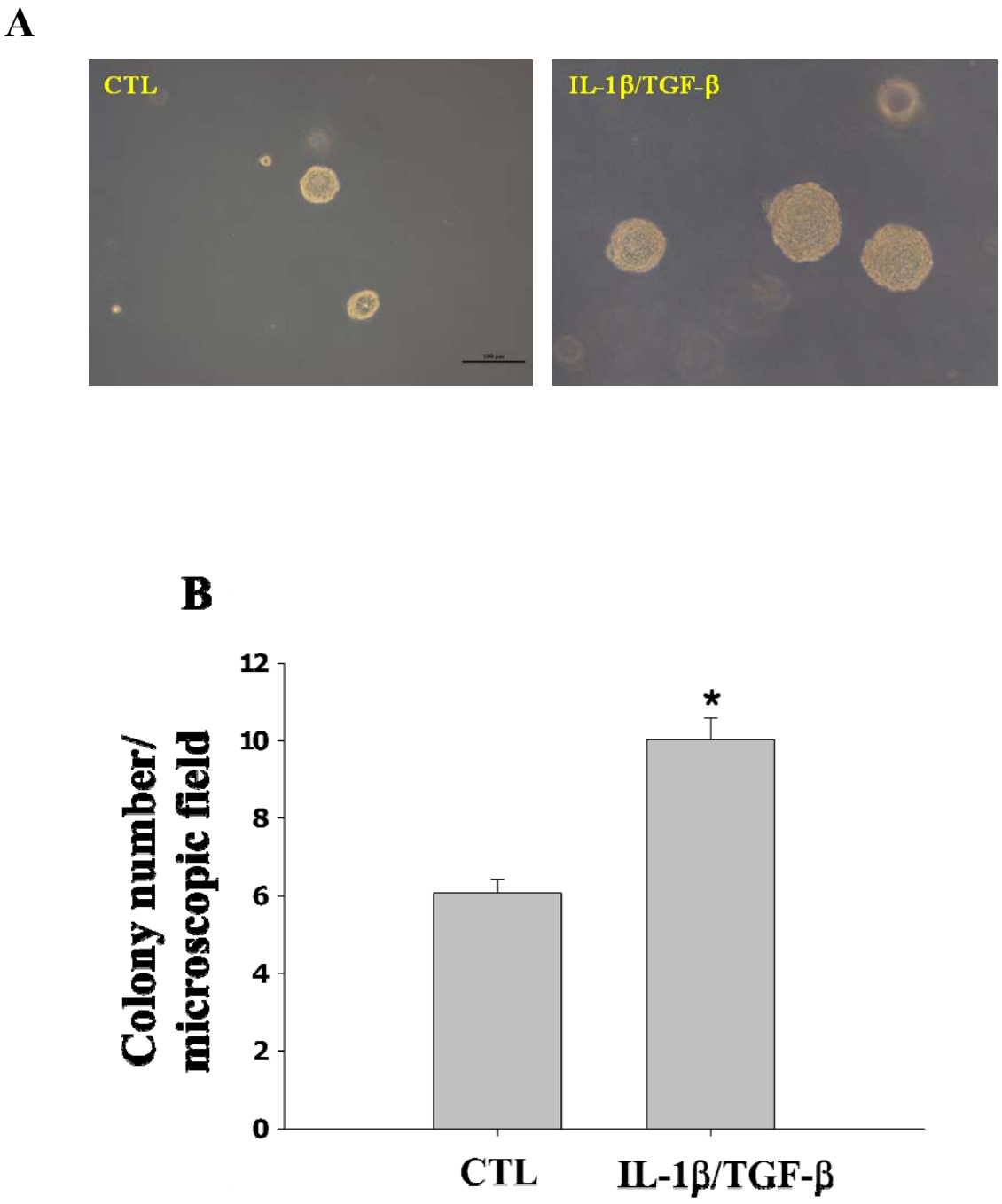

A
CTL
IL-1β/TGF-β
B
Colony number/ microscopic field
12
10
8
6
4
2
0
*
CTL
IL-1β/TGF-β

Figure 3.3

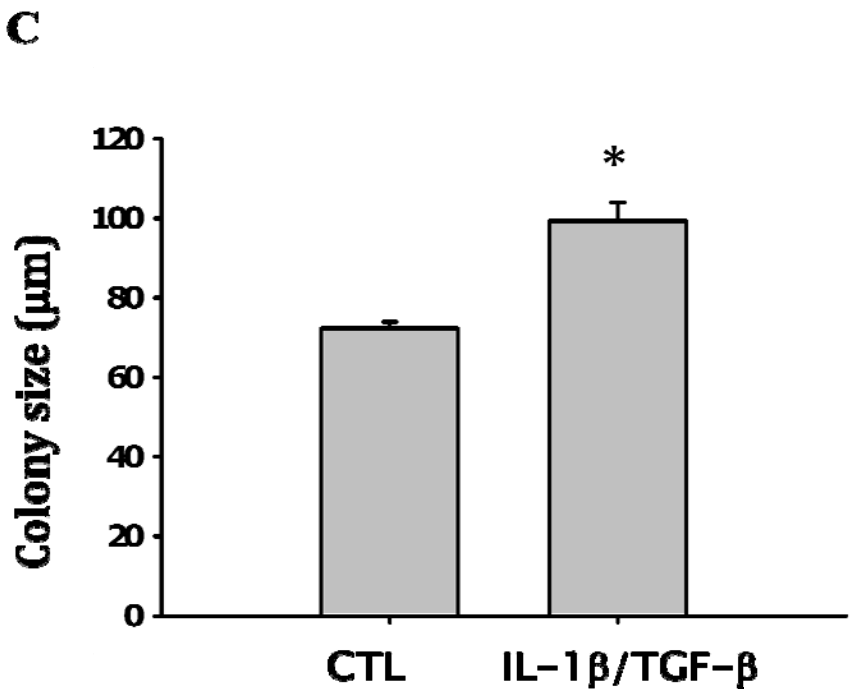


Figure 3.3 IL-1β/TGF-β-induced sphere cells form more and larger colonies than control cells. **A., B., and C.** Colony assay. Control cells or spherical cells induced by IL-1β/TGF-β were trypsinized, 10,000 cells of each type were plated in soft agar containing DMEM and 10% FBS and cultured for 14 days. **A.** Control cells form less and small colonies, while with IL-1β and TGF-β treatment, cells form more and larger colonies. **B., and C.** Quantitative measurement of the number and size of the colonies shows significant increases when cells are treated with IL-1β and TGF-β. Colonies were counted microscopically. Scale bar = 100μm. *P< 0.05. ** P< 0.01. Figures are representative of three independent experiments.

Figure 3.4

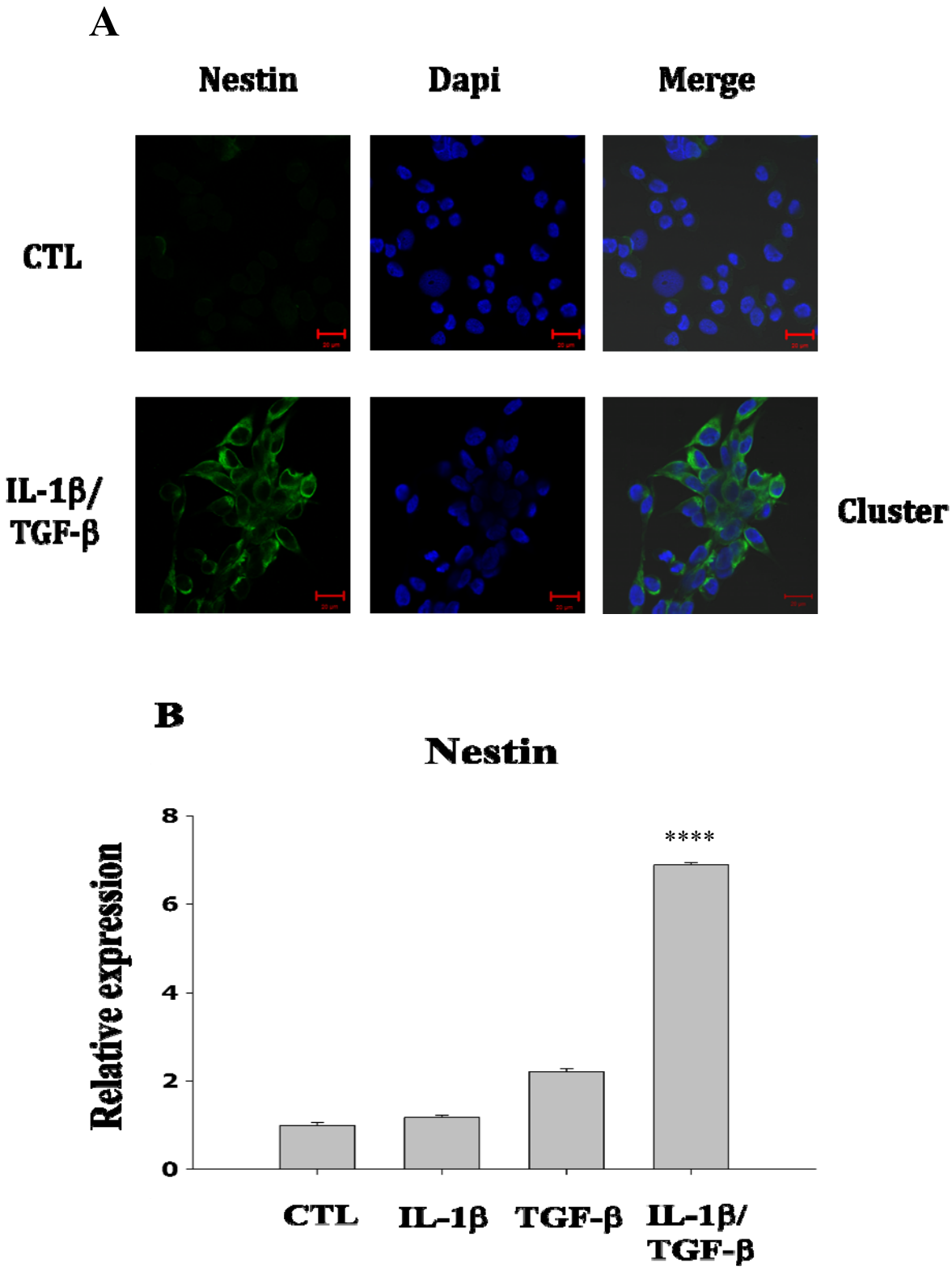
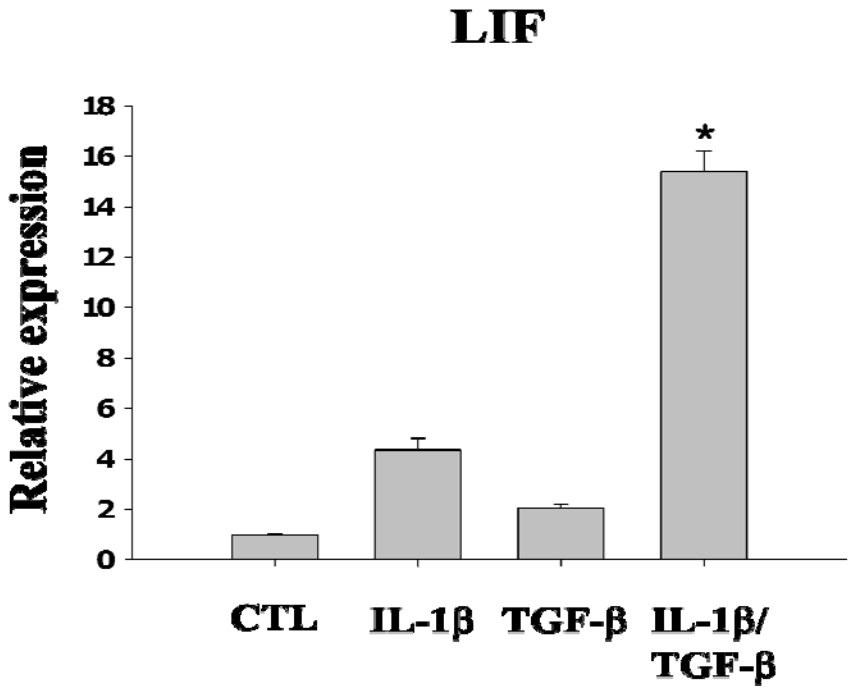

A
Nestin
Dapi
Merge
CTL
IL-1β/
TGF-β
Cluster
B
Nestin
Relative expression
8
6
4
2
0
****
CTL
IL-1β
TGF-β
IL-1β/
TGF-β

Figure 3.4

C


LIF
Relative expression
18
16
14
12
10
8
6
4
2
0
*
CTL
IL-1β
TGF-β
IL-1β/
TGF-β

D

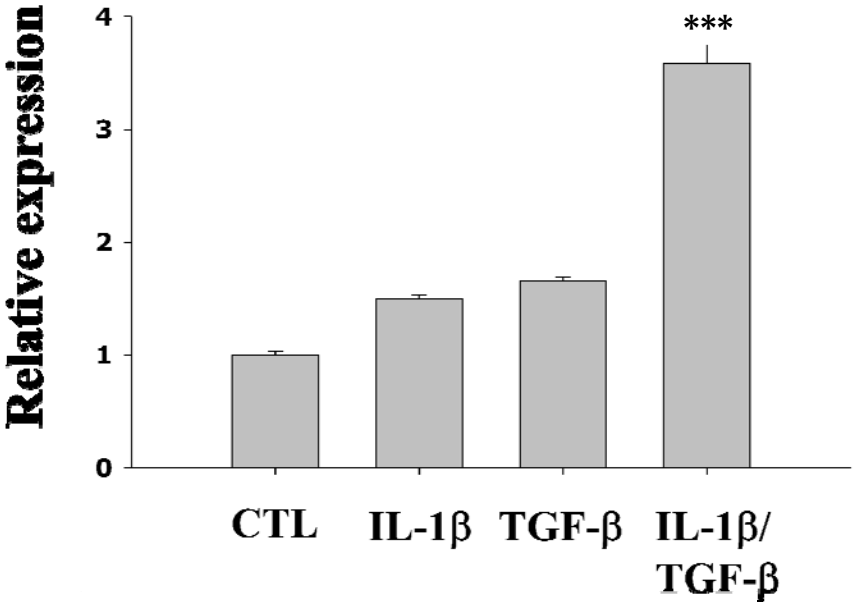

Bmi1
Relative expression
4
3
2
1
0
***
CTL
IL-1β
TGF-β
IL-1β/
TGF-β

Figure 3.4

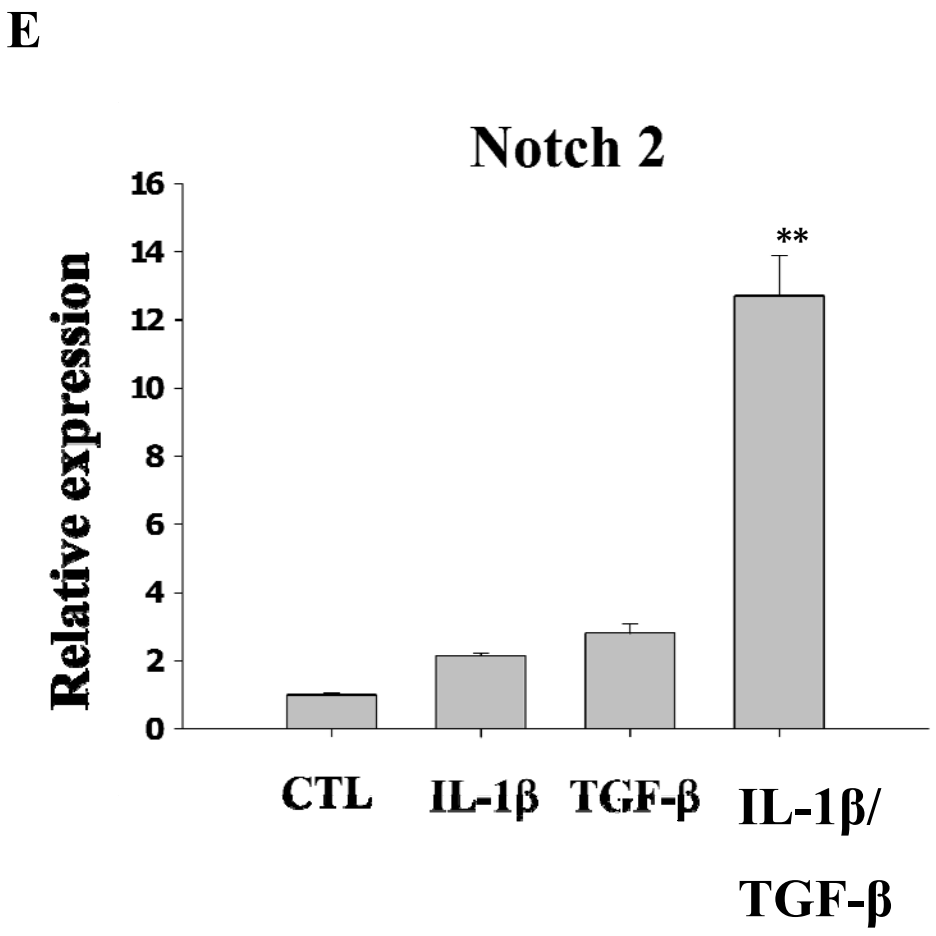


Figure 3.4 IL-1β/TGF-β-stimulated cells express stem cell markers.

A. Immunocytochemistry for Nestin was performed on LN229 cells, control cells or cells pretreated with IL-1β and TGF-β for seven days in SFM. With IL-1β and TGF-β treatment, more Nestin is expressed. Nuclei were counterstained with DAPI. Scale bar = 20mm. **B-E**. qRT-PCR analysis was performed to determine the mRNA level of stem cell markers Nestin **(B)**, LIF **(C)**, Bmi1 **(D)** and Notch2 **(E)** in LN229 cells in SFM in the absence or presence of IL-1β/TGF-β for seven days. With IL-1β and TGF-β treatment, more Nestin, LIF, Bmi1, Notch2 is expressed. β-actin mRNA levels were used as an internal normalization control. Scale bar = 20μm.*P<0.05; **P<0.01; ***P< 0.005; ****P< 0.001. Figures are representative of three independent experiments.

Figure 3.5

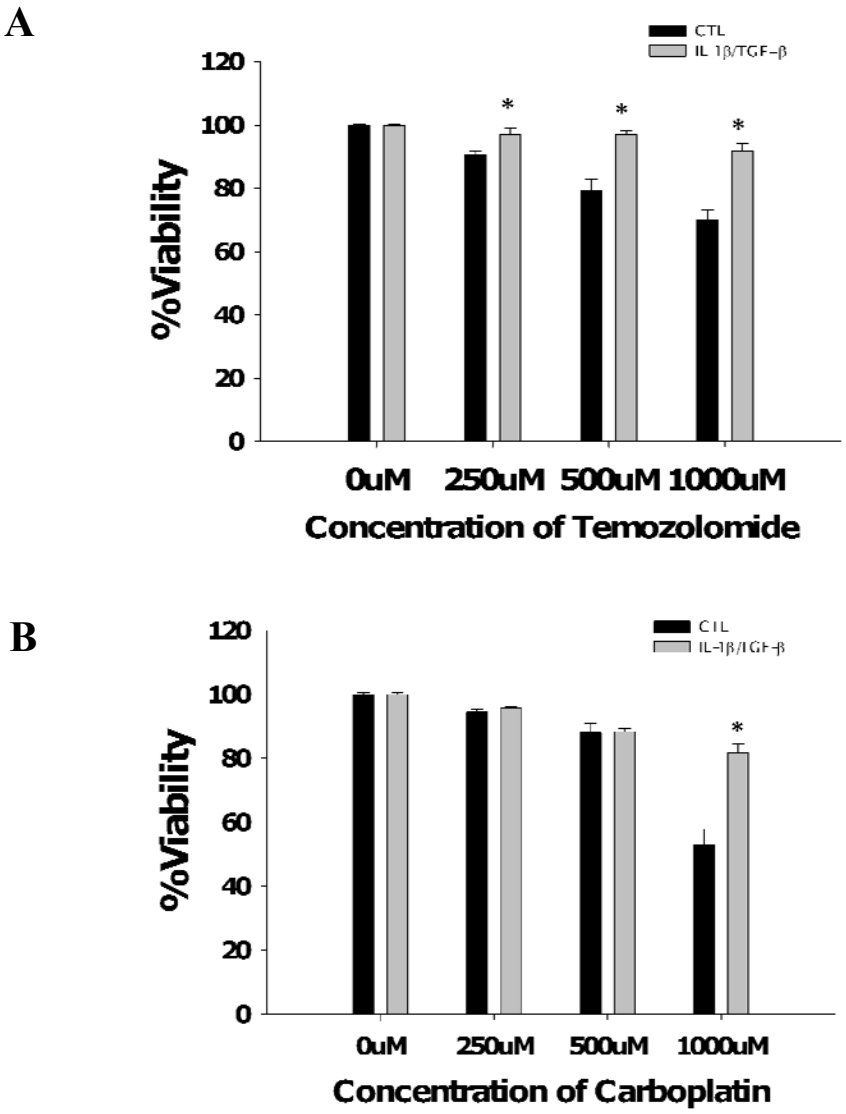
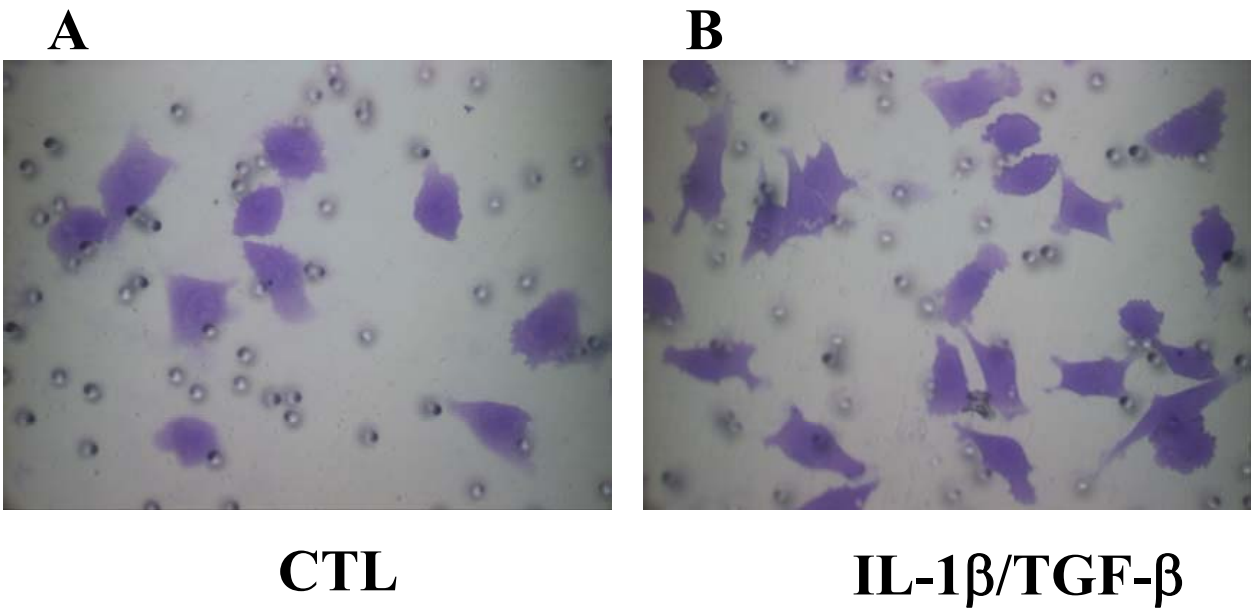


Figure 3.5 IL-1β/TGF-β-induced sphere cells develop drug resistance. LN229 cells (1000 cells/well) were cultured in SFM in the absence or presence of IL-1β and TGF-β for five days followed by the addition of different concentrations of Temozolomide or Carboplatin to the cultures which were further incubated for two days. The cells were dissociated, stained with trypan blue and counted under the microscope. **A.** With IL-1β and TGF-β , cells show significantly stronger resistant ability to Temozolomide. **B.** With IL-1β and TGF-β , cells also show significantly stronger resistant ability to Carboplatin. The viability was determined by the percentage of live cells over the sum of live and dead cells. * $P< 0.05$. Figures are representative of three independent experiments.

Figure 3.6


A
B
CTL
IL-1β/TGF-β

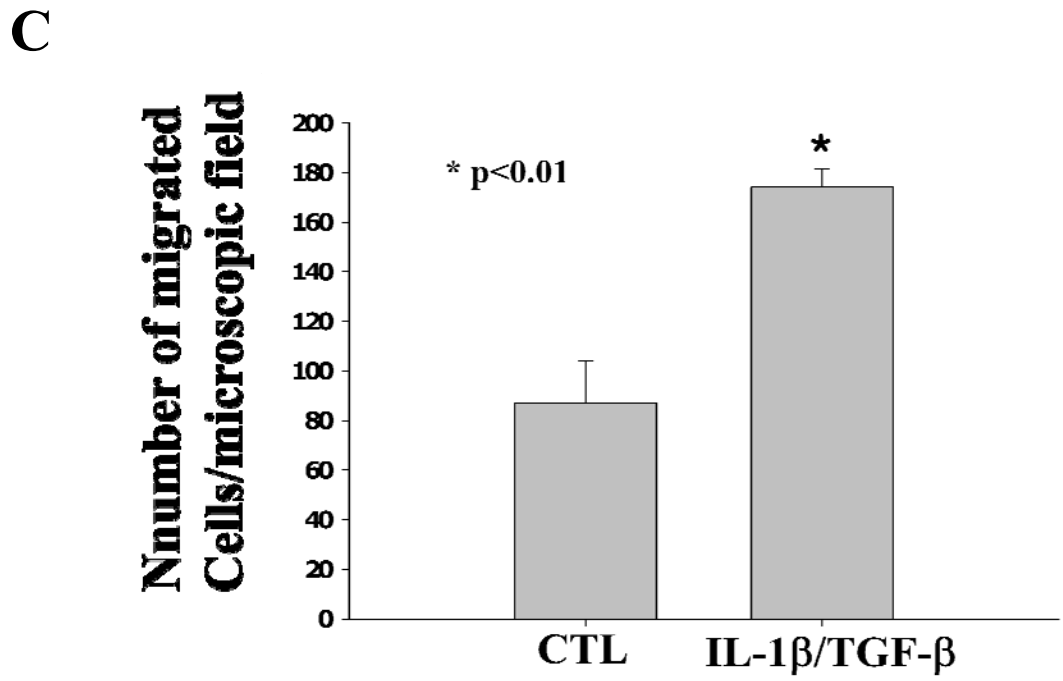

C
Nnumber of migrated Cells/microscopic field
200
180
160
140
120
100
80
60
40
20
0
* p<0.01
*
CTL
IL-1β/TGF-β

Figure 3.6

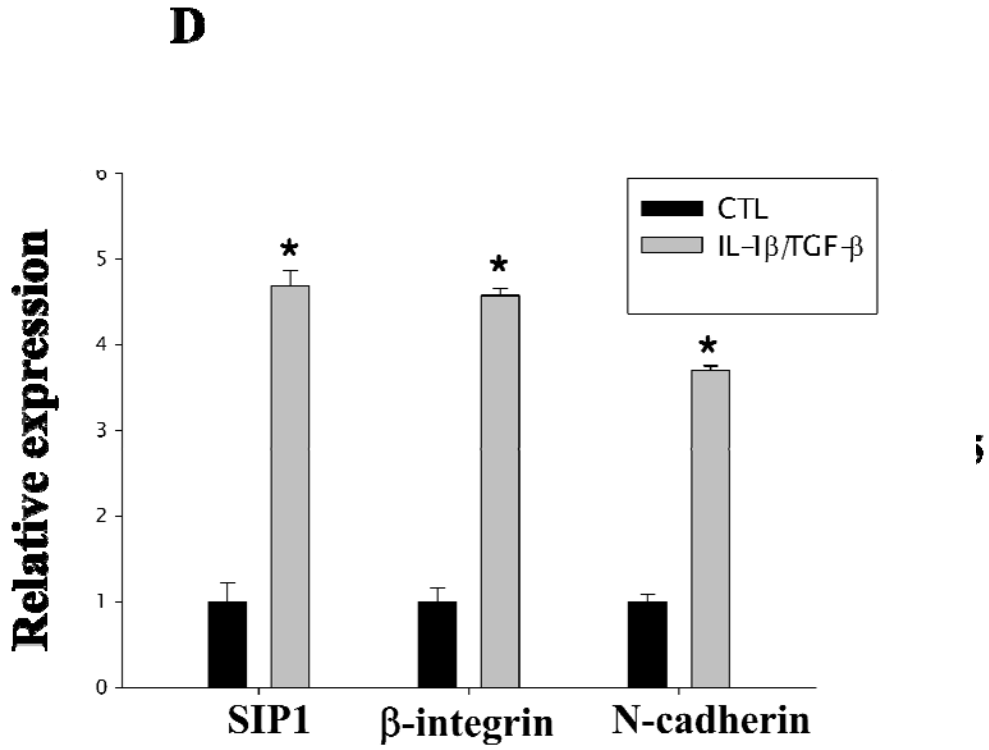
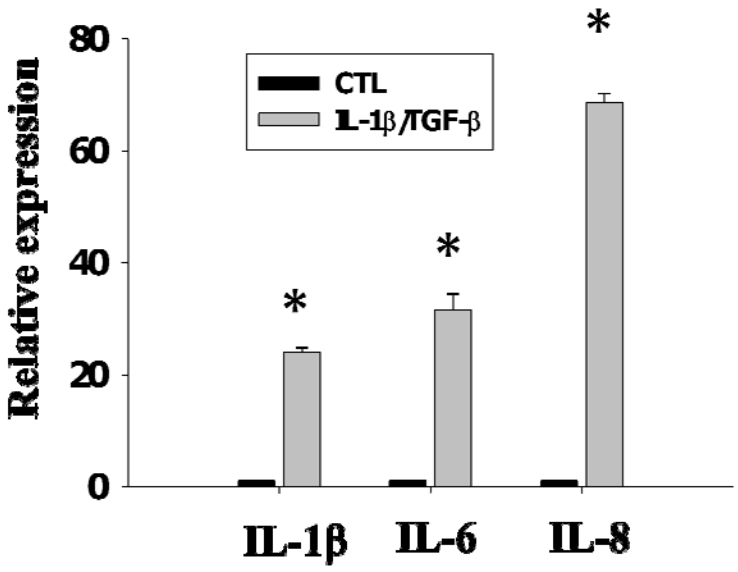


Figure 3.6 IL-1β/TGF-β-induced sphere cells have increased invasion and invasive gene expression. A., B., and C. Invasion assay. Control or spherical cells induced by IL-1β and TGF-β were trypsinized and $5x10^4$ cells of each group were plated on the top well of Matrigel Invasion chambers. Medium with 20% FBS was added to the lower chamber as chemoattractant. After 48h of incubation, non-invaded cells were removed from the top chamber and invaded cells at the lower surface were fixed and stained with 0.005% crystal violet before counting under a microscope. **A**. Fewer cells have migrated and stained in control treatment. B. More cells have migrated and stained with IL-1β and TGF-β treatment. **C.** Quantitative number of the migrated cells show significant increases when cells are treated with IL-1β and TGF-β. **D**. qRT-PCR analysis was performed to determine the mRNA level of invasive genes SIP1, β-integrin and N-Cadherin in LN229 cells in SFM in the absence or presence of IL-1β/TGF-β for seven days. β-actin mRNA levels were used as an internal normalization control. *P< 0.01**.** Figures are representative of three independent experiments.

Figure 3.7

A



B

Cytokine	CTL	IL-1β/TGF-β
IL-1β (pg/ml)	0	399.5±0.2
IL-8 (ng/ml)	378.7±39.8	1044.9±70.2

Figure 3.7

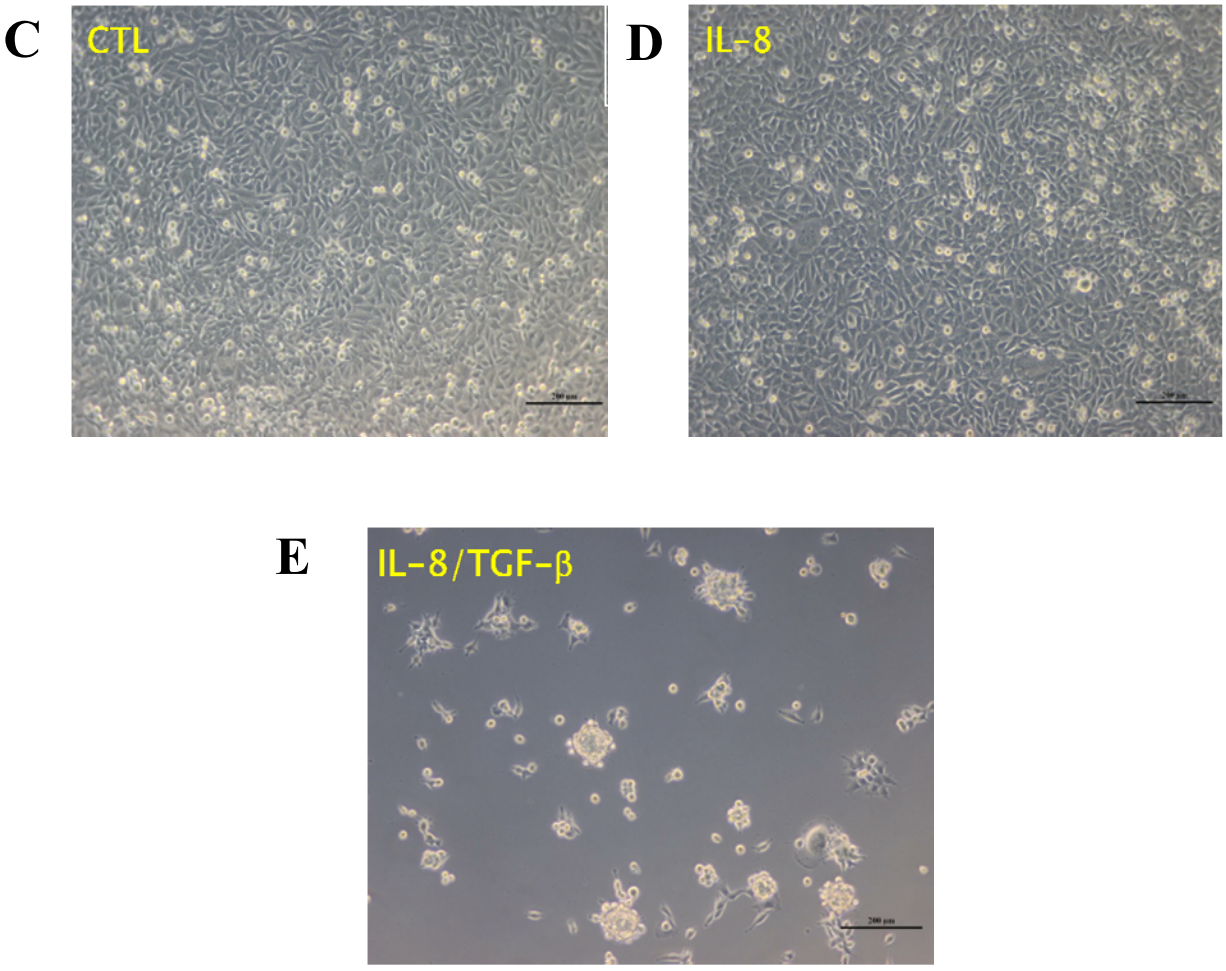


Figure 3.7 IL-1β/TGF-β induces inflammatory cytokines secretion. A. qRT-PCR analysis was performed to determine the mRNA level of cytokine genes IL-1β and IL-8 in LN229 cells in SFM in the absence or presence of IL-1β/TGF-β for seven days. β-actin mRNA levels were used as an internal normalization control. **A.** Quantitative measurement of the genes expression of IL-1β, IL-6 and IL-8 show significant increases when cells are treated with IL-1β and TGF-β. **B.** ELISA. Quantitative measurement of the proteins expression of IL-1β and IL-8 shows significant increases when cells are treated with IL-1β and TGF-β. **C., D., and E**. LN229 cells are capable of neurosphere formation when cells were treated with IL-8 and TGF-β. Cells were cultured in SFM in the absence or presence of IL-8/TGF-β for seven days. Representative images of LN229 cells were taken under a microscope after seven days of culture. * $P < 0.05$. Figures are representative of three independent experiments.

Figure.3.8

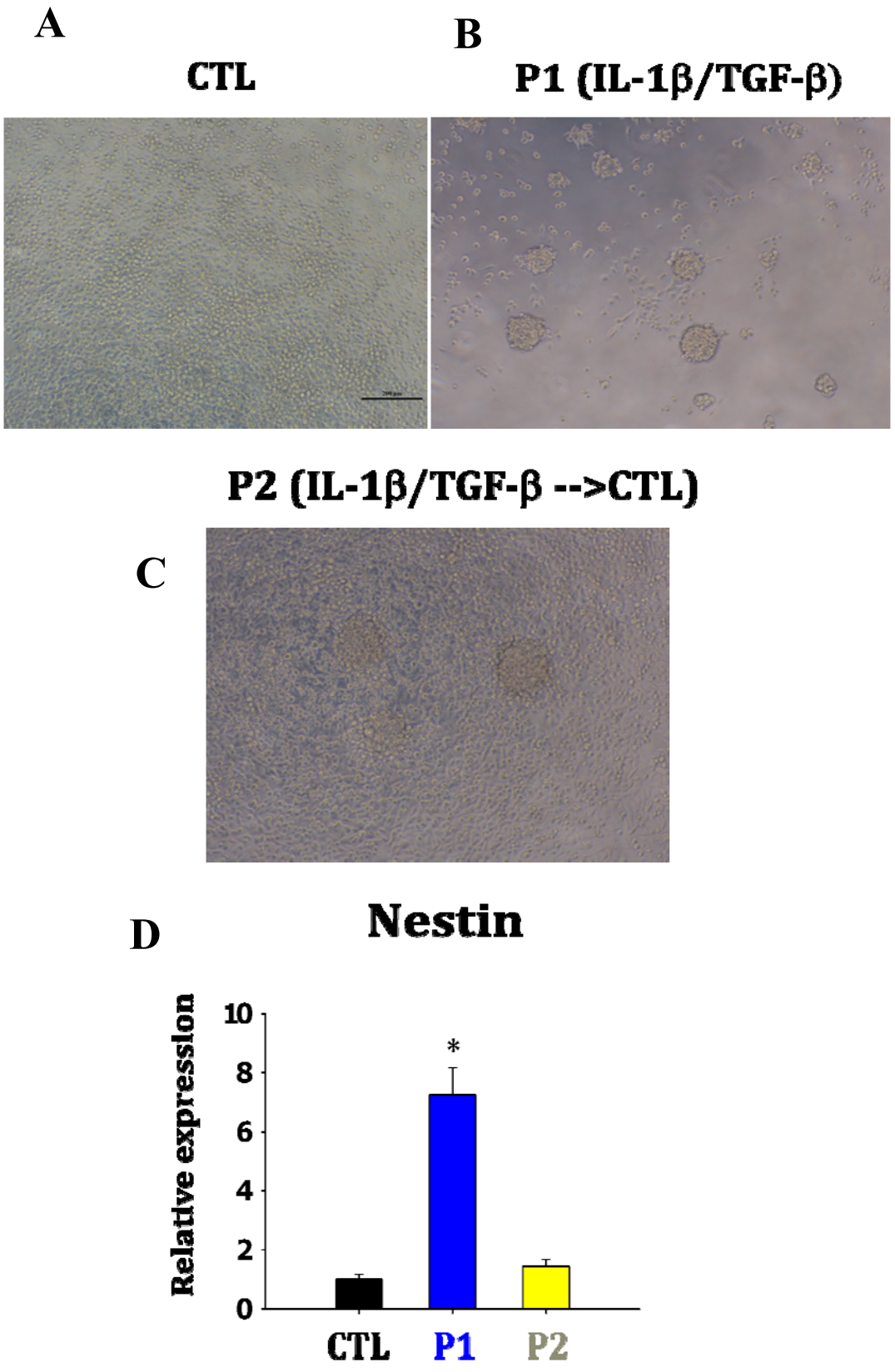
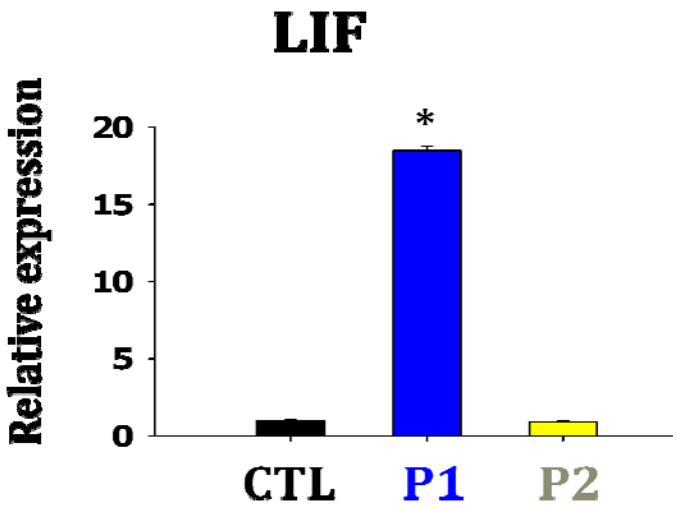

A
CTL
B
P1 (IL-1β/TGF-β)
P2 (IL-1β/TGF-β -->CTL)
C
D
Nestin
Relative expression
10
8
6
4
2
0
*
CTL
P1
P2

Figure.3.8

E


LIF
Relative expression
20
15
10
5
0
*
CTL
P1
P2

F

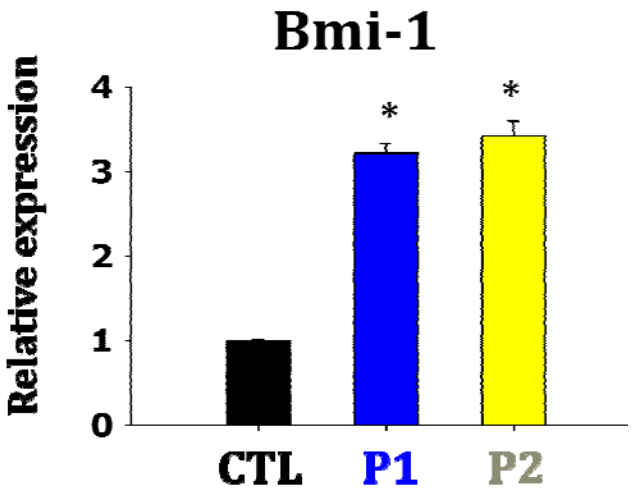
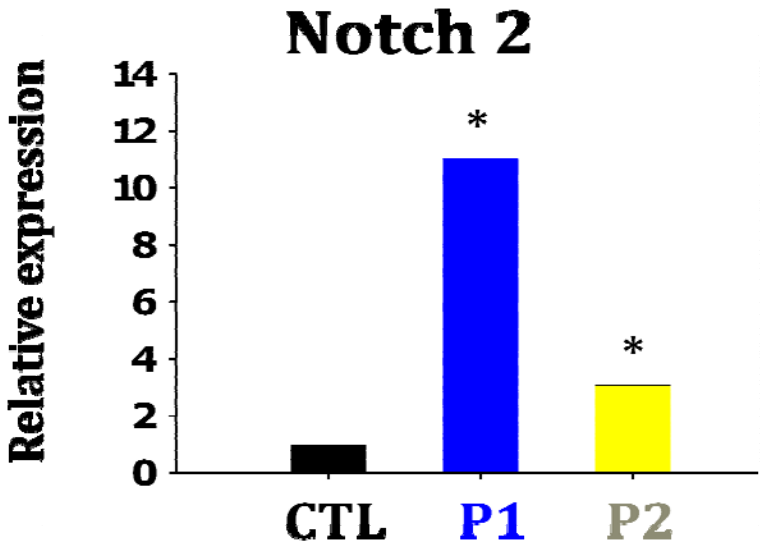

Bmi-1
Relative expression
4
3
2
1
0
*
*
CTL
P1
P2

Fig.3.8

G


Notch 2
Relative expression
14
12
10
8
6
4
2
0
*
*
CTL
P1
P2

H

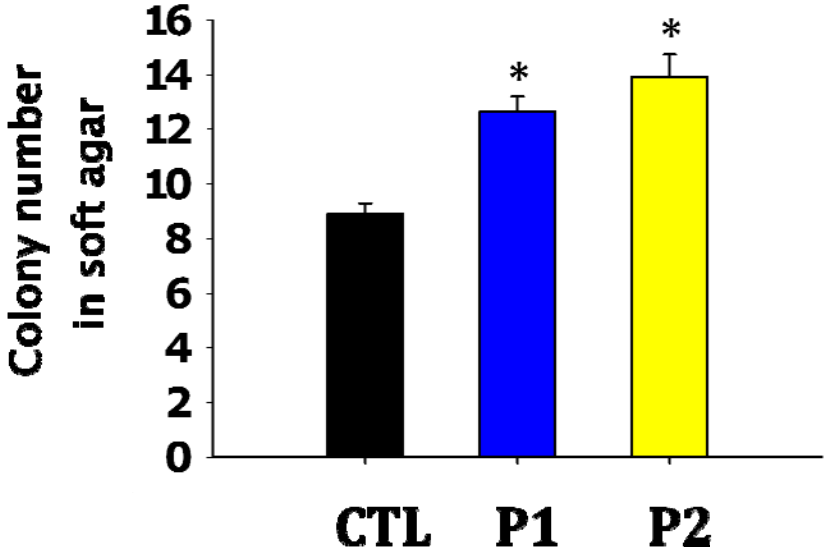

Colony number in soft agar
16
14
12
10
8
6
4
2
0
*
*
CTL
P1
P2

Fig.3.8

I

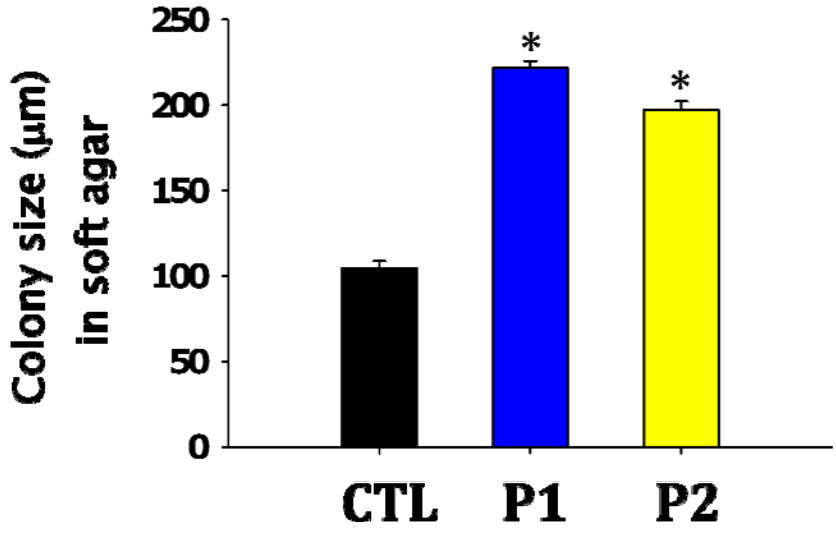


Figure 3.8 Combination of IL-1β/TGF-β induces P2 cells that are still able to form cell clusters in SFM without IL-1β/TGF-β stimulation. LN229 cells were cultured in SFM in the absence or presence of IL-1β and TGF-β for seven days (P1). P1 **(B)** cells are found as neurospheres. Control cells or spherical cells were trypsinized and 1,000 dissociated cells of each type were plated in SFM in the absence of IL-1β/TGF-β for an additional seven days (P2). P2 **(C)** cells form several cell clusters, while control cells **(A)** are found as monolayer cells. Images were taken microscopically. Scale bar = 200µm. qRT-PCR analysis was performed to determine the mRNA level of stem cell marker genes Nestin **(D),** LIF **(E)**, Bmi-1 **(F)** and Notch2 **(G)** in LN229 cells in SFM in the absence or presence of IL-1β/TGF-β for seven days. β-actin mRNA levels were used as an internal normalization control. **H., and I.** P2 cells induced by IL-1β and TGF-β form more and larger colonies compared to control cells. Control cells or spherical cells that were induced by IL-1β/TGF-β in SFM for seven days were trypsinized and replated in SFM in the absence or presence of IL-1β/TGF-β for another seven days.10,000 cells of each type were trypsinzed and plated in soft agar containing DMEM and 10% FBS and cultured for 14 days. Colonies were counted microscopically. *P< 0.05. Figures are representative of three independent experiments.

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