HER-2/neu-mediated Down-regulation of Biglycan Associated with Altered Growth Properties

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Background: Oncogenic transformation has been shown to down-regulate components of the extracellular matrix.

Results: HER-2/neu-mediated oncogenic transformation causes silencing of biglycan gene expression. Reconstitution of biglycan expression led to an impaired proliferation and migration of oncogenic transformed cells.

Conclusion: HER-2/neu-mediated silencing of Bgn expression may promote tumor cell proliferation and migration.

Significance: Biglycan represents a putative therapeutic target for the treatment of HER-2/neu+ tumor cells.

The extracellular matrix protein biglycan (Bgn) is a leucine-rich proteoglycan that is involved in the matrix assembly, cellular migration and adhesion, cell growth, and apoptosis. Although a distinct expression of Bgn was found in a number of human tumors, the role of this protein in the initiation and/or maintenance of neoplastic transformation has not been studied in detail. Using an in vitro model of oncogenic transformation, a down-regulation of Bgn expression as well as an altered secretion of different Bgn isoforms was found both in murine and human HER-2/neu oncogene-transformed cells when compared with HER-2/neu- cells. This was associated with a reduced growth, wound closure, and migration capacity. Vice versa, silencing of Bgn in HER-2/neu- fibroblasts increased the growth rate and migration capacity of these cells. Bgn expression was neither modulated in HER-2/neu+ cells by transforming growth factor-β1 nor by inhibition of the phosphoinositil 3-kinase and MAP kinase pathways. In contrast, inhibition of the protein kinase C (PKC) pathway led to the reconstitution of Bgn expression. In particular, the PKC target protein cAMP response element binding protein (CREB) is a major regulator of Bgn expression as the silencing of CREB by RNA interference was accompanied by ~5000-fold increase in Bgn-mRNA expression in HER-2/neu- cells. Thus, Bgn inhibits the major properties of HER-2/neu-transformed cells, which is inversely modulated by the PKC signaling cascade.

The extracellular matrix (ECM)4 protein biglycan (Bgn) belongs to the class I of small leucine-rich proteoglycans (SLRPs) that are characterized by a typical cluster of cysteine residues at the N terminus. The protein core of BGN consists of ~370 amino acids with two covalently linked glycosaminoglycan (GAG) side chains containing chondroitin sulfate and/or dermatan sulfate. In addition to the O-linked GAG chains, two N-linked oligosaccharide moieties are attached to the polypeptide chain (1). Furthermore, the core protein contains 12 leucine-rich repeat domains. Bgn specifically binds to collagen fibrils, thereby modulating the fibril diameter (2). In addition, Bgn as a family member of the SLRPs is functionally involved in matrix assembly, cellular migration, and adhesion, cell growth, and apoptosis (3, 4). Under physiological conditions the expression of Bgn is found in most tissues and is in particular highly expressed in articular cartilage (5). Bgn expression is associated with morphological changes of cellular hypertrophy and actin stress fibers characteristic for epithelial to mesenchymal trans-differentiation (6, 7). Furthermore, Bgn is up-regulated in fibrotic diseases and in stroma cells of solid tumors (8, 9). Interaction of tumor with stroma cells supports the growth and survival of neoplastic cells. A variety of stroma cells in the surrounding environment exists, such as fibroblasts, endothelial cells, macrophages, pericytes, lymphocytes, and mesenchymal stem cells, which facilitate tumor progression by producing growth factors, angiogenic molecules, and cytokines as well as modulating ECM components (10, 11). The quality and integrity of the ECM can influence tumor growth, disease progression, and formation of metastases (12). Most of the studies have focused on the SLRPs lumican and decorin, which are typically located around the tumor microenvironment and have been shown to possess antitumor activity. Whereas lumican blocks melanoma progression by regulation of cell migration, proliferation, and induction of apoptosis (13, 14), decorin represents a powerful cell growth and migration inhibitor by modulating tumor stroma deposition and cell signaling pathways including a reduced cyclin-dependent kinase and TGF-β activity (15, 16).

Oncogenic transformation is often associated with the modulation of the expression of ECM proteins, e.g. up-regulation of small leucine-rich proteoglycan; XTT, tetrazolium salt; ChABC, chondroitinase ABC.
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Generation of Bgn-overexpressing Cells, Bgn, and CREB-silenced Cells—Bgn cDNA was amplified by RT-PCR from the parental NIH3T3 cell line. The PCR product was cloned into the expression vector pIREsneo (Clontech, Heidelberg, Germany) as previously described (31). For down-modulation of Bgn and CREB expression plasmids containing small hairpin RNA (shRNA) sequences targeting Bgn or CREB were synthesized and generated by SABioscience (Frederick, MA). An unspecific, non-silencing shRNA expression vector served as control. For transfections, 1.5 μg/well Scal-digested murine Bgn-specific or CREB-specific shRNA-encoding plasmid (SABioscience) were employed for lipofection with PolyFect (Qiagen, Hilden, Germany) according to the manufacturer's instructions. shRNA-transfected cells were selected in 500 μg/ml G418 (PAA) or 2.5 μg/ml puromycin (Sigma-Aldrich) containing Eagle’s minimal essential medium respectively. Clones exhibiting at least 50% silencing of Bgn or CREB expression in murine fibroblasts were employed for further analysis.

RNA Isolation and Real Time Quantitative RT-PCR—Total cellular RNA was isolated using the NucleoSpin RNA II kit (Macherey-Nagel, Dueren, Germany) as previously described (32). Residual genomic DNA was digested with DNase I (Invitrogen) followed by transcription of 2 μg of total RNA into cDNA using the Revert H Minus First Strand cDNA synthesis kit (MBI Fermentas, St. Leon-Roth, Germany) and oligo(dT)18 primer according to the manufacturer’s instructions. Comparative quantification of gene expression was performed as previously described (32). The target-specific primers used for quantitative PCR are listed in supplemental Table S1.

Migration Assays—Migration assays were performed as previously described (32). Briefly, 5 × 10^4 cells were plated into the upper insert of the transwell diffusion chamber with a pore size of 8 μm (Corning Costar, Corning, NY). A gradient of 0.5–10% (v/v) FCS was used as chemoattractant. After 20 h the number of migrating cells was determined using the Cell Titer Glo Luminescence cell viability assay (Promega, Madison, WI) according to the manufacturer’s instructions. Luminescence was measured with a Lumat LB 9507 luminometer (Berthold Technologies, Bad Wildbad, Germany) and normalized against the luminescence of 5 × 10^4 cells directly seeded into the bottom of the transwell plate.

Scratch Assay—The scratch assay mimicking the directional cell migration was employed. Briefly, cells were grown to density in 6-well plates. The cell monolayer was wounded by scratching with a Pasteur pipette and washed twice with PBS before fresh medium containing 0.5% FCS was added. Images were captured before and at different time points after wound creation over 24 h. The images were compared, and the migration rates were quantified as recently described (33) using the MetaVue software (BioVision Technologies, Exton, PA).

Proliferation Assay—Cell proliferation was determined by measuring the conversion of the tetrazolium salt (XTT) to formazan using the Cell Proliferation kit II (Roche Applied Science) as described by the manufacturer. Briefly, 2.5 × 10^3 or 5 × 10^3 cells for poly-Heme-coated dishes were seeded in 96-well plates in triplicate. Cell growth was analyzed at the indicated time points by spectrophotometric determination of formed formazan at 490 nm after a 4-h incubation of the cells with the
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XTT substrate mixture. The experiment was carried out in independent assays, and the results are expressed as means of the optical density. In addition, the proliferation of cells on poly-Heme-coated dishes was measured as described above.

Western Blot Analyses—Cells were harvested as previously described (31), proteins were solubilized according to Laemmli (34), and protein concentration was determined with the BCA protein quantification kit (Pierce) according to the manufacturer’s instructions. For the analysis of supernatants, proteins were precipitated with 4 volumes of acetone for 12–16 h at −20 °C, and the protein pellet was processed as described above. 50 μg protein/lane were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Schleicher & Schüll). After blocking for 1 h in Tris-buffered saline containing 0.1% Tween 20 and 5% (w/v) skim milk (Difco) at room temperature, membranes were incubated overnight at 4 °C with the respective primary antibodies (mAb) directed against Bgn (Abcam, Cambridge, UK), pCREB/CREB, pHER-2/neu, pSmad 2/Smad 2 (Cell Signaling), and β-actin (Sigma). The following day the immunoblots were developed using the ECL method as recently described (31). Chemiluminescence signals were detected with a CCD camera (LAS3000) (Raytest, Straubenhardt, Germany). For determination of different protein expression levels, the respective area of the signal was integrated using the AIDA image analyzer (Raytest) and subsequently normalized to β-actin.

Flow Cytometry—For flow cytometric analysis 5 × 10^5 cells were stained with FITC-labeled anti-HER-1, anti-HER-3, and phosphatidylethanolamine-labeled anti-HER-2/neu mAb (BD Biosciences) using a standard protocol. The geometric mean fluorescence intensity was determined as recently described (25).

Statistical Analysis—Statistical analysis was performed with Student’s t test for pairwise comparison analysis. Significance was accepted if p values were ≤0.05. Data were expressed as the mean ± S.D.

RESULTS

Expression of Bgn in HER-2/neu-transformed Cells—Expression of Bgn was assessed in different model systems of HER-2/neu and HER-2/neu+ cells using quantitative PCR as well as Western blot analyses. In HER-2/neu-overexpressing murine fibroblasts, Bgn transcription was ~50-fold reduced when compared with parental HER-2/neu− NIH3T3 cells (Fig. 1A). The HER-2/neu-mediated reduction of the Bgn-transcript level resulted in a residual Bgn protein expression of 1.7% (Fig. 1B) when compared with NIH3T3 cells as determined by semi-quantitative densitometric analyses of Western blots (Fig. 1C). The effect of HER-2/neu on Bgn expression was further confirmed in melanoma cells that were transfected with either wt HER-2/neu (Est E2) or mut HER-2/neu (Est E2A) coding for a loss of function variant of this receptor tyrosine kinase. Indeed, transfection with wt HER-2/neu led to a significant decrease of Bgn-mRNA expression (Fig. 1D). More interesting, lack of HER-2/neu tyrosine kinase activity (Est E2A) had no effect on Bgn expression, suggesting that HER-2/neu-mediated signal transduction is essential for Bgn suppression and also relevant

FIGURE 1. HER-2/neu-mediated reduction of Bgn expression. A, mRNA expression of Bgn in parental HER-2/neu− and HER-2/neu+ cells is shown. Quantitative RT-PCR analyses of Bgn in NIH3T3 and HER-2/neu+ cells were performed with oligo-(dT)18-primed cDNA and normalized to β-actin. The expression level of NIH3T3 cells was used as control and set to 1. B, Western blot analysis of Bgn in parental HER-2/neu− (NIH3T3) and HER-2/neu+ cells is shown. 50 μg of protein/cell line was separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane before immunostaining was performed with a Bgn-specific antibody as described under “Experimental Procedures.” Equal protein loading was controlled by subsequent immunostaining using a β-actin-specific antibody. A representative result of three independent biological replicates is shown. C, densitometric analysis of Bgn expression in HER-2/neu− cells is shown. Areas of immunoblot signals were integrated using the AIDA image analyzer software and subsequently normalized to β-actin. The graphic shows the means and S.D. from three independent experiments. D, mRNA expression of Bgn in HER-2/neu− and HER-2/neu+ human melanoma cell lines is shown. Human melanoma cell line Est 039 and Est 049 transfected with p-empty, p-E2, and p-E2A were analyzed for Bgn transcription using quantitative PCR. Bgn mRNA expression was normalized against β-actin expression, and the parental cell line was set to 1.
in human cells (Fig. 1D). It is noteworthy that the overexpression of HER-2/neu did not alter the expression of other HER family members (supplemental Fig. 1).

Establishment of Bgn Model Systems—To analyze the function of Bgn, the expression vector pIRESneo-Bgn was transfected into the Bgn-deficient HER-2/neu cells by lipofection. Although Bgn protein expression was reconstituted in HER-2/neu cells (Fig. 2A), the Bgn expression level in HER-2/neu cells was lower than that of HER-2/neu cells. The Bgn overexpression in HER-2/neu cells was further associated with an altered morphology (data not shown). In addition, Bgn expression was about 2-fold decreased in the Bgn/H11001 NIH3T3 cells by Bgn-specific shRNA, whereas transfection with a scrambled shRNA did not decrease the Bgn expression (Fig. 2B).

Analysis of Secreted Bgn Isoforms—The processing and secretion of glycosylated biglycan into the medium of parental NIH3T3 of HER-2/neu as well as of Bgn overexpressing HER-2/neu cells were determined by immunoblot analyses. As shown in Fig. 2C, NIH3T3 cells synthesized different major Bgn isoforms with a molecular mass range of ~150–220 kDa and one of ~100 kDa. Immunoblot analyses of the supernatant of Bgn-transfected HER-2/neu and non-transfected HER-2/neu cells detected three isoforms ranging between ~100 and 150 kDa (Fig. 2C). Treatment of the supernatant with the deglycosylating enzymes ChABC and peptide N-glycosidase F leads to the detection of a signal of ~38 kDa corresponding to the mass of the core protein that is accompanied by a reduction of the signal intensities of the proteins >150 kDa (supplemental Fig. 4). Thus, not only Bgn gene expression but also Bgn processing leading to its secretion into the microenvironment is altered upon oncogenic transformation.
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Impact of Bgn on Proliferation and Anchorage-independent Growth—To study the influence of restored Bgn expression on the proliferation of HER-2/neu+ cells, XTT-based proliferation assays were performed. An increased proliferation of HER-2/neu+ cells was observed accompanied by a decreased doubling time of ~4 h when compared with parental cells. Overexpression of Bgn in these HER-2/neu+ cells resulted in an anti-proliferative effect with an increased doubling time of the Bgn transfectants from 18 to 22 h (Table 1). Vice versa, the anti-proliferative activity of Bgn was confirmed by Bgn silencing in NIH3T3 cells (Table 1), demonstrating that Bgn expression interferes with cell proliferation.

Because different Bgn isoforms were detectable in the supernatant of Bgn-transfected HER-2/neu+ cells, the role of fully secreted Bgn isoforms on the proliferation of HER-2/neu+ cells was studied by the addition of 100 nM bovine Bgn to the medium. As shown in Fig. 3A, a significant prolonged proliferation rate of HER-2/neu cells was detected after the addition of bovine biglycan to the medium, which was more pronounced by removal of the GAG chains by incubation of Bgn with ChABC before the treatment of the cells. This result suggests that the core protein has anti-proliferative activity (Fig. 3A), whereas incubation with ChABC alone did not affect the growth rate of the cells (Fig. 3A).

The influence of Bgn on the anchorage-independent growth of HER-2/neu+ transformants as well as of the Bgn-expressing HER-2/neu+ cells was analyzed on poly-Heme-coated cell culture dishes, and the growth was recorded as described under “Experimental Procedures.” As shown in Fig. 3B, expression of Bgn diminished the anchorage-independent growth of HER-2/neu+ cells in a non-significant manner about 40%, whereas the parental NIH3T3 cells were not able to grow anchorage-independent. This result suggests that Bgn has the ability to revert this oncogenic property of the transformed phenotype.

Impact of Bgn on Migration—To analyze the influence of Bgn overexpression in HER-2/neu+ cells on their migration, wound closure potential as well as transwell assays were performed. A decreased directional migration was found in Bgn-transfected HER-2/neu+ cells when compared with control HER-2/neu+ cells (Fig. 4, A and B). Using a FCS gradient as attractant, the migration rate significantly decreased about 2-fold from ~45% for migrating HER-2/neu+ and vector-transfected control cells to 15–25% depending on the Bgn-transfected clones analyzed (Fig. 4C). To confirm the effect of Bgn on the migration potential, parental Bgn+ and corresponding shBgn-transfected NIH3T3 cells were also subjected and compared in migration assays. Bgn suppression in NIH3T3 cells increased the wound closure ability from 40% in the control cells to 45–70% in the transfectants depending on the respective clone analyzed (Fig. 4, D and E). Furthermore migration through transwell plates was enhanced from 28% in parental cells to 42–52% in cells with silenced Bgn expression (Fig. 4F). These results indicate that Bgn expression negatively interferes with the migration of cells.

Molecular Mechanisms of Deregulated Bgn Expression in HER-2/neu+ Cells—Previous studies could show a TGF-β-inducible Bgn expression (35). To determine whether a perturbation of the TGF-β signaling cascade is responsible for the diminished Bgn expression in HER-2/neu-transformed cells, parental NIH3T3 and HER-2/neu+ cells were stimulated with TGF-β8 in the presence and absence of an inhibitor of this signaling cascade, respectively. After 48 h the induction of this pathway was confirmed by the analysis of phosphorylated SMAD2. The addition of TGF-β increased SMAD2 phosphorylation in both parental NIH3T3 and HER-2/neu+ cells.

### Table 1

| Cell line       | Bgn | Doubling time (h) |
|-----------------|-----|------------------|
| HER-2/neu       | −   | 18.6 ± 1.1       |
| Vector          | −   | 18.3 ± 1.8       |
| Clone 1         | +   | 22.6 ± 2.1       |
| Clone 2         | +   | 21.0 ± 0.7       |
| Clone 3         | +   | 24.2 ± 2.4       |
| NIH3T3          | −   | 23.0 ± 2.1       |
| Scramble        | +   | 24.2 ± 2.2       |
| shBgn clone 1   | −   | 19.8 ± 2.5       |
| shBgn clone 2   | −   | 19.5 ± 1.8       |

* p < 0.01
* c Not significant.
* + p < 0.05.

![Figure 3](image-url)

**Figure 3. Influence of Bgn on the proliferation and the anchorage-independent growth.** A, shown is impaired proliferation after exogenous Bgn addition. 100 nM bovine Bgn (untreated or deglycosylated with chondroitinase ABC) or 100 milliunits of ChABC in 1% FCS-containing medium was added after 24 h to 2.5 × 10^4 HER-2/neu+ cells/well. The relative proliferation was analyzed after 4 days using the XTT assay as described under “Experimental Procedures.” Proliferation of HER-2/neu+ cells was set to 1. The graph summarizes means and S.D. of seven independent experiments carried out in triplicate. n.s., not significant.
whereas SMAD2 activation was suppressed in the presence of the inhibitor A8301 (Fig. 5A). Regarding Bgn, its expression was increased by TGF-β in HER-2/neu− NIH3T3 cells, but it was not affected in HER-2/neu+ cells. Thus, HER-2/neu transformation inhibited the stimulating capacity of TGF-β for the induction of Bgn expression by an unknown mechanism.

Because MAPK and PI3K/Akt pathways are altered upon HER-2/neu transformation, both signaling cascades were blocked by specific inhibitors before Bgn expression was analyzed. However, inhibition of these pathways did not affect Bgn expression levels in the oncogenic transformants (supplemental Figs. 2 and 3). In contrast, treatment with the PKC inhibitor RO-31-8220 led to the restoration of Bgn expression after 48 h. The deactivation of the PKC pathway could be demonstrated by the diminished phosphorylation of CREB, which was accompanied by a reduction of total CREB expression after 48 h (Fig. 5B). In contrast, inhibition of the PKA pathway by H89 treatment had only a marginal effect on Bgn expression (Fig. 5B). Based on the fact that CREB has two potential binding sites in the Bgn
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FIGURE 5. CREB-mediated control of Bgn expression in HER-2/neu + transfectants. A, inhibited induction of Bgn expression in HER-2/neu + cells is shown. NIH3T3 cells and HER-2/neu + transfectants were treated with TGF-β and A8301 as described under “Experimental Procedures.” After 48 h cells were harvested and proteins isolated. A representative result of three independent experiments is shown. B, reconstituted Bgn expression after inhibition of the PKC pathway is shown. Expression analyses of Bgn after inhibition of the PKC and PKA pathway are shown. HER-2/neu + cells were treated with the inhibitors H89 and RO-31-8220 for 24 and 48 h, and the amounts of phosphorylated CREB (Ser133) and total CREB as well as Bgn were determined by Western blot analyses. A representative result of three independent experiments is shown. C, silencing of CREB in HER-2/neu + transfectants is shown. HER-2/neu + cells were transfected with CREB-specific shRNA and scramble shRNA. Selected clones were analyzed by Western blotting for phospho-CREB expression, CREB, and pCREB. D, reconstituted Bgn-mRNA expression after CREB silencing is shown. mRNA expression analysis of CREB-shRNA-transfected HER-2/neu + cells is shown. Quantitative RT-PCR analyses of shCREB-transfected HER-2/neu + cells were performed with oligo-dt primed cDNA and normalized to β-actin. The expression level of HER-2/neu + cells transfected with scramble shRNA was used as control and set to 1. The diagram shows the means ± S.D. of two biological replicates. E, enhanced CREB activation in HER-2/neu + transfectants is shown. CREB activation in NIH3T3 and HER-2/neu + cells was determined by immunoblot analyses of the Ser133 epitope of CREB as described under “Experimental Procedures.” A representative result of three independent experiments is shown.

Promoter/gene, the influence of this transcription factor on the Bgn expression was further analyzed by RNA interference studies. As shown in Fig. 5C, transfection of HER-2/neu + cells with CREB-specific shRNA led to a more than 60% reduction of CREB expression in HER-2/neu cells that was accompanied by an ~5000-fold increase of Bgn mRNA expression in shCREB-transfected HER-2/neu + cells, whereas transfection with a scrambled shRNA had no effect on Bgn transcription (Fig. 5D). Furthermore, HER-2/neu + cells expressed higher levels of phosphorylated CREB when compared with parental NIH3T3 cells (Fig. 5E). In summary, the results of these experiments identified for the first time an involvement of the PKC pathway for the induction of Bgn expression and CREB as a molecular mediator of Bgn transcription.

DISCUSSION

Small leucine-rich proteoglycans like decorin and Bgn are major components of the extracellular matrix and have been shown to influence signal transduction cascades and different cellular processes like migration and invasion (4). The role of Bgn in regulating cellular proliferation was addressed only in a limited number of studies (36). Recent studies using cDNA and proteomic technologies demonstrated a down-regulated mRNA expression of Bgn and decorin in osteosarcoma, leukemia, intrahepatic cholangiocarcinoma, and melanoma (21–23, 37). In contrast, increased expression of Bgn was detected in gastric cancer (38) and colorectal cancer (39), suggesting a distinct role of this ECM molecule in different tumor entities. Moreover in in vitro models of oncogenic transformation, Bgn expression is suppressed (19, 20). To shed light into this discrepancy and to increase the knowledge of the pathophysiological role of Bgn, its influence on cell proliferation and migration properties was analyzed.

In the current study, Bgn mRNA expression was down-regulated on the mRNA and protein level in HER-2/neu + transfectants when compared with parental untransformed cells. In addition, human melanoma cells transfected with a HER-2/neu-overexpressing vector demonstrated a down-regulated Bgn mRNA expression, whereas a transduction of the dominant-negative HER-2/neu vector into these cells did not affect Bgn transcription. This result clearly indicates a regulatory function of HER-2/neu for Bgn expression and the relevance of this process also for human tumors. It is noteworthy to mention that both HER-2/neu variants showed a comparable cell surface expression, suggesting a correct processing of the inactive protein variant (supplemental Fig. 1) confirming our earlier results (26).

The expression of Bgn in HER-2/neu + fibroblasts was induced by transfection with a Bgn expression vector. However, immunoblot analysis of soluble Bgn in the supernatant of parental NIH3T3 cells compared with HER-2/neu + and Bgn-transfected HER-2/neu + cells showed the secretion of two additional isoforms at a molecular mass of ~180 and 220 kDa. In line with our results Tufvesson et al. (40) detected Bgn isoforms of a similar mass range in the cell culture medium of
human lung fibroblasts. Because the treatment of the supernatant with ChABC led to a reduction of these signals, we propose that these two additional isoforms exert their distinct migration behavior in response to the variable lengths of their GAG chains. These data are in line with the report of Tiede et al. (41) who identified increased GAG chain lengths in cardiac fibroblasts after treatment with nitric oxide and platelet-derived growth factor BB. Therefore, the HER-2/neu-induced aberrant signal transduction may lead to an impaired post- and/or cotranslational processing of Bgn. This posttranslational modification might have several cell biological consequences, as the truncated GAG chains could lead to a decreased anchorage and may interfere with cellular signaling events by the binding of growth factors, which provide an explanation for the altered proliferation and migration capacity of HER-2/neu and parental NIH3T3 cells. It is noteworthy that the major Bgn isoforms of Bgn-transfected as well as parental HER-2/neu cells consist of two variants of ~100 kDa, whereas these isoforms were nearly not detectable in NIH3T3 cells. The molecular and functional reasons are unknown until now. We suppose that this isoform is the same as the previously described monoglycanated isoform in skin fibroblasts of a patient with Ehlers-Danlos syndrome, whereas healthy control fibroblasts lack this isoform (42).

Transgenic overexpression of Bgn inhibited the HER-2/neu-induced cell proliferation, anchorage-independent growth on poly-Heme-coated cell culture dishes, and migration. This is in line with previous reports demonstrating that the SLRPs lumican and decorin (14, 18, 43) suppress the oncogene-mediated transformation process. Overexpression of these molecules not only inhibit the proliferation of transformed cells in vitro but also their tumorigenicity in vivo. Furthermore, Weber et al. (8) demonstrated a G1 arrest of pancreatic cancer cells when treated with 20 nM Bgn. Thus, in the HER-2/neu models analyzed, Bgn seems to be involved in the suppression of the transformed phenotype comparable to the activity of decorin and lumican. In addition, not only overexpression but also treatment of HER-2/neu+ cells with recombinant Bgn caused a significant reduction in their proliferation capacity. These findings confirm a function of Bgn in controlling the HER-2/neu-mediated growth properties. In agreement to our data, Datsis et al. (44) could show that Bgn impairs the migration of osteosarcoma cells. In addition, Bgn expression in these cells is negatively regulated via the parathyroid hormone and fibroblast growth factor 2. These authors also demonstrated a reduction of secreted Bgn but did not detect altered growth properties in the osteosarcoma cells with silenced Bgn expression, suggesting a cell and tissue type-specific role for Bgn function. The lack of fully processed Bgn isoforms might also be responsible for the increased doubling times of Bgn-transfected HER-2/neu+ cells when compared with HER-2/neu+ cells, as an induction of EGFR expression by glycosylated bovine Bgn was shown (45).

To define the responsible pathways for the down-regulation of Bgn expression, our studies were extended to several canonical signal transduction cascades like the MAPK and the PI3K/Akt pathway. Targeted inhibition of both pathways did not reconstitute Bgn expression in HER-2/neu-transfomed cells (supplemental Figs. 2 and 3). However, glycosylated bovine Bgn has been demonstrated to induce EGFR expression via the PI3K/AKT pathway in human chondrocytes and has been postulated to bind to the EGFR (45). Regarding the effect of cytokines, TGF-β has been shown to mediate proteoglycan synthesis via the PI3K/Akt pathway. Bgn levels were increased upon TGF-β treatment of cells (35). This pathway led to TGF-β-increased Bgn expression in parental NIH3T3 cells but not in HER-2/neu-transformed cells, suggesting that Bgn synthesis is not controlled by the smad and non-smad signaling pathways (Fig. 5A). This discrepancy was not due to impaired expression of TGF-β receptors and might be explained by other HER-2/neu-mediated effects, which still have to be defined. In contrast, treatment with an inhibitor of the PKC cascade restored the expression of the ECM molecule in HER-2/neu+ cells. To further elucidate the mechanisms of transcriptional Bgn control, the transcription factor CREB, representing a direct target of PKC signaling (46), was silenced via RNA interference in HER-2/neu cells as the Bgn promoter contains binding motif(s) for this molecule. Indeed, shRNA-mediated knock down of CREB led to a several thousand-fold increase in the Bgn-mRNA expression. These results suggest for the first time a regulatory role of the PKC signaling pathway for Bgn and in particular for CREB. High levels of CREB expression have been shown to be associated with the malignant behavior of tumor cells and further support angiogenesis and resistance to apoptosis (47), whereas CREB-specific silencing altered growth properties and apoptosis of oncogene-transformed cells (46).

Due to the limitations of the murine fibroblast model, further in vitro and in vivo experiments are required to determine the functional role of Bgn in human oncogene-transformed cell systems as well as in malignancies. However, modulation of Bgn expression might be a suitable strategy for therapeutic intervention.

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