

Decorin-Induced Growth Inhibition Is Overcome through Protracted Expression and Activation of Epidermal Growth Factor Receptors in Osteosarcoma Cells

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Abstract

Decorin is an established natural oncosuppressive factor whose action is being studied in detail. Recently, decorin gene therapy formulations using adenoviral vectors have been shown in several animal models with very promising results. The present study describes the first exception to the established oncosuppression model using human osteosarcoma cells. MG-63 osteosarcoma cells were found to constitutively produce decorin, and furthermore, to be resistant to decorin-induced growth arrest. On the contrary, decorin seemed to be beneficial to osteosarcoma cells because it was necessary for MG-63 cell migration and acted as a mediator, counteracting the transforming growth factor- β 2-induced cytostatic function. Efforts to determine how MG-63 cells could overcome the decorin-induced cytostatic effect established that decorin in MG-63 cells does not induce p21 expression nor does it cause protracted retraction and inactivation of the epidermal growth factor receptor. Conversely, epidermal growth factor receptor seemed to be overexpressed and continuously phosphorylated. In view of the proposed design of decorin-based anticancer therapeutic strategies, our study provides new data on pathways that cancer cells might employ to overcome the established decorin-induced growth suppression. (Mol Cancer Res 2008;6(5):785–94)

Introduction

Decorin belongs to the family of small leucine-rich proteoglycans. It has a small protein core of 50 kDa bearing a chondroitin or dermatan sulfate glycosaminoglycan chain attached in close proximity to its NH₂-terminal domain. Decorin is secreted mainly by cells of mesenchymal origin

and has been implicated in the control of extracellular matrix assembly as well as in the modulation of cell attachment, migration, and growth. Specifically, decorin has been shown to modulate extracellular matrix assembly through interaction with tropoelastin, stabilization of collagen fibrils, and acceleration of the mineralization process (1-3). Decorin can affect cell adhesion and migration through the inhibition of fibronectin binding and the interference of integrin interaction with extracellular matrix proteins (4).

Decorin is rarely expressed by cancer tissue as has been shown using an analysis of a variety of tumors of epithelial origin (colon, pancreas, prostate, and breast; refs. 5-7). However, it is expressed in the tumor stroma by mechanisms involving hypomethylation of the decorin promoter or induction by tumor-secreted growth factors. Analysis of cancer cell lines from the uterus, skin, soft tissues, and bone marrow showed that they do not express decorin (8, 9), whereas normal lung and dermal fibroblasts produced it in large amounts (10, 11). Forced expression of decorin in cancer cell lines *in vitro* caused a severe cytostatic effect. Decorin has been shown to interact with epidermal growth factor (EGF) receptor (EGFR; refs. 12-14) and erb2 (15), causing a transient receptor phosphorylation which is followed by the induction of endogenous cyclin-dependent kinase inhibitor p21(waf1) expression and a subsequent arrest at the G₁ phase (16, 17) or caspase-3-dependent apoptosis (18).

In vivo, decorin deletion in mice, in combination with p53, results in the accelerated appearance of lymphomas as compared with p53 alone (19). Furthermore, increased decorin expression *in vivo*, achieved with gene transfer techniques, causes growth inhibition in various tumors (20-22) and it specifically prevents metastasis in a breast cancer animal model (23). Summarizing the data on the relation of decorin with cancer, today decorin is believed to be a powerful natural anticancer agent produced by normal host cells against cancer cells. Targeted expression of decorin in cancer tissues is proposed as a new anticancer therapeutic strategy (18).

Osteosarcoma is the most frequent bone sarcoma in children and adolescents. The bone cells (osteoblasts) produce osteoid, a complex of mineralized extracellular matrix proteins (24). The pathogenesis of osteosarcoma implicates qualitative and quantitative changes in the proteoglycans component of the extracellular matrix (25, 26). In a preliminary study, it was found that proteoglycan expression profiles differ between osteosarcoma and normal osteoblastic cells. In the present study, we focused on decorin and discovered that MG-63 cells

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expressed decorin mRNA in large amounts and secreted significant amounts of protein, in contrast with Saos-2 and normal osteoblastic cells, in which it was absent. The aim of this study was to investigate how decorin biosynthesis was affected by growth factors related to osteosarcoma pathogenesis [transforming growth factor- β 2 (TGF- β 2), basic fibroblast growth factor (bFGF), and platelet-derived growth factor-BB (PDGF-BB)], why MG-63 cancer cells produce decorin, which is a well-documented anticancer agent, and how MG-63 cells could escape the documented decorin-induced cytostatic effect on cancer cells. In the present study, we showed that MG-63 cells produced high amounts of decorin and were resistant to decorin-induced growth arrest through the sustained expression and activation of the EGFR. Furthermore, MG-63 cells were found to use decorin to counterbalance the growth-limiting effects of TGF- β 2. These data provide a prototype exception model for the well-documented cytostatic effect of decorin, indicating a novel mechanism by which a cancer cell can overcome its cytostatic effect.

Results and Discussion

MG-63 Cells Produce Significant Amounts of Decorin

Initially, we aimed to analyze the expression of decorin in human cells of osteoblastic origin. We used the well-characterized MG-63 and Saos-2 cell lines as well as normal osteoblastic cells (PDL) isolated from a human periodontal ligament to compare the decorin status of osteoblastic osteosarcoma with normal osteoblastic cells. We tested for the presence of decorin mRNA in serum-free cultures using real-time PCR verified by sequencing. Significant amounts of decorin mRNA were found in the MG-63 cells, whereas in Saos-2 and PDL cells, it was virtually absent (Fig. 1A). Analysis of culture supernatants after concentration and puri-

fication by ion exchange chromatography verified the secretion of fully glycosylated decorin by MG-63 cells (Fig. 1B). Previous work by Iozzo et al. (8) in MG-63 cells indicated the presence of decorin mRNA but failed to detect secreted decorin. The finding of secreted decorin could be explained by the higher sensitivity of the methodologic approach we used.

TGF- β 2, bFGF, and PDGF-BB Significantly Enhance the Transcription of the Decorin Gene as well as the Extracellular Secretion of Decorin in MG-63 Cells

The next question addressed was if it would be possible to induce decorin expression in human osteoblastic cells, irrespective of its starting basal level expression status. TGF- β 2, bFGF, and PDGF-BB are considered key players in the pathogenesis of osteosarcoma (27-30). The effects of these growth factors on decorin biosynthesis at the gene and protein levels were examined *in vitro* under serum-free culture conditions. Significant up-regulation of both mRNA and protein were identified in the MG-63 cells (Fig. 1A and B). PDGF-BB treatment caused the most prominent effect (3-fold induction of protein secretion) followed by TGF- β 2 and bFGF (2-fold). The treatment had no effect on Saos-2 and PDL cells, in which no decorin up-regulation was identified. Therefore, decorin is not only present in MG-63 cells, but it can be further induced by major effectors of osteosarcoma pathogenesis.

MG-63 Osteosarcoma Cells Are Not Sensitive to Decorin-Induced Growth Suppression as Opposed to Saos-2

Decorin is a well-studied natural anticancer agent with cytostatic properties for a wide array of cancer-specific cell lines *in vitro* and *in vivo* (8, 9). The constitutive expression of a potentially growth-inhibiting factor in a cancer cell line is certainly intriguing. To examine the role of decorin expression

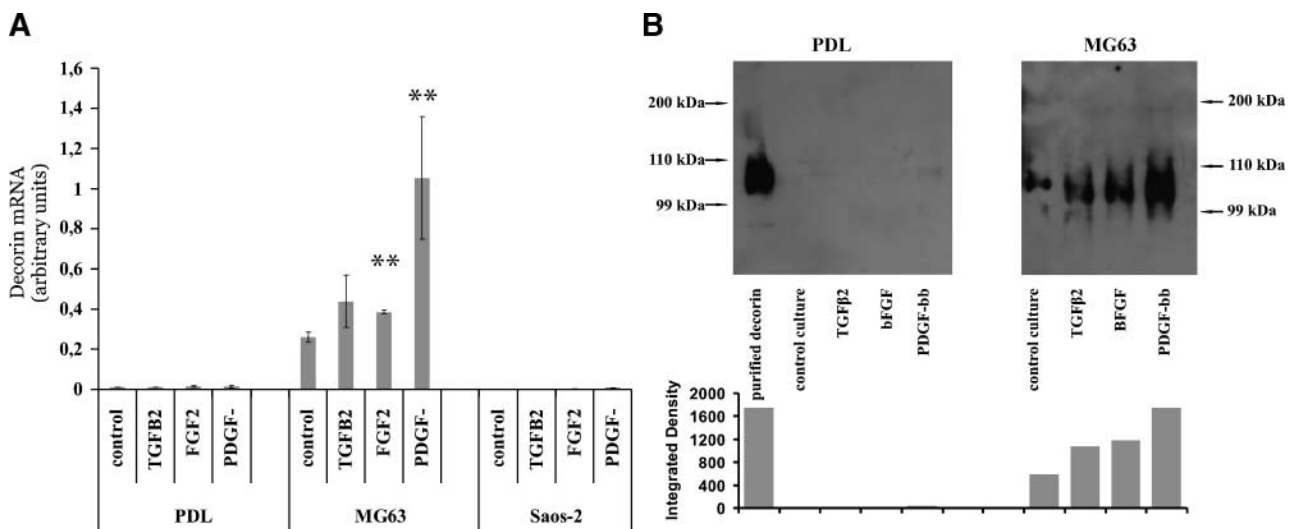


FIGURE 1. Comparison of decorin mRNA (**A**) and protein (**B**) expression between Saos-2, MG-63, and normal osteoblastic cells (PDL). Prior to stimulation with growth factors, cells were serum-starved for 24 h. TGF- β 2, bFGF, and PDGF-BB, at the final concentration of 10 ng/mL, were added in fresh serum-free medium. Proteoglycans were extracted after 48 h of growth factor treatment whereas mRNA were extracted after 24 h. The measurement of decorin mRNA was done using real-time PCR and normalized by glyceraldehyde-3-phosphate dehydrogenase expression. All mRNA measurements were done in triplicate. The secreted proteoglycans were concentrated by ion exchange chromatography and decorin was identified by Western blotting using the 6B6 antibody (Seigakaku). The blots were quantified digitally and the presented values are the band-integrated densities. Columns, mean of triplicate values; bars, SD; **, $P < 0.01$.

in MG-63 cells, we analyzed the effect of both the increased presence of decorin (by exogenous addition) and the absence of decorin [inhibition of endogenous decorin by short interfering RNA (siRNA)] on osteosarcoma cell growth. Initially, MG-63 and Saos-2 cells were treated with increasing amounts of decorin and their proliferation rates were determined. The results obtained showed that exogenous decorin had no effect on MG-63 cell proliferation even at the highest concentration used (100 $\mu\text{g}/\text{mL}$). On the contrary, decorin caused a dose-dependent inhibition in the non-decorin-expressing Saos-2 cells (Fig. 2A), in agreement with a previously published report (8).

Inhibition of Decorin Expression Has No Effect on MG-63 Cell Growth but Reduces Cell Motility

Inhibition of decorin mRNA and protein by siRNA transfection was efficiently achieved by up to 80% within 24 hours of treatment and by up to 90% at 48 hours (Fig. 2B). Inhibition of decorin mRNA by increasing doses of decorin-specific siRNA after 48 hours of treatment had no significant effect on MG-63 cell growth as compared with the treatment with a control siRNA (Fig. 2C).

Decorin has been previously shown to significantly affect cell motility through interference with the interaction of cells with matrix proteins (4). Next, we analyzed the effect of endogenous decorin production on the mobility of MG-63 cells using a “wound healing” assay. Control siRNA-treated cells closed 30% of the inflicted gap within 24 hours of the initiation of treatment. MG-63 cells treated with decorin siRNA showed significantly reduced mobility in the same time period (Fig. 2D). Saos-2 cells, which do not express decorin, seemed to migrate with equal efficiency in the presence or the absence of decorin siRNA, indicating that the decorin siRNA itself does not have any intrinsic cell mobility-inhibiting action.

Endogenous Decorin Counteracts the Cytostatic Effect of TGF- β 2

Aiming to define indirect ways with which decorin can affect MG-63 cell function, we examined the possibility of binding and affecting the mitogenic function of TGF- β 2, PDGF-BB, and bFGF. It has been previously shown that decorin was able to bind efficiently to TGF- β 2 and PDGF-BB as well as down-regulate their function through the reduced phosphorylation of their corresponding receptors (31, 32). bFGF has also been shown to interact with glycosaminoglycans, particularly of the heparan sulfate family (33). Initially, we determined the effect of these growth factors on MG-63 cell growth in a dose-response assay. bFGF and PDGF-BB caused a dose-dependent increase of cell growth (Fig. 2E). TGF- β 2 caused a minor inhibition, which appeared at 1 ng/mL but remained constant even after a 20-fold dose increase. In order to examine whether the resistance of MG-63 cells to TGF- β 2 growth inhibition was mediated by decorin, cells were treated with decorin siRNA to block endogenous production. In the absence of endogenous decorin, MG-63 cells became sensitive to a dose-dependent growth inhibition by TGF- β 2 (Fig. 2F).

To summarize, the results thus far have shown that MG-63 cells are not sensitive to decorin-induced growth arrest, nor do they require decorin for their growth. On the contrary, decorin

seems to be necessary for MG-63 cell migration and to mediate the counteraction of the TGF- β 2-induced cytostatic function. Nevertheless, decorin is an established cytostatic factor affecting a wide array of cancer-specific cell lines. The surprising finding that MG-63 cells express decorin constitutively raises the question of how MG-63 cells overcome the decorin-induced cytostatic effect.

Decorin Cannot Induce p21 Transcriptional Up-Regulation in MG-63 Cells

A previously described method of overcoming the decorin cytostatic effect was through the deletion of the *p21* gene (8). To analyze the p21 status of MG-63 and Saos-2 cells, we used serum starvation, which is known to cause growth arrest, and quantified the p21 mRNA with real-time PCR during a period of 48 h. Expression of p21 was present in both MG-63 and Saos-2 cells and became up-regulated by serum starvation (Fig. 3A), peaking at 36 h, coinciding with the inhibition of cell growth (data not shown). Exogenous addition of decorin (20–100 $\mu\text{g}/\text{mL}$) failed to induce additional or accelerated p21 expression when compared with the untreated cells in both MG-63 (Fig. 3B) and Saos-2 (Fig. 3C) cells. The absence of p21 expression was verified at the protein level by confocal microscopy experiments when comparing the staining of decorin-treated and p21-transfected MG-63 (Fig. 3D). The same lack of p21 induction at the protein level was observed in Saos-2 cells. Therefore, decorin cannot cause the growth arrest of MG-63 cells by up-regulating p21 expression.

Decorin Causes Protracted Expression and Phosphorylation of EGFR in MG-63 Cells

The main mediator of the decorin-induced cytostatic effect has been shown to be the direct binding of decorin to EGFR or Erb2, which results in autophosphorylation of the receptors followed by protracted internalization and blockade of the signaling pathway (15, 34). Both MG-63 and Saos-2 cells express EGFR and erb2 as determined by real-time PCR quantification. The former express mainly EGFR (Fig. 4A), whereas the latter express erb2 (Fig. 4B). No significant changes were observed after decorin treatment on the mRNA expression of EGFR or erb2 in both MG-63 and Saos-2 cells. When the protein levels of EGFR were examined in MG-63 cells, it was discovered that the number of EGFRs per cell increased significantly after treatment with decorin and peaked 24 hours after the initiation of treatment. The constitutive EGFR expression in MG-63 was also gradually increased, probably through the action of the accumulated endogenously produced decorin (Fig. 4C and E). These results could be explained by either a decreased internalization/degradation of EGFR or a *de novo* EGFR synthesis by increased translation of the already existing levels of mRNA. When the phosphorylation status of EGFR was analyzed, it was found that decorin induced an immediate (30 min) phosphorylation of EGFR which later subsided (24 hours), and again appeared at the 48-hour time point (Fig. 4C and F). When MG-63 cells were not treated with decorin, the phosphorylation of EGFRs was achieved only at the 48-hour time point, probably induced by the accumulated endogenously produced decorin. In the decorin-sensitive Saos-2 cells, we observed an immediate increase of phosphorylation

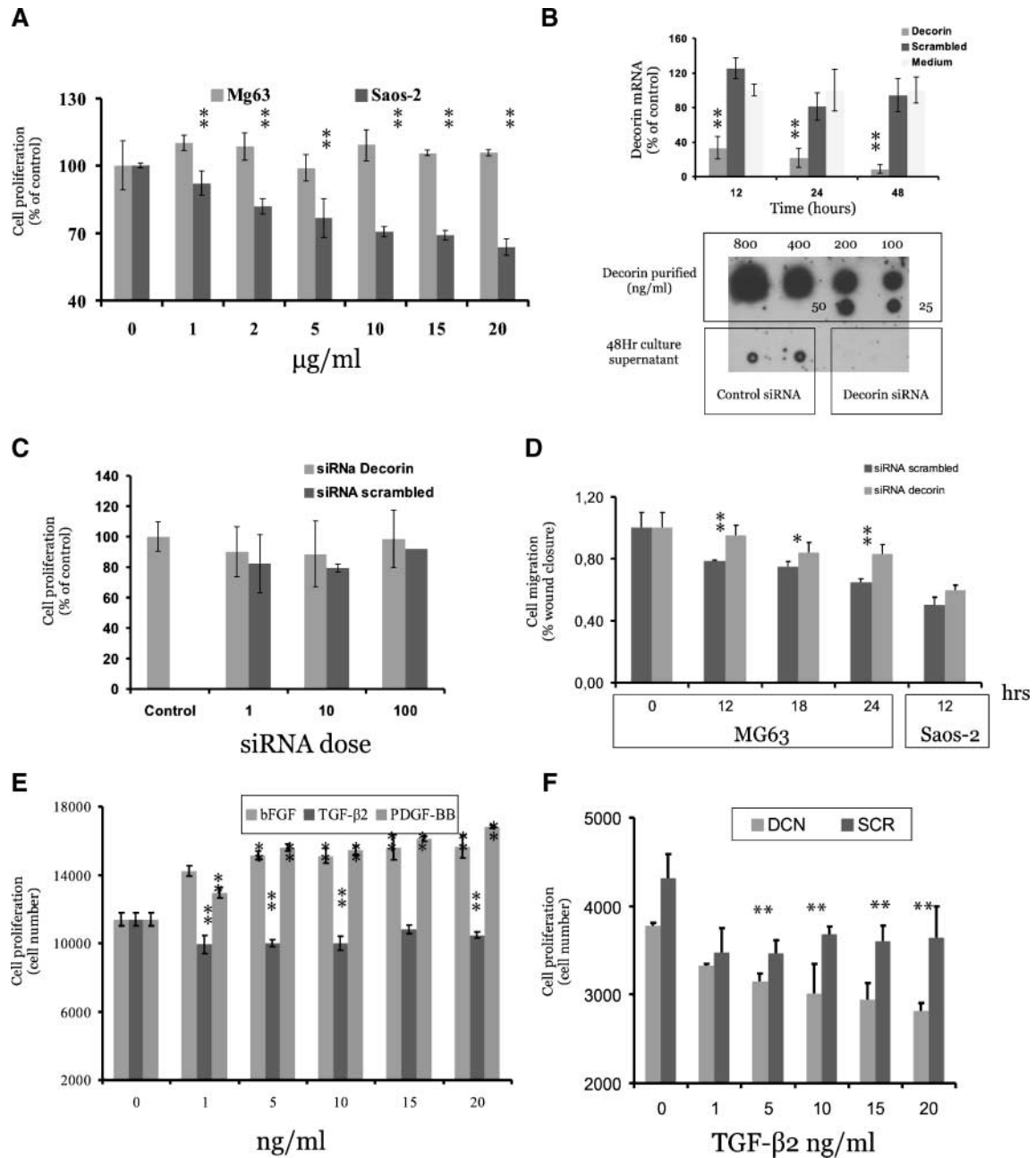


FIGURE 2. **A.** Measurement of the effects of increasing amounts of decorin on the growth of MG-63 and Saos-2 cells. The cell growth changes were measured after a 24-h serum starvation and a 48-h treatment period using a fluorescence-based assay (Cyquant, Molecular Probes, Invitrogen). Growing cells from nonconfluent cultures were harvested and seeded in 96-well plates (Costar) at a density of 10^4 cells per well in 200 μ L of DMEM (10% FBS). The cells were allowed to rest overnight. Prior to stimulation, the cells were incubated in serum-free medium for 24 h. This was then replaced with fresh medium supplemented with TGF- β 2, bFGF, PDGF-BB (10 ng/mL), or decorin (20 μ g/mL). After 48 h of incubation, the cells were lysed and their numbers were calculated using the Cyquant fluorometric assay. A separate standard curve for each cell line was used to convert fluorescence units to cell numbers. All measurements were done in triplicate. **B.** MG-63 cells were transfected with decorin or scrambled siRNA and the levels of decorin mRNA were quantified by real-time PCR in three time points. Columns, percentage of change from the untransfected control culture. siRNA specific for decorin as well as scrambled controls was purchased from Invitrogen. Cells were transfected using the LipofectAMINE reagent (Invitrogen) according to the manufacturer's instructions. **C.** MG-63 cells were transfected for 6 h with increasing amounts of decorin or scrambled siRNA and then cultured for an additional 42 h before cell numbers were measured with the Cyquant method (Cyquant, Molecular Probes). Columns, percentage of change from the control culture. **D.** Cells were transfected with decorin or scrambled siRNA and measured for their ability to restore the inflicted wound *in vitro*. Growing cells from nonconfluent cultures were harvested and seeded at 10^5 /well in 24-well culture plates and cultured in DMEM supplemented with 10% FBS overnight. The cells were serum-starved for 24 h, transfected with siRNA (scrambled control or decorin), and the cell layer was wounded by scratching with a sterile 10 μ L pipette tip. Detached cells were removed by washing twice with media. The wound closure in serum-free media was monitored using a digital image processor connected to a microscope at six different positions across the wound and each treatment was measured in triplicate. The wound area was quantified by digital image analysis (ImageJ 1.4.3.67 Launcher Symmetry Software). **E.** Serum-starved MG-63 cells were treated for 48 h with increasing amounts of PDGF-BB, TGF- β 2, and bFGF and their growth was measured using the fluorescence-based assay. **F.** Analysis of MG-63 growth after transfection with decorin (DCN) or scrambled (SCR) siRNA and treatment with increasing amounts of TGF- β 2. Columns, mean of triplicate values; bars, SE; **, $P < 0.01$.

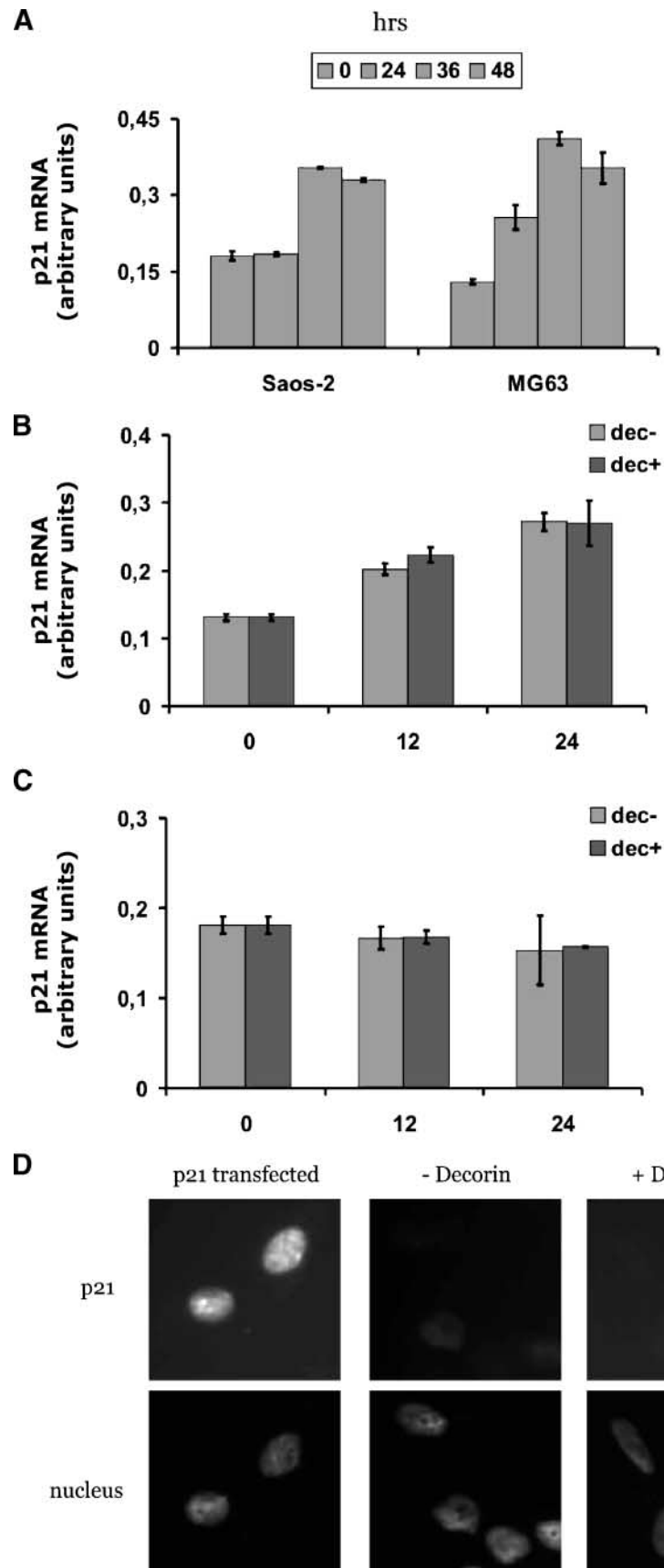


FIGURE 3. Analysis of p21 mRNA expression. **A.** Saos-2 and MG-63 cells were cultured *in vitro* in the absence of serum for 48 h and their mRNA analyzed at several time points by real-time PCR (as described in Fig. 1). Analysis of p21 mRNA expression in MG-63 (**B**) and Saos-2 (**C**) cells after *in vitro* treatment with decorin (20 μ g/mL) in the presence of serum. Columns, mean of triplicate values; bars, SE. **D.** MG-63 cells were analyzed for p21 protein expression by confocal microscopy after decorin treatment (+Decorin) or not (-Decorin) for 24 h using transfected p21-overexpressing cells as positive controls. For confocal laser microscopy, cells were grown and treated on sterile coverslips, blocked, and stained for p21 expression (primary anti-p21, secondary Invitrogen Alexa 488) using Hoechst as a counterstain for nuclear visualization. The coverslips were mounted (ProlongGold, Invitrogen) and examined with a confocal laser scanning microscope (TCS NT, Leica) equipped with an ArKr laser.

after 30 minutes of decorin treatment and a subsequent protracted retraction of the phosphorylated receptor (Fig. 4D and G), in agreement with previously published data (13, 34). Because it has been established that decorin-induced down-regulation of EGFR expression and signaling is responsible for the growth-inhibiting effects of decorin, it can be deduced that the observed protracted expression and phosphorylation of EGFR are related to the insensitivity of MG-63 cells to the decorin-induced growth arrest.

The Effects of Decorin on Osteosarcoma Cell Proliferation and Migration Require Active EGFR Signaling

Both decorin and EGF deliver signals using the EGFR signaling cascade. To analyze, in more detail, the functional relationship of decorin and EGFR in MG-63 versus Saos-2 cells, we employed a specific pharmacologic EGFR inhibitor. Gefitinib is a well-described specific inhibitor of EGFR phosphorylation (35). Indeed, our pilot experiments showed that gefitinib could dose-dependently inhibit the phosphorylation of

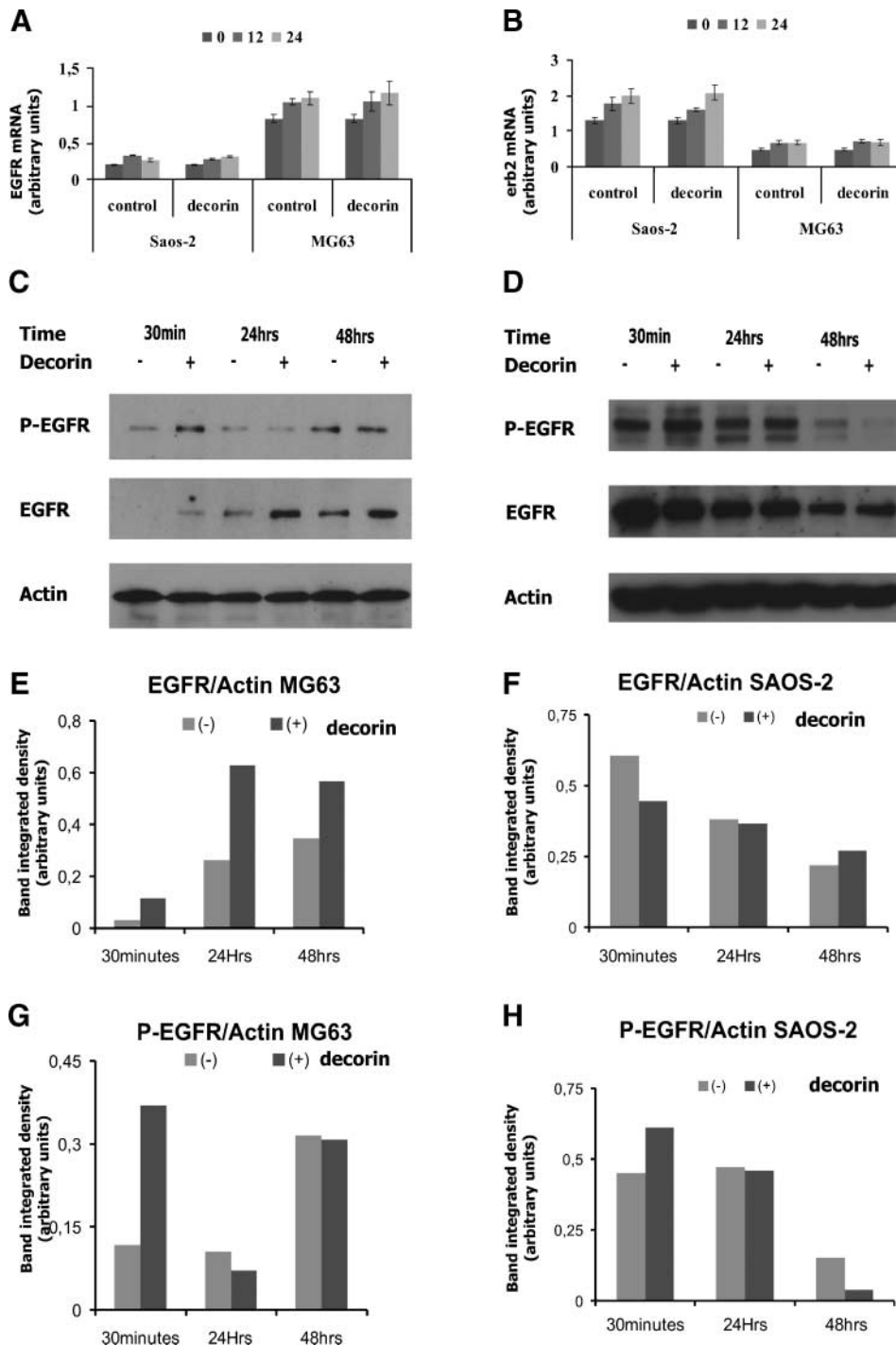


FIGURE 4. EGFR expression and phosphorylation. Analysis of EGFR (A) and erb2 (B) mRNA expression in MG-63 and Saos-2 cells by real-time PCR (described in Fig. 1). mRNA expression was measured in the presence or absence of decorin (20 μ g/mL) after 0, 12, and 24 h of treatment. For the analysis of EGFR phosphorylation, MG-63 (C) and Saos-2 (D) cells were treated with decorin (20 μ g/mL) for 12, 24, and 48 h. The cell content of EGFR, phosphorylated EGFR, and actin was visualized by Western immunoblotting analysis. The films were digitally analyzed to quantify the resulting bands and used to calculate the ratios of EGFR/actin (E and F) and phosphorylated EGFR/actin (G and H) in MG-63 and Saos-2 cells, respectively.

EGFR in both cell lines (Fig. 5A and B) without any macroscopically detected cell toxicity. Both osteosarcoma cell lines, when treated with EGF, were shown to proliferate faster and increase cellular motility (Fig. 5C-F). When comparing the effects of decorin and EGF on cell growth, it should be noted that in Saos-2 cells, they cause opposite effects which are both dependent on EGFR signaling (Fig. 5C). In MG-63 cells, although EGF increased cell growth, decorin had no effect (Fig. 5D). When analyzing the effects of EGF on cell motility, we found that in Saos-2 cells, EGF caused a minor increase of cell motility which was completely inhibited by gefinitib. In MG-63 cells, the effect of EGF on cell motility was more pronounced. It should be noted that the untreated MG-63 cells showed a relatively high basal level of motility which was also dependent on active EGFR signaling, indicating that it might be caused by the increased levels of decorin which these cells produce. Concurring with this, the basal level MG-63 cell motility could be abolished with the inhibition of decorin synthesis and secretion by siRNA (Fig. 2D).

A major finding of our experiments was the absence of decorin-induced protracted retraction of EGFR in MG-63 cells. When comparing the effect of decorin and EGF on the levels of EGFR in MG-63 cells (after 24-48 hours of treatment), we reported a striking difference. EGF abolishes EGFR expression whereas decorin, although it uses the same receptor, has the opposite effect (Fig. 5G). The reduction of EGFR receptors by EGF has been previously described in detail (36). Specifically, it has been shown that upon EGF binding, EGFR is endocytosed (37), ubiquitinated, and marked for destruction in the lysosome (38). On the other hand, decorin seems to bind EGFR and to increase its expression over time. When assessing the effect of decorin on EGFR levels in the presence of gefinitib, we found that EGFR expression increased equally efficiently (Fig. 5H), establishing that the decorin-induced increase of EGFR levels did not require active EGFR signaling.

Conclusions

Decorin is a multifunctional molecule of the extracellular matrix. Among the multitude of its assigned functions, the most intriguing is the ability to inhibit the growth and the metastasis of a wide range of cancer cells *in vitro*. A very elegant set of experiments by Iozzo et al., working in human squamous carcinoma cells *in vitro* and *in vivo*, has recently established that decorin directly interacts with EGFR (12-14) and erb2 (15), inducing protracted receptor internalization (39) which results in the attenuation of receptor-mediated intracellular signaling (34) and induction of apoptosis (18). The present study describes the first exception to the established model examining human osteosarcoma cells. MG-63 cells constitutively produced decorin and they were not sensitive to decorin-induced growth arrest. On the contrary, decorin seemed to be beneficial to osteosarcoma cells because it was necessary for MG-63 cell migration and acted as a mediator, counteracting the TGF- β 2-induced cytostatic function. The attempt to determine how MG-63 cells can overcome the decorin-induced cytostatic effect established that decorin in MG-63 cells did not induce p21 expression, nor did it cause protracted retraction and

inactivation of the EGFR. Conversely, EGFR seemed to be overexpressed and continuously phosphorylated. Furthermore, we have shown that the mechanism resulting in increased EGFR levels did not require active EGFR signaling, therefore raising the possibility that it might be achieved by the interaction and stabilization of extracellular decorin and EGFR. The exact mechanism by which decorin prevents the ubiquitination and lysosome-dependent destruction of EGFR, which has been described in detail for the EGF signaling pathway, needs to be addressed in further studies. In view of the proposed design of decorin-based anticancer therapeutic strategies (20-23), our study provides new data on pathways that cancer cells might employ to overcome the established decorin-induced growth suppression. The identification of such pathways is critical in order to potentially improve the strategy design and maximize its therapeutic efficacy.

Materials and Methods

Materials

Monoclonal antibodies against the protein core of decorin as well as antimouse secondary antibody were purchased from Seigakaku. Anti-p21 was purchased from Santa Cruz Biotechnology. Fetal bovine serum, DMEM, penicillin, streptomycin, and L-glutamine were all obtained from Biochrom KG. DEAE-Sephacel and Decorin were purchased from Sigma Chemical, Co. TGF- β 2, PDGF-BB, and bFGF were from R&D Diagnostics (Invitrogen). All other chemicals used were of the best available grade.

Cell Culture

Human periodontal ligament osteoblastic fibroblasts were obtained from explant cultures of PDL tissues. MG-63 and Saos-2 were obtained from AATC. All cell lines were grown at 37°C in a humidified atmosphere of 5% CO₂ (v/v) in DMEM supplemented with 10% fetal bovine serum, 4 mmol/L of L-glutamine, 2 g/L of sodium bicarbonate, 100 IU/mL of penicillin, and 100 μ g/mL of streptomycin. Prior to stimulation with growth factors, cells were serum-starved for 24 h. TGF- β 2, bFGF, and PDGF-BB, at the final concentration of 10 ng/mL, were added in fresh serum-free (MG-63) medium. Proteoglycans were extracted after 48 h of growth factor treatment whereas mRNA were extracted after 24 h.

RNA Isolation and Real-time PCR

Total RNA was isolated from 1.2×10^6 cells by the TRIzol method (Invitrogen) according to the manufacturer's instructions. The yield and the purity of the RNA preparations were estimated by measuring the A260/A280 ratio. One microgram of total RNA was used for cDNA synthesis using the ThermoScript RT-PCR System (Invitrogen) according to the manufacturer's instructions. Specific PCR primers for the respective targets were selected (glyceraldehyde-3-phosphate dehydrogenase forward 5'-GGAAGGTGAAGGTCGGAGTCA-3' and reverse 5'-GTCATTGATGGCAACAATATCCACT-3'). The glyceraldehyde-3-phosphate dehydrogenase mRNA was used as a normalizer for the cell content. For the real-time PCR reaction, we used the QuantiTech SYBR Green master mix (Qiagen). The real-time PCR reactions were carried out in an ABI 7000 cyler

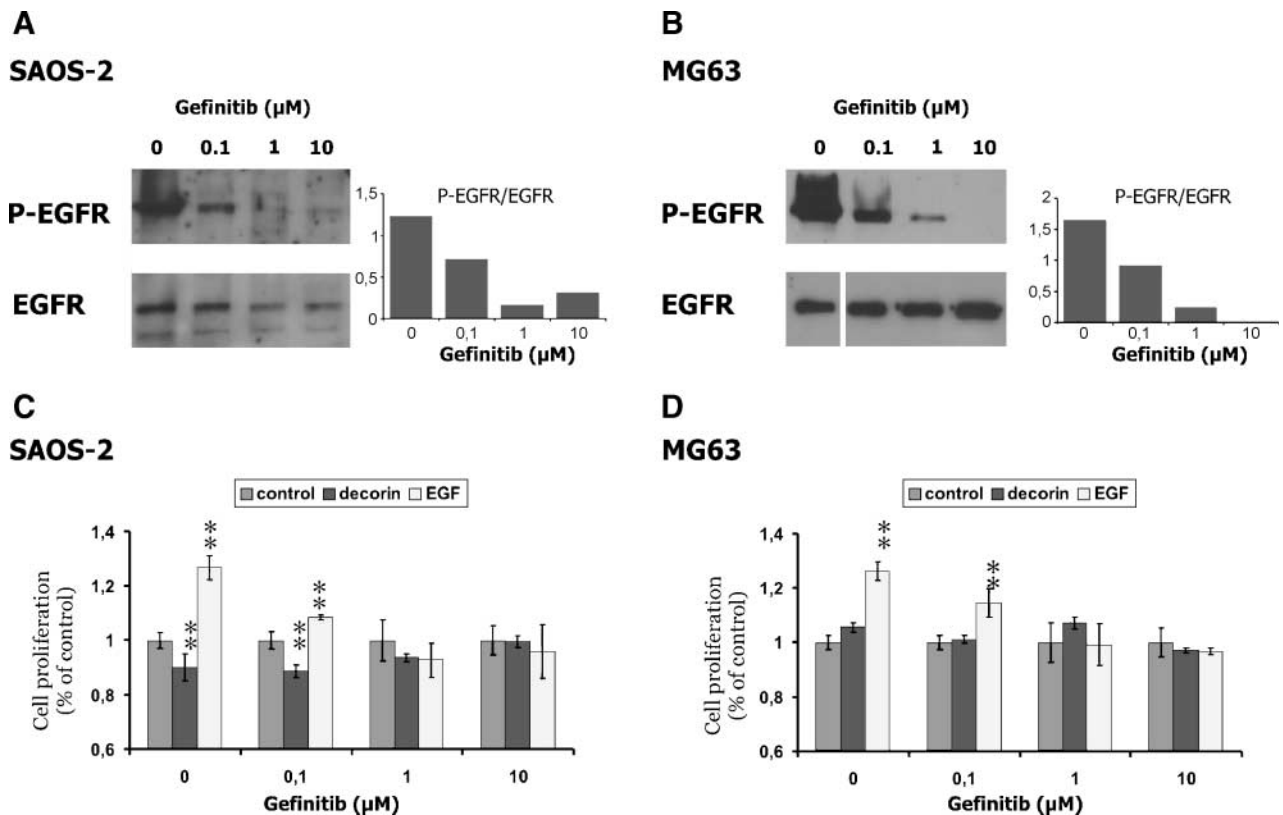


FIGURE 5. Comparative analysis of EGF and decorin function on MG-63 and Saos-2 cells. Serum-starved Saos-2 (**A**) and MG-63 (**B**) cells were treated with EGF (50 ng/mL) for 30 min in the presence of increasing amounts of gefitinib. The cells were lysed and equal amounts of protein/lysate were analyzed for phosphorylated EGFR versus the total EGFR by Western blotting. The bands were quantified using image analysis software (ImageJ 1.4.3.67 Launcher Symmetry Software) and the ratio of phosphorylated/total EGFR is presented as a bar chart next to each gel. Saos-2 (**C**) and MG-63 (**D**) cells were serum-starved for 24 h in 96-well culture plates and treated with decorin (20 $\mu\text{g/mL}$) or EGF (50 ng/mL) for 48 h in the presence of increasing amounts of gefitinib in serum-free medium. The total cell number, after the treatment, was measured using a fluorescence-based assay (Cyquant, Invitrogen). Saos-2 (**E**) and MG-63 (**F**) cells were treated with EGF (50 ng/mL) and gefitinib (10 μM) in serum-free media and the changes of the wounding area were measured during 24 h as described in the wound-healing assay of Fig. 2D. The presented values represent the percentage change from the wounding area at the time of wounding. Columns, mean of triplicate values from the proliferation and wound healing assays; bars, SE; *, $P < 0.05$; **, $P < 0.01$. **G.** Western analysis of the EGFR levels in MG-63 cells after 24 to 48 h of treatment with EGF (50 ng/mL) or decorin (20 $\mu\text{g/mL}$) in serum-free media. **H.** Western analysis of the EGFR levels in gefitinib-treated (10 μM) MG-63 cells with or without decorin (20 $\mu\text{g/mL}$) in serum-free medium for 24 to 48 h. **G** and **H**, columns, the ratio of EGFR/actin measured after digital image analysis (bottom).

and all measurements were done in triplicate. A melting curve analysis was employed to verify the presence of correct product through comparison with the predicted melting point temperature. Finally, we did an extension step at 72 °C for 10 min and the PCR products were visualized by agarose electrophoresis and ethidium bromide staining to verify the correct size of each product.

Isolation and Purification of Secreted Decorin

Culture supernatants from a 75 cm² flask were concentrated by passing them through Viva spin columns with a 10 kDa cutoff (Sartorius) and transferred to 0.01 mol/L of formamide and 0.05 mol/L of sodium acetate buffer containing protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 5 mmol/L NEM, 5 mmol/L benzamide, and 1% sodium cholate). Proteoglycans present in the extract and the cell medium were then separated by ion exchange chromatography on DEAE-Sephacel columns. Proteoglycans were eluted by 1.0 mol/L of NaCl in formamide buffer (17) and precipitated with four volumes of ethanol.

SDS-PAGE and Western Blotting

Following the respective treatments, cells were lysed with 50 mmol/L of Tris-HCl, 0.5 mol/L of EDTA, 1% Triton X-100, 0.1% NaCl, and protease/phosphatase inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 5 mmol/L NEM, 5 mmol/L benzamide, 1% sodium cholate, and 1 mmol/L orthovanadate). The protein content of the cell lysates was determined by the bicinchoninic acid protein assay kit (Pierce). Cell extracts or purified proteoglycans were electrophoresed on 8% or 10% polyacrylamide gels. Transfer to nitrocellulose membranes was done in 10 mmol/L CAPS (pH 11) and 10% methanol, using the EC140 Mini Blot Module (ThermoEC). The membranes were blocked with PBS containing 0.1% Tween 20 (PBS-T) and 5% (w/v) low-fat milk powder, O/N at 4 °C. Incubation with primary monoclonal antibody diluted in PBS-T and 2% (w/v) low-fat milk powder was done for 1 h at room temperature. The immune complexes were visualized with the SuperSignal West Pico Chemiluminescent substrate (Pierce), according to the manufacturer's instructions.

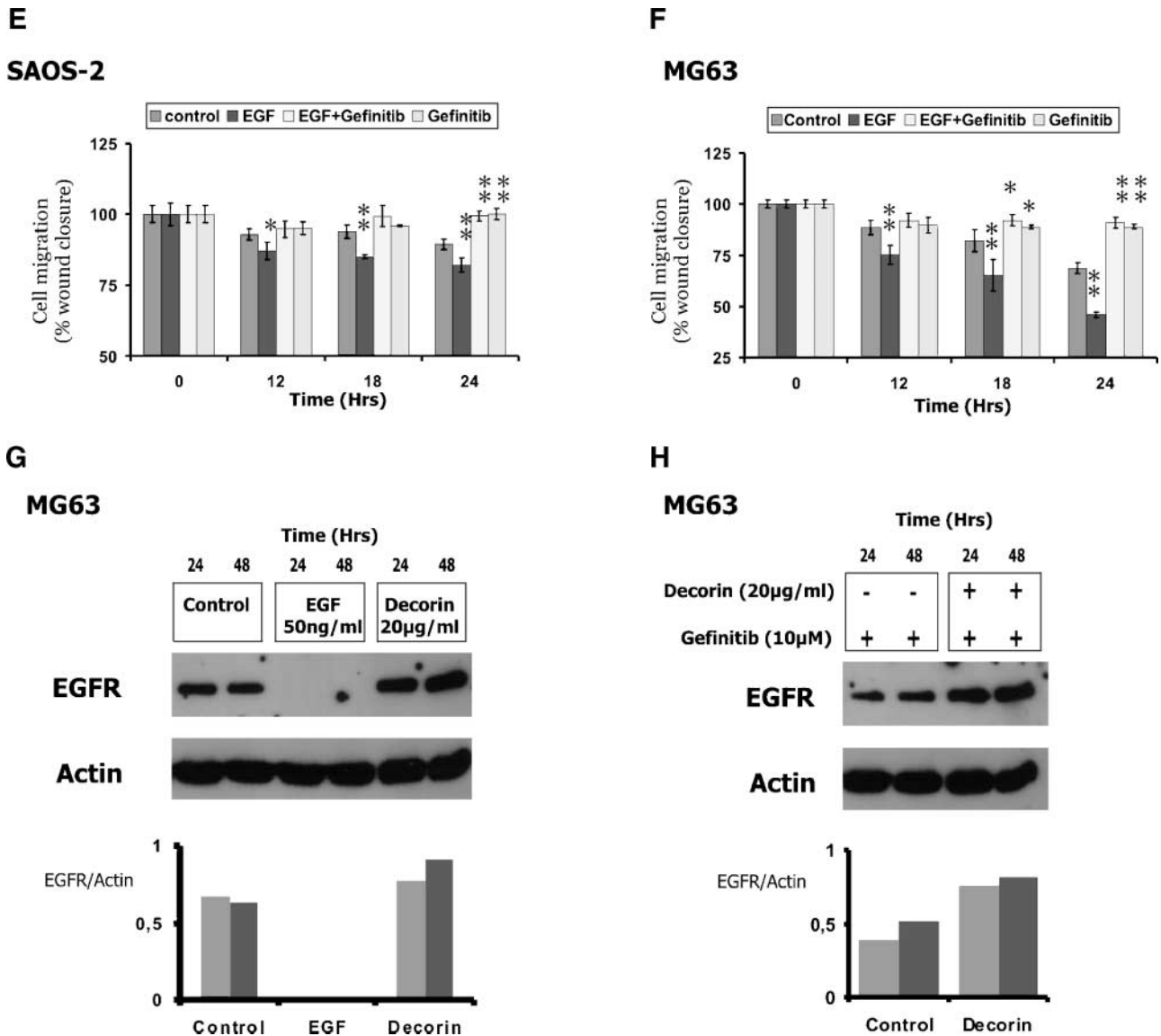


FIGURE 5 Continued.

For the dot blot analysis, MG-63 cells were transfected with decorin and scrambled control siRNA and cultured for 48 h. The culture supernatant was harvested and 30 µL of the supernatant was spotted in duplicate on nitrocellulose membrane using a dot blot device (Bio-Dot Apparatus; Bio-Rad). On the same membrane, a standard curve was included using purified decorin diluted in culture medium (spotted 30 µL of standard dilutions, 800-25 ng/mL). Blocking, incubation with antibodies, and visualization were done as previously described for Western blotting.

Transfection

siRNA specific for decorin as well as scrambled control was purchased from Invitrogen. Cells were transfected using the LipofectAMINE reagent (Invitrogen) according to the manufacturer's instructions.

Proliferation Assay

Growing cells from nonconfluent cultures were harvested and seeded in 96-well plates (Costar) at a density of 10⁴ cells per well in 200 µL of DMEM (10% fetal bovine serum). The cells were allowed to rest overnight. Prior to stimulation, the cells were incubated in a serum-free medium for 24 h. This was then replaced with fresh medium supplemented with TGF-β2, bFGF, PDGF-BB (10 ng/mL), or decorin (20 µg/mL). After 48 h of incubation, the cells were lysed and their number was calculated using the Cyquant fluorometric assay (Molecular Probes, Invitrogen) according to the manufacturer's instructions. Fluorescence was measured in a Fluorometer (Biotek) using the proposed excitation and emission filters. A separate standard curve for each cell line was used to convert fluorescence units to cell numbers. All measurements were done in triplicate.

Wound Healing Assay

Growing cells from nonconfluent cultures were harvested and seeded in 24-well culture plates 10^5 cells/well to produce overnight confluent layers in DMEM supplemented with 10% fetal bovine serum. The cells were starved for 24 h in DMEM without serum. The cell layer was wounded by scratching with a sterile 10 μ L pipette tip. Detached cells were removed by washing twice with media. The cells were treated with decorin (20 μ g/mL) in serum-free medium. The wound closure was monitored at six different positions across the wound using a digital image processor connected to a microscope and image analysis software (ImageJ 1.4.3.67 Launcher Symmetry Software).

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