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Dose-Dependent Differential Upregulation of CCN1/Cyr61 and CCN3/NOV by the Gap Junction Protein Connexin43 in Glioma Cells

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Abstract Gap junctions form channels that allow exchange of materials between cells and are composed of transmembrane protein subunits called connexins. While connexins are believed to mediate cellular signaling by permitting intercellular communication to occur, there is also increasing evidence that suggest connexins may mediate growth control via a junction-independent mechanism. Connexin43 (Cx43) is the most abundant gap junction protein found in astrocytes, and gliomas exhibit reduced Cx43 expression. We have previously observed that restoration of Cx43 levels in glioma cells led to increased expression of CCN3 (NOV) proteins. We now report that overexpression of Cx43 in C6-glioma cells (C6-Cx43) also upregulates the expression of CCN1 (Cyr61). Both CCN1 and CCN3 belong to the *C*yr61/*C*onnective tissue growth factor/*N*ephroblastoma-overexpressed (CCN) family of secretory proteins. The CCN proteins are tightly associated with the extracellular matrix and have important roles in cell proliferation and migration. CCN1 promotes growth in glioma cells, as shown by the increased proliferation rate of CCN1-overexpressing C6 cells. In addition to its effect on cell growth, CCN1 also increased the motility of glioma cells in the presence of extracellular substrates such as fibronectin. Gliomas expressing high levels of Cx43 preferentially upregulated CCN3 which resulted in reduced growth rate. CCN3 could also be observed in Cx43 gap junction plaques in confluent C6-Cx43H culture at the stationary phase of their growth. Our results suggest that the dissimilar growth characteristics between high and low Cx43 expressors may be due to differential regulation of CCN3 by varying levels of Cx43. J. Cell. Biochem. 103: 1772–1782, 2008.

Key words: connexin 43; CCN1; CCN3; gliomas; gap junctions

Gap junctions are composed of transmembrane connexin proteins which permit intercellular communication by forming a channel between two adjacent cells. As such, they are positioned to coordinate a variety of activities which can influence cell growth. Downregulation of gap junctional intercellular communication (GJIC) has been observed in a variety of tumor cell lines and cancer tissues [Mesnil, 2002]. Gliomas are brain tumors containing cells of glial origin; glial cells in the brain provide structural and functional support to neurons and possess extensive GJIC with channels formed primarily of connexin43 (Cx43) proteins. High-grade gliomas often have decreased Cx43 expression [Huang et al., 1999; Soroceanu et al., 2001] and overexpression of

Abbreviations used: Cx43, connexin 43; ECM, extracellular matrix; FBS, fetal bovine serum; GJIC, gap junctional intercellular communication; HRP, horseradish peroxidase.

This is the first report of Cx43 differentially regulating the expression and localization of two secretory, extracellular matrix-associated proteins CCN1 and CCN3. Our findings strengthen the role of gap junctions in cell growth control by directly regulating the expression of signaling molecules.

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Cx43 reverses the oncogenic phenotype of glioma cells [Huang et al., 1998; Xia et al., 2001].

The mechanism by which connexins act as growth inhibitors is unknown. Initial investigation of connexin-induced tumor suppression focused on its channel-forming ability [Mesnil, 2002; Mesnil et al., 2005]. While it is clear that gap junctions can selectively mediate intercellular exchange of ions and small molecules, there has been no identification of a direct intercellular mediator of growth control passing through gap junctions. On the other hand, many intracellular proteins have now been identified to interact with the connexin proteins and participate in signaling events reviewed in [Herve et al., 2004].

CCN3/NOV (nephroblastoma overexpressed) protein has been identified as a novel Cx43-cytoplasmic tail-associating protein [Fu et al., 2004; Gellhaus et al., 2004] and belongs to the *C*YR61/*C*onnective tissue growth factor/*N*ephroblastoma overexpressed (CCN) family of multi-modular proteins that mediate diverse cellular functions, including adhesion, migration, and proliferation [Brigstock, 2003; Perbal, 2004]. The CCN proteins are composed of five modules commonly found in extracellular matrix (ECM) proteins. The presence of a secretory signal peptide indicates that the CCN proteins exert some effect as secreted factors although recent data suggests they also possess intracellular functions [Perbal, 2004]. CCN1/CYR61 (cysteine-rich protein), another member of the CCN family, was first identified as an immediate early gene and has been clearly shown to be an angiogenic factor acting via the integrin receptors to promote cell adhesion, migration, and proliferation [Kireeva et al., 1996; Babic et al., 1998; Bleau et al., 2005].

CCN proteins are often implicated in tumor growth, invasion, and metastasis. Overexpression of CCN1 in human glioma cells increases their tumorigenecity [Xie et al., 2004] but in other cancer types, CCN1 appears to be tumor suppressive [Tong et al., 2004] and is linked to growth suppression in endometrial cancer cells and lung cancer [Tong et al., 2001; Chien et al., 2004]. In contrast, CCN3 is primarily tumor suppressive in many tumor types, including gliomas [Gupta et al., 2001].

We and others have previously reported that overexpression of Cx43 increases CCN3 expression [Fu et al., 2004; Gellhaus et al., 2004]. Here, we show that Cx43 also upregulates CCN1 protein. Overexpression of CCN1 contributed to increased growth and motility in glioma cells. Our results suggest differential regulation of CCN1 and CCN3 in gliomas depending on the expression levels of Cx43.

MATERIALS AND METHODS

Retroviral Infection of C6 Glioma Cells

To obtain C6 cells overexpressing CCN1/Cyr61 protein, CCN1 cDNA (a gift from Dr. L. Lau, University of Illinois College of Medicine, Chicago, IL) was cloned into the retroviral vector pAP2 containing an IRES-GFP, and C6 cells at 60% confluency were infected as described previously [Mao et al., 2000]. Both C6 parental and transfected cell lines were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Sigma) at 37°C in the presence of 5% CO_2. C6-overexpressing GFP or Cx43GFP were obtained as described previously [Fu et al., 2004].

Protein Isolation and Western Analysis

For detection of CCN proteins present in the cytoplasm, C6 cells were lysed in RIPA buffer containing 0.1% SDS, 1% IGEPAL, 0.5% Sarkosyl, 50 mM Tris–HCl (pH 8.0), 150 mM NaCl supplemented with MiniComplete protease inhibitors (Roche Applied Science) and phosphatase inhibitors (Sigma). Precipitation of proteins from conditioned medium was carried out as described previously [Fu et al., 2004]. Briefly, conditioned media was collected following culturing of C6 cells in serum-free media for 24 h. Methanol precipitation was performed on 200 μl of each sample. Proteins were then air-dried and resuspended in RIPA buffer. Equal loading of proteins from conditioned medium was ensured by normalizing the protein samples to the number of cells in the culture dish determined by a hemocytometer. Protein samples were then separated on a 12% polyacrylamide gel and western transferred onto PVDF membrane (Amersham). Rabbit anti-CCN1 (Santa Cruz), rabbit anti-CCN3 [Fu et al., 2004] and mouse anti-GAPDH (Hytest, Turku, Finland) were used as the primary antibodies, followed by either anti-rabbit-horseradish peroxidase (HRP) or anti-mouse-HRP (Cedarlane, Hornby, Canada) as secondary antibodies. Protein bands were detected with Super-Signal chemiluminescent substrate (Pierce).

Quantification of protein bands was carried out with Image J 1.36b software [Abramoff et al., 2004].

For serum stimulation experiments, glioma cells were serum deprived for 2–4 days. The quiescent status of the glioma cells was verified by cell cycle analysis. Under these conditions, the majority of the C6 or C6 transfected with Cx43 (82–88%) were driven into the G0/G1 phase, while only about 2–4% of the cells are in S-phase (data not shown).

Immunocytochemistry

Cells grown on coverslips were fixed in cold 80% methanol at −20°C for 10 min. Coverslips were blocked in 2% BSA in PBS for 30 min. Co-staining of CCN3 or CCN1 with Cx43 was carried out with rabbit anti-CCN3 [Fu et al., 2004] or rabbit anti-CCN1 (Santa Cruz) and mouse anti-Cx43 (Chemicon) as primary antibodies; followed by sequential incubation with secondary antibodies anti-rabbit-Alexa 568 and anti-mouse-Alexa 488 (Molecular Probes) for 1 h at room temperature. Coverslips were mounted onto glass slides using ProLong Gold antifade reagent with DAPI (Molecular Probes) and viewed with a Zeiss Axioskop microscope.

Cell Growth Analysis

C6 parental cells or C6 cells overexpressing Cx43 or CCN1 were seeded at a density of 10,000 cells per well of a 12-well plate in triplicates. Cells were counted daily using a Coulter Counter R Z1 series particle counter (Coutler Electronics, ON, Canada). Mean values were assessed for significance using analysis of variance (ANOVA), followed by Tukey-Kramer multiple comparison test to determine significance difference ($P < 0.01$).

Spheriods Migration Assays

To determine the effect of ECM on glioma motility, drops of cell suspension (20 μl) containing 40,000 cells were placed onto the lids of culture dishes, which were inverted over dishes containing DMEM medium [Del Duca et al., 2004; Suzuki and Iwaki, 2005]. Hanging drop cultures were incubated for 48 h and the resulting two-dimensional aggregates were transferred, using a Pasteur pipette, to culture dishes coated with fibronectin (5 μg/cm^2) or BSA. The distance traveled by migrating cells out of the spheroids was determined 24 h later. Average migration distance was measured by Axiovision software (Zeiss).

RESULTS

C6 glioma cells are characterized by low Cx43 expression and low intercellular communication [Zhu et al., 1991, 1992]. They have been extensively utilized as a model of glioblastoma, displaying similar cellular and molecular characteristics to human gliomas [Grobben et al., 2002]. Overexpression of Cx43 in these cells restored their intercellular communication and decreased their proliferation rate [Zhu et al., 1991, 1992; Naus et al., 1992]. When we further determined the changes in expression of cellular proteins due to Cx43 overexpression in C6 cells using a differential gene display strategy, CCN1 was detected as one of the genes that are upregulated due to Cx43 expression [Naus et al., 2000]. This observation was confirmed by standard Northern analysis of glioma cells overexpressing Cx43 [McLeod et al., 2001]. Among the Cx43-transfected clones, C6-Cx43H (Cx43-13 [Zhu et al., 1991]) expressed the highest level of exogenous Cx43, and correspondingly exhibited the highest level of CCN1 protein (Fig. 1A). Less CCN1 protein was detected in a lower Cx43-expressing subclone C6-Cx43L (Cx43-14 [Zhu et al., 1991]) (Fig. 1A). We also observed a correlation between CCN3 and Cx43 expression levels in gliomas (Fig. 1A). Quantification of protein expression revealed C6-Cx43H expressed 2.7-fold more Cx43 than C6-Cx43L (Fig. 1B). Correspondingly, C6-Cx43H also expressed 2.5-fold more CCN1 and 2.3-fold more CCN3 than C6-Cx43L (Fig. 1B). CCN3 is known to be glycoyslated [Manara et al., 2002] and was sometimes observed as multiple bands on protein gels (Fig. 1A).

CCN1 is an immediate early gene that has been shown to be activated by growth promoting stimuli such as serum [Latinkic et al., 1991]. To investigate whether the expression of Cx43 will alter the expression of CCN1 upon stimulation, we treated quiescent C6-Cx43H cells with serum and determined the CCN1 levels at various time points. An increase in CCN1 protein levels was detectable at 2 h, and this increase was sustained for at least 6 h after serum stimulation (Fig. 1C). The increase in protein expression mirrored closely with CCN1 RNA level expression, which peaked at 2 h

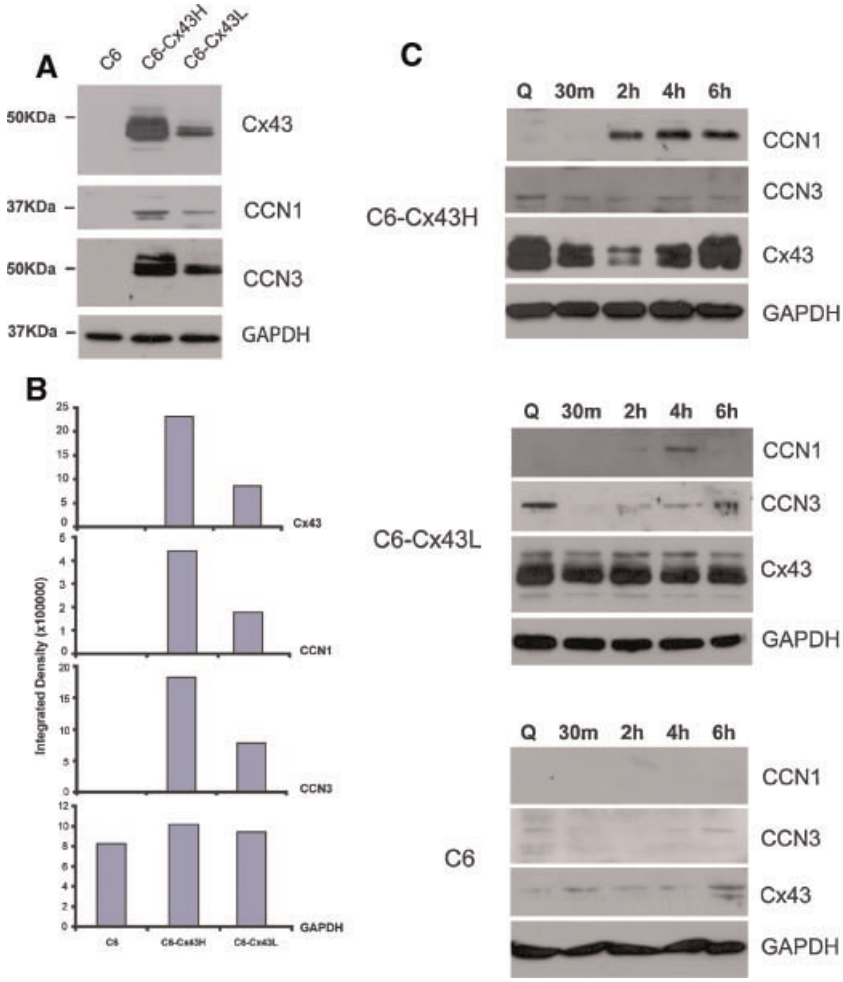

Fig. 1. Overexpression of Cx43 in glioma cells upregulates CCN1 and CCN3 protein levels. **A:** Western analysis on a 12% SDS–PAGE gel of lysates of confluent C6 cells overexpressing high (C6-Cx43H) and low (C6-Cx43L) levels of Cx43 with anti-CCN1 antibody and anti-CCN3 antibodies. **B:** Quantification of protein bands observed in (A) using ImageJ. **C:** Serum stimulation of C6-Cx43H upregulates CCN1 and downregulates CCN3. Western analysis on a 10% SDS–PAGE gel of C6-Cx43H, C6-Cx43L, and C6 parental cells at 30 min, 2 h, 4 h, and 6 h after addition of serum to quiescent (Q) cells for the expression of CCN1, CCN3, and Cx43. GAPDH was used to monitor equal protein loading.

after serum stimulation [McLeod et al., 2001]. In contrast, CCN3 had the highest expression in quiescent C6-Cx43H cells and its expression was barely detectable 2 h after serum stimulation (Fig. 1C). C6-Cx43L, which expressed lower levels of Cx43, also showed a similar trend of CCN1 and CCN3 expression upon serum stimulation although the results are less obvious than C6-Cx43H. The levels of CCN1 and CCN3 were too low in C6 cells to draw any conclusions. Low levels of endogenous Cx43 were observed in C6 cells which seemed to slightly increase after 6 h of serum stimulation. Exogenous Cx43, however, did not vary significantly for C6-Cx43L (Fig. 1C). Interestingly, there was a decrease of exogenous Cx43 2 h after serum stimulation in C6-Cx43H cells.

To delineate the growth effect mediated by CCN1 from other Cx43-dependent signaling pathways, we expressed exogenous CCN1 protein in native C6 cells (C6-CCN1). A protein with the expected size of 37 kDa was detected by anti-CCN1 antibody only in the lysate of CCN1-expressing cells (Fig. 2A). The secreted CCN1 protein in the conditioned medium was much smaller (~18 kDa) than the protein detected in the cellular lysate (Fig. 2A). To determine whether extracellular proteases are involved in the formation of the 18 kDa protein, we collected the conditioned medium of CCN1-expressing cells in the presence of a cocktail of protease inhibitors containing aprotinin, bebstatin, E-65, leupeptin, and pepstatin for the inhibition of serine, cysteine, aspartic, and

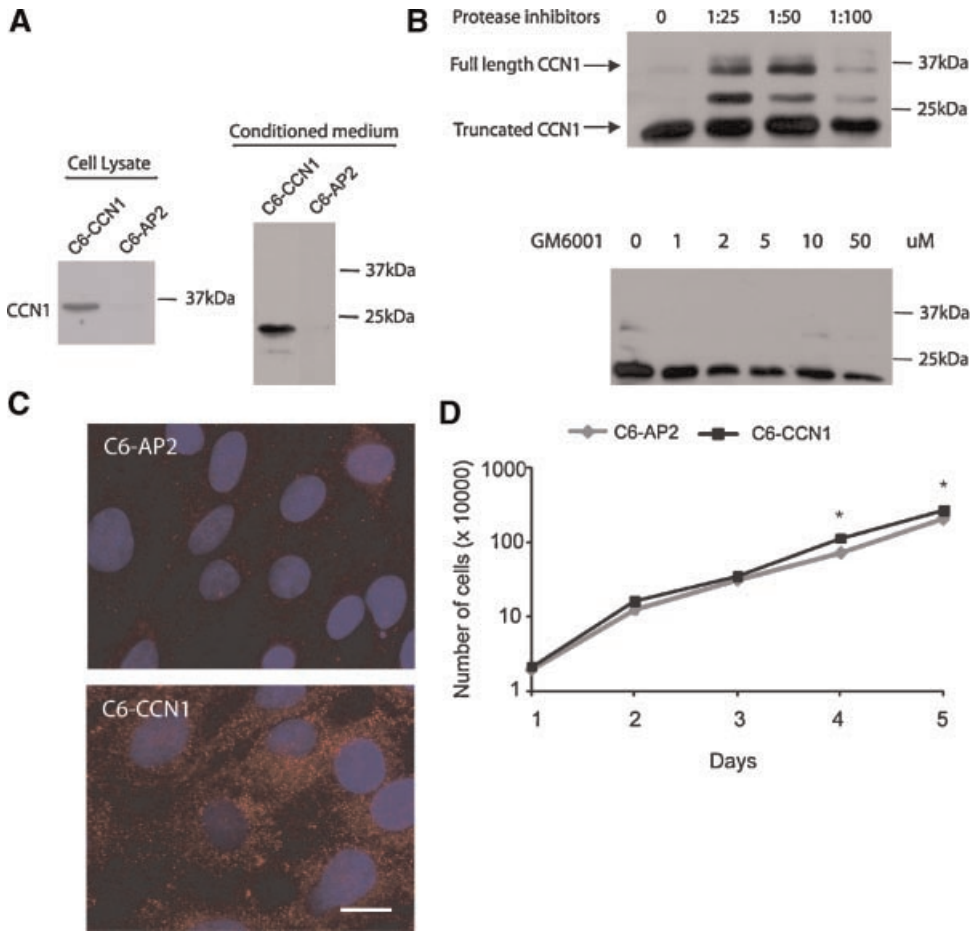

Fig. 2. Overexpression of CCN1 in glioma cells. **A:** Western analysis showing the expression of exogenous full length CCN1 (~37 kDa) in the lysate and a truncated CCN1 (~18 kDa) in the conditioned medium of C6-CCN1 cells. **B: Upper panel**, formation of full length secreted CCN1 in the presence of protease inhibitors. Addition of protease inhibitors in the culture medium facilitated the formation of a larger protein that corresponds to the size of full length CCN1. Western analysis of conditioned medium in the absence and presence of protease inhibitors of various concentrations. **Lower panel**, metalloproteases may not be responsible for the cleavage of CCN1. Various concentrations of GM6001, a broad range metalloprotease inhibitor, did not facilitate the formation of protein bands of higher molecular weight detectable by anti-CCN1 antibody. **C:** Immunoflourenscence of C6 cells transfected with either empty vector AP2 or CCN1 plasmid vector and stained with antibodies against CCN1. DAPI was used to visualize the location of nuclei (scale bar = 10 μm). **D:** Growth curve of C6 cells expressing empty vector (C6-AP2) or CCN1 (C6-CCN1) (*$P < 0.001$, one way ANOVA). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

aminopeptidases. We observed the appearance of proteins with higher molecular weight only in the presence of high concentration of protease inhibiters (1:25 or 1:50 dilution; Fig. 2B), suggesting that secreted CCN1 was cleaved by protease(s) secreted by the glioma cells. Our results also suggest metalloproteases were not involved in the cleavage since GM6001, a metalloprotease inhibitor, did not increase the formation of higher molecular weight bands (Fig. 2B). Intracellular CCN1 had a diffuse cytoplasmic distribution, as detected with anti-CCN1 antibody in C6-CCN1 cells (Fig. 2C). The distribution of exogenous CCN1 was very similar to the cytoplasmic localization of upregulated endogenous CCN1 in C6-Cx43H cells (Fig. 3C). We next investigated the growth characteristics of C6-CCN1. These CCN1-expressing cells had a modest increase in proliferation rate compared to the glioma cells expressing the control vector (Fig. 2D). Our result is in agreement with the observation made by Xie et al. [2004] that CCN1 enhances growth in human glioma cell lines.

We next determined the growth and motility of glioma cells overexpressing Cx43. Our previous work has already shown that overexpression of Cx43 decreases the growth and

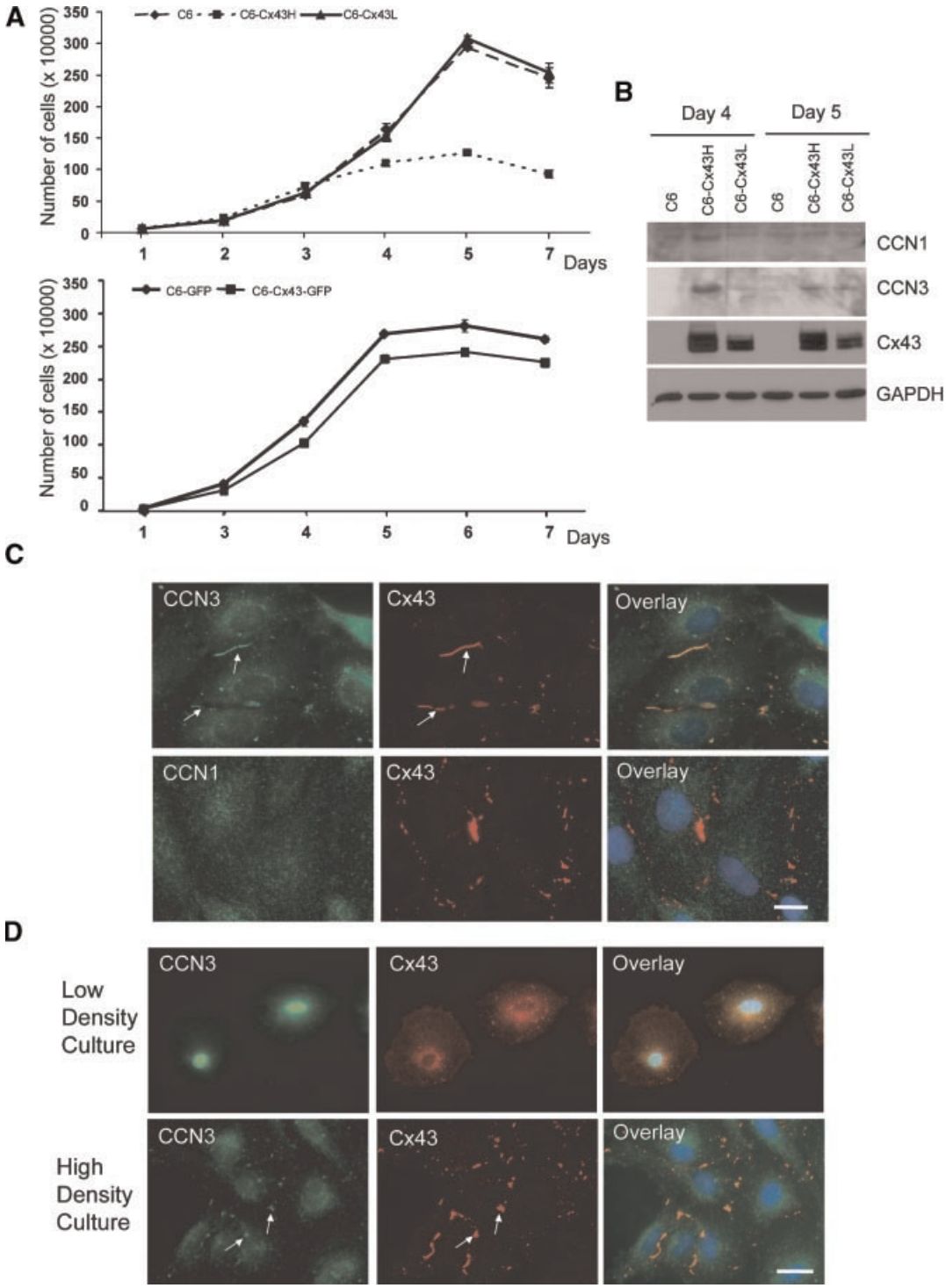

Fig. 3. The growth characteristics of high and low Cx43 overexpressors. **A: Upper panel**, growth curve of C6 cells overexpressing high (C6-Cx43H) and low (C6-Cx43L) levels of Cx43 proteins. C6-Cx43H had a much lower saturation density compared to parental C6 cells (*$P < 0.001$, one way ANOVA). **Lower panel**, growth curve of C6 cells overexpressing Cx43 tagged with GFP (C6-Cx43GFP) and control (C6-GFP) ($P < 0.01$ from day 4 onwards). **B:** Western analysis of the cell lysates of C6-Cx43H, C6-Cx43L, and C6 parental cells at day 4 and day 5 of the growth curve for the expression of CCN1, CCN3, and Cx43. GAPDH was used to monitor equal protein loading. **C:** Localization of CCN1 and CCN3 in gliomas expressing high levels of Cx43. Immunofluorenscence of C6-Cx43H cells co-stained with anti-Cx43 and either anti-CCN3 or anti-CCN1. Colocalization of CCN3 and Cx43 was observed (white arrows) at the gap junctional plaques between two cells. **D**: Immunofluorescence of C6-Cx43H cells co-stained with anti-Cx43 and anti-CCN3. Strong nuclear staining of CCN3 was observed in low-density culture. In high-density confluent culture, CCN3 staining was mainly in the cytoplasm and localized to Cx43 containing plaques. DAPI was used to visualize the location of nucleus (scale bar = 10 μm). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

proliferation of glioma cells over a time interval of 4 days in culture [Zhu et al., 1991, 1992; Naus et al., 1992]. While the growth rate of C6-Cx43H remained stationary from day 4 to day 7, C6-Cx43L had identical growth rate to parental C6 cells (Fig. 3A). Similarly, Cx43 transfected glioma cells consisting of pooled clones also showed reduced growth density, with growth rate intermediate between Cx43L and Cx43H (Fig. 3A). To understand the possible causes that contribute to different growth rates between the two Cx43-expressing gliomas, we determined the expression of CCN1, CCN3, and Cx43 in these cells from cultures in day 4 and day 5 of their growth respectively. We observed a significant increase of CCN3 in C6-Cx43H cells at day 4, and this increase coincided perfectly with the reduced growth rate of C6-Cx43H during this period (Fig. 3B). There was also a slight increase of CCN1 in C6-Cx43H at day 4 compared to day 5. Cx43 levels, in contrast, did not change between these 2 days for both C6-Cx43H and C6-Cx43L. Although C6-Cx43H increased the expression of both CCN1 and CCN3, the subcellular distribution of these two proteins in C6-Cx43H was quite different. A small population of CCN3 was localized to the gap junctional plaques in confluent C6-Cx43H cells (Fig. 3C; [Fu et al., 2004]). In contrast, CCN1 had a diffuse cytoplasmic distribution in the high Cx43-expressing gliomas and we did not observe any localization of CCN1 to the gap junctions (Fig. 3C). Interestingly, CCN3 was mainly located in the nucleus of C6-Cx43H cells from an early, subconfluent stage of growth in culture (Fig. 3D). In contrast, C6-Cx43H cells from a later stage of growth showed cytoplasmic CCN3 staining typically observed in confluent culture (Fig. 3C, D). C6-Cx43L also showed similar redistribution of CCN3 from low- to high-density culture (data not shown). Thus, our results indicate upregulation of CCN3 in high-density culture is correlated with redistribution of CCN3 from the nucleus to the cytoplasm, a process that appears to be facilitated by Cx43.

ECM proteins play an important role in the migration and invasion of gliomas in the brain [Demuth and Berens, 2004]. Fibronectin appears to be the preferred substrate for enhancement of glioma invasion [Ohnishi et al., 1997]. To investigate whether CCN1 mediates Cx43-dependent motility on fibronectin, we determined the motility of glioma cells overexpressing CCN1 or Cx43 by measuring the migration distance of cells moving from glioma spheroids [Del Duca et al., 2004; Suzuki and Iwaki, 2005]. This procedure involved the aggregation of glioma cells in the absence of adherent substrate, such as the lid of culture dishes (Fig. 4A). The resultant aggregates were then seeded onto fibronectin-coated surface. Both CCN1- and Cx43-expressing C6 cells showed increased motility on fibronectin (Fig. 4B), indicating that CCN1 played a role in Cx43-dependent motility in the presence of fibronectin.

DISCUSSION

Gap junctions regulate cell growth by permitting intercellular communication between adjacent cells although recent evidence also suggests that the cytoplasmic tail of the connexin subunit forming the gap junctions can associate with intracellular signaling molecules [Herve et al., 2004]. The recent identification of growth suppressive CCN3 [Perbal, 2006] as a Cx43-associating protein expands the possible pathways used by gap junctions to regulate cell growth [Fu et al., 2004; Gellhaus et al., 2004]. Here, we showed that overexpression of Cx43 also increased the expression of CCN1, a member of the CCN family associated with growth promotion.

We observed a direct correlation of Cx43 levels with CCN1 and CCN3 protein expression. Reduced Cx43 expression is associated with high-grade glioblastoma [Huang et al., 1999; Soroceanu et al., 2001]. A downregulation of CCN3 due to decreased Cx43 levels in high-grade tumors will be consistent with the increased growth of malignant gliomas. Indeed, CCN3 protein levels were decreased upon serum stimulation, confirming the anti-proliferative property of CCN3 and the observation made by others on the downregulation of CCN3 transcripts upon serum stimulation of starved cells [Scholz et al., 1996]. On the other hand, high CCN1 expression is observed in high-grade glioblastoma and is associated with poorer prognosis [Xie et al., 2004]. Expression of CCN1 protein in Cx43-expressing gliomas can be further increased with the addition of serum to quiescent cells, suggesting the role of CCN1 as an immediate early gene is unchanged in gliomas overexpressing Cx43. Furthermore, overexpression of CCN1 in glioma cells

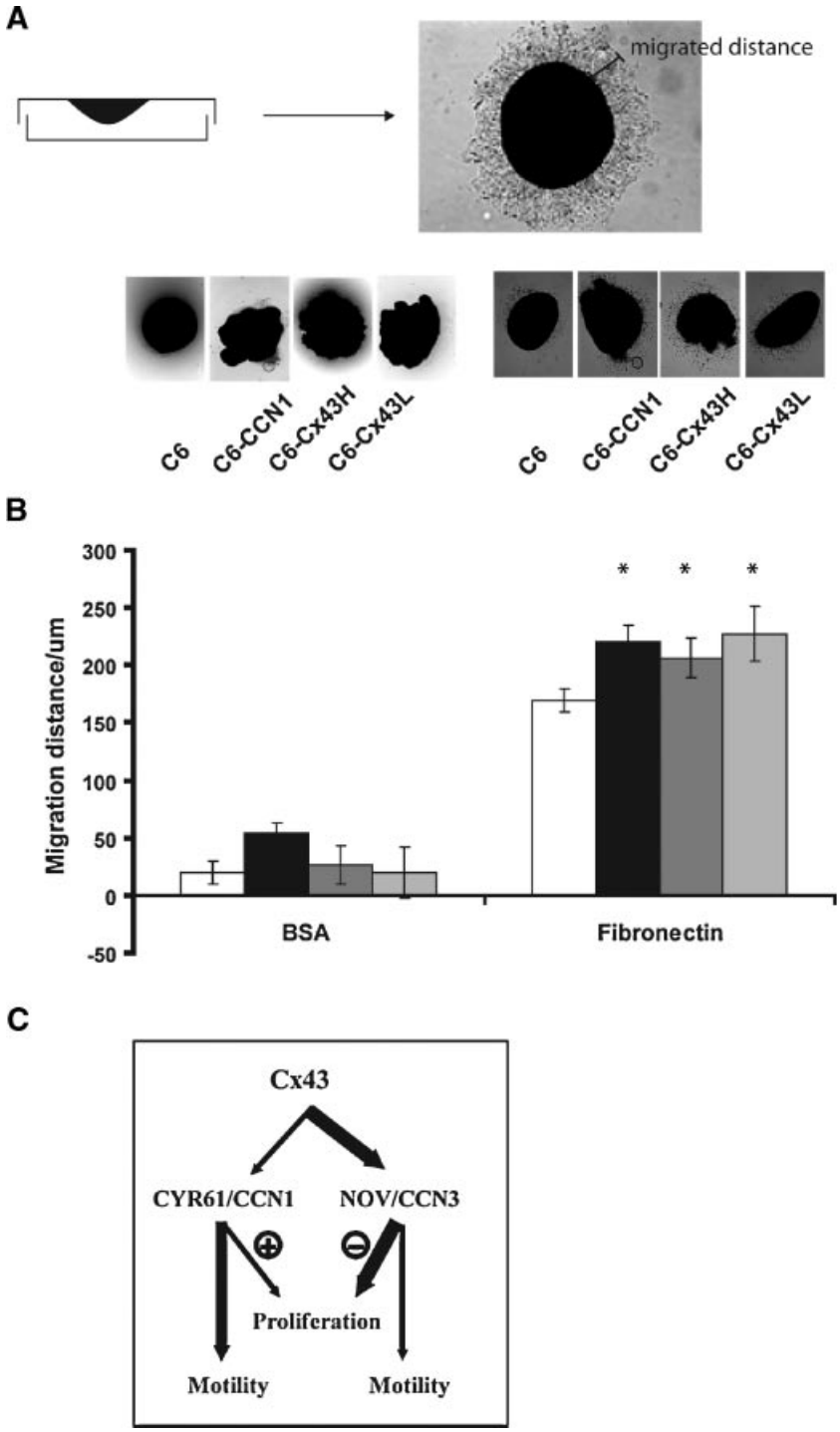

Fig. 4. The motility characteristics of high and low Cx43 overexpressors. **A:** A schematic diagram showing the determination of the motility of glioma cells overexpressing CCN1 or Cx43 by measuring the migration distance of cells moving from glioma spheroids seeded onto fibronectin. The spheroid was formed by growing glioma cells under non-adhering condition. **B:** Spheriod assays for C6 cells expressing CCN1 (C6-CCN1), high levels of Cx43 (C6-Cx43H), low levels of Cx43 (C6-Cx43L) or control C6 cells in the presence of fibronectin. BSA in PBS was used as a control (*$P < 0.05$, Student's *t*-test). **C:** A schematic diagram showing the regulation of both CCN1 and CCN3 by Cx43 in cell proliferation and motility. CCN1 is growth promoting while CCN3 is growth suppressive. Cx43 seems to affect growth suppression by altering the protein levels and localization of CCN3 (thick arrows). On the other hand, CCN1 might mediate Cx43-dependent cell motility.

increased their rate of proliferation after 3 days in culture, when serum became limiting. This agrees with the previous findings with human glioma lines [Xie et al., 2004] that CCN1 has a growth-promoting role in gliomas. Taken together, our results suggest an interesting role of Cx43 in simultaneously regulating a growth-promoting and a growth-suppressing member of the CCN family. One possible way Cx43 may regulate gene expression of multiple signaling molecules is via affecting the levels of transcription factors. For instance, Cx43 is capable of associating with the transcription factor ZONAB [Penes et al., 2005]. A large-scale gene analysis has similarly shown a substantial decrease of transcription factors in Cx43 null heart tissue [Iacobas et al., 2005].

Although the majority of CCN3 is believed to be secreted and is proposed to be a ligand for integrin receptors [Lin et al., 2003, 2005], at least two forms of intracellular CCN3 have been detected in tumor cells [Perbal, 2004]. Interestingly, the overexpression of a non-secretory CCN3 is growth inhibitory in epithelial BHK cells [Planque et al., 2006]. We and others have shown that there is a direct association between Cx43 and CCN3, and that the C-terminal cytoplasmic tail of Cx43 is necessary for its interaction with CCN3 [Fu et al., 2004; Gellhaus et al., 2004]. Indeed, we could detect a small proportion of CCN3 localizing to the gap junctional plaques in C6-Cx43H. There is also a strong link between intracellular CCN3 localization with Cx43 and cell growth. Reduced growth rate of high Cx43-expressors C6-Cx43H was accompanied by an increase in CCN3 level and the redistribution of CCN3 from the nucleus to the cytoplasm and membrane. Cx43 has previously been shown to play a role in sequestering CCN3 away from the nucleus. The upregulation of Cx43 using an inducible system in trophoblast Jeg3 cells induces the relocalization of CCN3 from the nucleus to the membrane sites containing Cx43 plaques [Gellhaus et al., 2004]. In contrast, we have not detected any direct association of CCN1 with Cx43 in vitro (data not shown) or by immunostaining. One reason might be due to the lack of any known intracellular CCN1 isoform that is available to interact with Cx43 [Perbal, 2004].

In addition to its effect on cell growth, overexpression of Cx43 also altered the motility characteristics of the gliomas. Both CCN1 and Cx43 overexpressors exhibited enhanced motility in the presence of fibronectin, an ECM substrate commonly found in the brain. It is not surprising that overexpression of CCN1, either exogenously or via overexpression of Cx43, enhanced motility of glioma cells in fibronectin. CCN1 is known to activate signaling cascades by binding to members of the integrin family; specifically, CCN1 acts through integrins $\alpha V\beta 3$ and $\alpha 6\beta 1$ [Bleau et al., 2005]. $\alpha V\beta 3$ is one of the fibronectin-binding integrin. In addition, the $\beta 1$ integrin subunit is also generally expressed more strongly in glioma multicellular spheroids in vitro [Knott et al., 1998; Rooprai et al., 1999].

It is interesting to observe the cleavage of secreted CCN1 in C6 glioma cells. Proteases that are expressed in high-grade astrocytomas include serine protease plasminogen activator (PA)/plasmin system, cysteine protease cathepsin B, and matrix metalloprotease MMP2 and MMP9 [Chintala et al., 1996; Chintala and Rao, 1996]. The application of a cocktail of inhibitors on serine and cysteine proteases partially prevented the cleavage of full-length CCN1 protein, indicating plasmin or cathepsin B may be responsible for the cleavage. Interestingly, plasmin in breast cancer cells has been shown to release a truncated form of CCN1 (28 kDa) into the surrounding medium [Pendurthi et al., 2005]. Although increased protease MMP secretion has been observed in Cx43-overexpressing glioma cells [Zhang et al., 2003], our results showed that MMP was not involved in the cleavage of CCN1 in glioma cells.

In conclusion, we show that Cx43, a gap junction protein, potentially affects cell growth and motility by altering the levels of CCN proteins. Interestingly, a correlation of Cx43 overexpression levels in C6 glioma clones and in vivo tumorigenicity has been observed previously [Omori and Yamasaki, 1998], suggesting a "threshold" level of Cx43 is required to activate growth inhibitory pathways. Our findings suggest Cx43 regulates CCN3 levels to control cell proliferation, and CCN1 may be more important in Cx43-mediated motility. It has also been suggested that the apparent opposite effects of CCN1 and CCN3 in tumor growth control may help to achieve "balance" in normal cell growth and development [Bleau et al., 2005]. While the cross-talk between CCN1 and CCN3 in maintaining optimal growth and motility is yet to be determined, the reduced growth rate of glioma cells expressing high

levels Cx43 indicates the dominant role of CCN3-mediated growth suppression (Fig. 4C). As the upregulation of CCN1 and CCN3 appears to be dependent on the "dosage" of Cx43, the relative levels of the two CCN proteins determine the overall growth and motility characteristics of the cells. Optimal cell growth probably involves precise regulation and a disruption of this fine balance may result in uncontrolled growth and aberrant motility, as observed in gliomas.

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