Uteroglobin (UG) Suppresses Extracellular Matrix Invasion by Normal and Cancer Cells That Express the High Affinity UG-binding Proteins*

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Gopal C. Kundu, Asim K. Mandal, Zhongjian Zhang, Giuditta Mantile-Selvaggi, and Anil B. Mukherjee‡

From the Section on Developmental Genetics, Heritable Disorders Branch, NICHD, National Institutes of Health, Bethesda, Maryland 20892-1830

Uteroglobin (UG) is a steroid-inducible, multifunctional, secreted protein with antiinflammatory and antichemotactic properties. Recently, we have reported a high affinity UG-binding protein (putative receptor), on several cell types, with an apparent molecular mass of 190 kDa (Kundu, G. C., Mantile, G., Miele, L., Cordella-Miele, E., and Mukherjee, A. B. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 2915–2919). Since UG is a homodimer in which the 70 amino acid subunits are connected by two disulfide bonds, we sought to determine whether UG monomers also interact with the 190-kDa UG-binding protein and if so, whether it has the same biological activity as the dimer. Surprisingly, we discovered that in addition to the 190-kDa species, another protein, with an apparent molecular mass of 49 kDa, binds reduced UG with high affinity and specificity. Both 49- and 190-kDa proteins are readily detectable on nontransformed NIH 3T3 and some murine cancer cells (e.g. mastocytoma, sarcoma, and lymphoma), while lacking on others (e.g. fibrosarcoma). Most interestingly, pretreatment of the cells, which express the binding proteins, with reduced UG dramatically suppresses extracellular matrix (ECM) invasion, when such treatment had no effect on fibrosarcoma cells that lack the UG-binding proteins. Tissue-specific expression studies confirmed that while both 190- and 49-kDa UG-binding proteins are present in bovine heart, spleen, and the liver, only the 190-kDa protein is detectable in the trachea and in the lung. Neither the 190-kDa nor the 49-kDa protein was detectable in the aorta. Purification of these binding proteins from bovine spleen by UG-affinity chromatography and analysis by SDS-polyacrylamide gel electrophoresis followed by silver staining identified two protein bands with apparent molecular masses of 40 and 180 kDa, respectively. Treatment of the NIH 3T3 cells with specific cytokines (i.e. interleukin-6) and other agonists (i.e. lipopolysaccharide) caused a substantially increased level of 125I-UG binding but the same cells, when treated with platelet-derived growth factor, tumor necrosis factor-α, interferon-γ, and phorbol 12-myristate 13-acetate, did not alter the UG binding. Taken together, these findings raise the possibility that UG, through its binding proteins, plays critical roles in the regulation of cellular motility and ECM invasion.

Some of the most important biological processes, such as inflammation, wound healing, embryo implantation, and cancer cell metastasis, involve cellular migration and invasion of the extracellular matrix (ECM). However, the mechanisms that regulate these processes are not clearly understood. Recent studies have focused on understanding the receptor-mediated pathways that play critical roles in regulating cellular motility and invasion of the ECM.

More than three decades ago, a steroid-inducible secreted protein in the uterus of pregnant rabbits was discovered and was named blastokinin by Krishnan and Daniel (1) and uteroglobin (UG) by Beier (2). Several years later it was recognized that this protein, detectable in nearly all vertebrates, inhibits phospholipase A2 (EC 3.1.1.4) activity (3–7). It is now recognized as one of the most potent endogenous immunomodulatory/antiinflammatory agents produced by the secretory epithelia of all organs that communicate with the external environment (8). While UG was first discovered in the rabbit uterus (1, 2), it is also expressed in many extraterine tissues including the thymus, pituitary gland, the respiratory and gastrointestinal tracts, pancreas, mammary gland, prostate, and the seminal vesicle (9). UG is also present in the blood (10, 11) and in urine (12), although it is not found in the kidney. Because of its variation in tissue of origin and multifunctional nature, this protein is known by several different names, such as the progesterone-binding protein (13), Clara cell 10 kDa (CC10 kDa) protein (14, 15), urine protein-1 (12, 16–18), polychlorinated biphenyl-binding protein (19), and retinol-binding protein (20).

Several years ago, we reported the presence of a UG-like protein in the human uterus, lungs, and the prostate (21) and subsequently, the human UG cDNA (22) and the gene (23, 24) were cloned and characterized. Additionally, cloning of the cDNAs and genes encoding UG in the mouse (7, 25), rat (26), and the hamster (27) has demonstrated a remarkable structural similarity of this gene in various species. Moreover, the results of immunohistochemical analyses suggested that a protein immunoreactive to UG antibody is expressed in the mucosal epithelial cells of virtually all vertebrate classes (24), raising the possibility that this protein plays important physiological roles.

The UG gene consists of three exons, two introns, and a 5′-flanking region containing several steroid hormone-response elements, which regulate its differential, tissue-specific expres-

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‡ To whom all correspondence should be addressed: Section on Developmental Genetics, National Institutes of Health, Bldg. 10, Rm. 9S241, Bethesda, MD 20892-1830. Tel.: 301-496-7213; Fax: 301-402-6632; E-mail: mukherja@cc1.nichd.nih.gov.

** The abbreviations used are: ECM, extracellular matrix; UG, uteroglobin; hUG, human UG; DSS, dextran sodium sulfate; IFN-γ, interferon-γ; LPS, lipopolysaccharide; PAGE, polyacrylamide gel electrophoresis; HBSS, Hanks’ balanced salt solution; PBS, phosphate-buffered saline; Fn, fibronectin.
sion. For example, while progesterone up-regulates UG production in the uterus, its induction in the fallopian tubes is dependent upon estrogen. Prolactin appears to enhance the progesterone-induced UG gene expression in the uterus (28, 29). Moreover, it is now clear that prolactin transcriptionally augments progesterone-induced UG gene expression in the endometrium via a specific protein that binds at the 5′-flanking region of the UG gene (30, 31). In the prostate and in the seminal vesicle, the UG gene is regulated by testosterone, while in the lungs it is constitutively expressed, although a 3-fold stimulation of expression by glucocorticoid treatment of rabbits has been reported. Recently, it has been found that administration of IFN-γ to mice stimulates UG production in the lungs and an IFN-γ response element has been identified in the 5′-flanking region of the mouse UG promoter (32). Furthermore, it has been reported that treatment of cultured human bronchial epithelial cells with IFN-γ induces UG mRNA and protein (33). These results further suggest a potential role of UG in the regulation of immunological/inflammatory processes.

Both natural and recombinant human UGs have an anamnous electrophoretic mobility in that they migrate like a 10-kDa protein in SDS-PAGE, when their calculated molecular mass is 15.8 kDa. We have recently achieved a high level expression of recombinant rabbit and human UGs (34, 35) in Escherichia coli that facilitated the comparison of their structural features as determined by x-ray crystallography (36) and by multidimensional NMR (37). The crystallographic structure of the recombinant human UG has been further refined at 2.2-Å resolution. The results of these and other studies (36) showed that rabbit and human UGs are indistinguishable proteins both structurally and functionally (34, 35), although there is only 68% amino acid sequence identity between them. A highly allergenic protein, FeldII, found in domestic cats, that has local amino acid sequence similarity to UG, has been reported (38).

Recently, a mammary gland protein, mammaglobin, was also found to have some sequence similarity with that of UG and reported to be overexpressed in human breast cancer cells (39). Structural similarities of UG with a sperm-binding protein found in the rat seminal vesicles (40), C2 chain of the rat prostate steroid-binding protein (41), colicin A (42), and the CAP domain of haloalkane dehalogenase (43) have been reported. The significance of these sequence similarities remains unclear.

The structural features of a protein may explain, in part, its chemical and biological properties (44). Thus, several groups of investigators have focused on delineating the quaternary structures of the rabbit UG (45–48). The x-ray diffraction studies (49–53) resolved the structural features of several crystal forms of rabbit UG. The results showed that UG is a homodimer in which each monomer consists of 70 amino acids. The two monomers are oriented in an antiparallel fashion and are covalently connected by two disulfide bonds (Cys\(^{a} \rightarrow \) Cys\(^{b}\) and Cys\(^{c} \rightarrow \) Cys\(^{d}\)). The 70 amino acids in each of the monomers form four α-helices, and there is a β-turn between α-helices 2 and 3. The appearance of the UG dimer is globular and contain a relatively large central hydrophobic cavity. Several Van der Walls interactions and hydrogen bonding exist between the two monomers and the dimer is stabilized by carboxymethylolation of the disulfide bonds (45). It is now clear that the α-helix-3 of UG, from all species studied so far, are exposed to the solvent and has the potential to interact with other molecules (e.g. binding proteins). Interestingly, Robinson et al. (54) have reported an active transport of UG from the endometrium of the uterus into the blastocele of the preimplantation rabbit embryos, raising the possibility that a UG-binding protein (carrier) may be present on the trophoblasts. We have recently reported that UG binds to a protein with a molecular mass of 190 kDa, on several cell types, including the trophoblasts with high affinity and specificity (55).

Here, we report that (a) using reduced \(^{125}\)I-labeled human UG (hUG) and affinity cross-linking with disuccinimidyl suberate (DSS) we identified a UG-binding protein with a molecular mass of 49 kDa, in addition to the previously reported 190 kDa species; (b) purification of the binding proteins from bovine spleen by UG affinity chromatography and analysis by SDS-PAGE and silver staining identified two proteins with apparent molecular masses of 40 and 180 kDa, respectively; (c) both of these proteins are readily detectable in the heart, and the liver but only the 190-kDa protein band is detectable in the trachea and the lung, while in the aorta they are totally absent; (d) treatment of the NIH 3T3 cells with specific cytokines (e.g. interleukin-6) and other agents (e.g. lipopolysaccharide) appreciably enhances the intensity of radioactive UG-binding protein bands while treatment of the same cells with platelet-derived growth factor, tumor necrosis factor-α, IFN-γ, and phorbol 12-myristate 13-acetate failed to do so; and (e) both nonreduced and reduced UG dramatically suppressed the invasion of ECM by NIH 3T3, murine mastectomy, sarcoma, and lymphoma but not of fibrosarcoma cells. Interestingly, while mastectomy, sarcoma, and lymphoma cells appeared to express the UG-binding proteins, they were undetectable in fibrosarcoma cells. Taken together, these results raise the possibility that hUG plays critical roles in regulating cellular invasiveness of both normal and some cancer cells, at least in part, via its specific, high affinity binding proteins.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant hUG was expressed in E. coli and purified to homogeneity according to the method of Mantile et al. (35). DSS was purchased from Pierce. BioCoat Matrigel™ invasion chambers were from Collaborative Biomedical.

**Cell Cultures**—The cells (NIH 3T3, mouse mastectomy, sarcoma, lymphoma, and fibrosarcoma) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Life Technologies, Inc.), 100 units/ml penicillin, 100 μg/ml streptomycin, and 10 μg/ml of glutamine in a humidified atmosphere of 5% CO\(_2\) and 95% air at 37 °C.

**ECM Invasion Assay**—Chemoinvasion assays were performed as described previously (55, 56). Briefly, confluent cells (NIH 3T3, mouse mastectomy, sarcoma, lymphoma, and fibrosarcoma) were harvested with trypsin and EDTA and then centrifuged. The cells were resuspended in Dulbecco’s modified Eagle’s medium/bovine serum albumin. The lower compartment of the invasion chamber was filled with fibroblast-conditioned medium, which was used as a chemottractant. The upper compartment was overlaid with polyethylene pthalate membrane precoated with Matrigel™ basement membrane matrix. The cells (1.6 \( \times \) 10\(^5\)/well) were seeded in the upper compartment of the prehydrated Matrigel-coated invasion chambers in the absence or presence of reduced hUG and incubated at 37 °C for 24 h in a humidified incubator. The cells that invaded the Matrigel and attached to the lower surface of the filter were stained with Giemsa. The upper surface of the filter was scraped with moist cotton swabs to remove Matrigel and nonmigrated cells. The chamber was washed with water, the migrated cells were counted under an inverted microscope, and photomicrographs (120 \( \times \)) were taken by using a Zeiss photomicroscope, Axiovert 405 M.

**Binding Studies**—The UG (20 μg) was radiiodinated using sodium \(^{125}\)I-iodide (carrier and mCi; carrier free) and IDO-DOA-BEADS. The reaction was carried out in 150 μl of PBS, pH 7.4 at 25 °C for 10 min and \(^{125}\)I-UG was purified by Sephadex G-25 spun column chromatography (1200 \( \times \) g for 4 min). The specific activity of purified carrier-free \(^{125}\)I-UG was 25 μCi/μg. The confluent cells (NIH 3T3, mouse mastectomy, sarcoma, lymphoma and fibrosarcoma), in 12-well plates, were washed once with PBS, pH 7.4 and then incubated with varying concentrations of reduced \(^{125}\)I-UG in 1 ml of Hanks’ balanced salt solution (HBSS), pH 7.8.
containing 0.1% bovine serum albumin in the absence or presence of excess unlabelled reduced hUG at room temperature for 2 h. The UG was reduced in presence of 10 mM dithiothreitol at 37 °C for 15 min. The reaction was stopped by rapid removal of unbound 125I-hUG, and the cells were washed three times with PBS, pH 7.4, and solubilized in 1 N NaOH followed by addition of an equal volume of 1 N HCl. The radioactive activity was measured by gamma counter (ICN Biomedicals, model 10/600 plus) with a counting efficiency of approximately 80%. The specific binding was calculated by subtracting the nonspecific binding from the total binding. The binding data were analyzed by Scatchard plot using the LIGAND computer program.

Affinity Cross-linking Experiments—Confluent cells (NIH 3T3, mouse mastocytoma, sarcoma, lymphoma, and fibrosarcoma) grown in six-well plates, were washed with PBS, pH 7.4 and incubated with reduced 125I-hUG (3.0 nM) in 2.0 ml of HBSS, pH 7.6, containing 0.1% bovine serum albumin in the absence or presence of unlabeled reduced UG (1 μM) for 2 h at room temperature. After washing with PBS, the cells were incubated further with 0.20 mM DSS in 2.0 ml of HBSS, pH 7.6, for 20 min. The reaction was terminated by adding 50 mM Tris-HCl buffer, pH 7.5, and cells were scraped, collected by centrifugation at 10,000 × g for 15 min, and lysed in 60 μl of 1% Triton X-100 solution containing 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 2 mM EDTA. The supernatants (30 μl) obtained by centrifugation at 10,000 × g for 15 min were suspended in sample buffer containing 5% β-mercaptoethanol, boiled for 5 min, and electrophoresed on 4–20% gradient SDS-polyacrylamide gel (Bio-Rad). The gels were briefly stained with Coomassie Blue, dried in a Bio-Rad gel dryer, and autoradiographed using Kodak X-Omat AR x-ray film.

Tissue-specific Expression—The membrane samples were prepared from bovine heart, spleen, trachea, lung, liver, and the aorta. The membrane samples containing equal amount of total proteins from different tissues were incubated with reduced 125I-hUG (3.0 nM) in HBSS, pH 7.6, containing 0.1% bovine serum albumin in the absence or presence of unlabeled reduced UG for binding as mentioned above. The samples were cross-linked with DSS, lysed, electrophoresed, and autoradiographed. In separate experiments, the NIH 3T3 cells were treated with different cytokines and other agents. The treated cells were then incubated with reduced 125I-hUG (3.0 nM) in HBSS, pH 7.6, for binding, cross-linked, electrophoresed, and autoradiographed as described above.

UG Receptor Purification—The bovine spleen tissue (10 g) was homogenized in 10 mM NaHCO3 buffer, pH 8.0. The homogenate was centrifuged at 600 × g for 10 min at 4 °C. The supernatant was centrifuged at 24,000 × g for 60 min. The pellets were solubilized with 50 mM Tris-HCl buffer, pH 7.4, containing 1% Triton X-100, 10 mg/ml leupeptin, 2 mM EDTA, and 0.4 mM phenylmethylsulfonyl fluoride by stirring at 4 °C for 6 h. The supernatant was collected by centrifugation at 24,000 × g for 90 min and applied to CNBr-activated Sepharose 4B-coupled UG affinity column. The Sepharose 4B-coupled UG affinity column was prepared according to the instruction of the manufacturer. The UG receptor protein was eluted from the column using 0.1 M glycine HCl, pH 3.0, containing 0.1% Triton X-100, 10 mg/ml leupeptin, 2 mM EDTA, and 0.4 mM phenylmethylsulfonyl fluoride and neutralized immediately with 2 mM Tris-HCl, pH 8.0. The fraction containing the UG-binding proteins was detected by 125I-Ug binding and affinity crosslinking assay. The homogeneity of the purified receptor was checked by SDS-PAGE followed by silver staining (Bio-Rad).

RESULTS AND DISCUSSION

We have previously demonstrated that recombinant human UG in its natural dimeric conformation binds to a protein on human trophoblast cells with a molecular mass of 190 kDa with high affinity and specificity (55). Since UG is a homodimer in which the 70 amino acid subunits are linked by two disulfide bonds, we sought to determine whether both reduced and nonreduced UG interacted with the same 190-kDa UG-binding protein. Thus, we tested both the nonreduced and recombinant human UG on nontransformed NIH 3T3 and cancer cells (mastocytoma, sarcoma, lymphoma, and fibrosarcoma) cell lines. Scatchard analysis of steady state binding of 125I-UG (reduced) indicates the presence of a single class of specific binding with dissociation constant (Kd) of 20 nM using NIH 3T3 cells (Fig. 1). We have also examined the 125I-UHG (reduced) binding on mastocytoma, sarcoma, and lymphoma cells, and the Kd values were 20–25 nM, but there was no such binding when fibrosarcoma cells were used (data not shown). The nonreduced 125I-UHG also bound the receptors with Kd of 30–35 nM in mastocytoma, sarcoma, and lymphoma cells, respectively (data not shown).

For further characterization of the UG binding sites on these cells, we performed affinity cross-linking studies with DSS using 125I-hUG (reduced) in the absence or presence of unlabeled UG. The results uncovered a radiolabeled 49-kDa protein band (Fig. 2) in addition to the previously identified 190-kDa band (55). Both 49- and 190-kDa radioactive protein bands (Fig. 2) were virtually unrecognizable when 1 μM unlabeled human UG was added to the reaction mixture prior to binding and affinity cross-linking (Fig. 2). As expected, in the absence of DSS no protein bands were visible in the autoradiogram (Fig. 2, lane 1). When nonreduced 125I-UHG was used, we detected only a 190-kDa protein band using NIH 3T3 (55), mastocytoma, sarcoma, and lymphoma cells (data not shown). Neither the 49-kDa nor the 190-kDa bands were visualized when fibrosarcoma cells were incubated with nonreduced or reduced 125I-UHG for cross-linking (data not shown).

In an attempt to purify the UG-binding proteins, we used bovine tissues and performed affinity cross-linking to determine if such tissues express these proteins. Like the cell types described above, we detected both the 49- and 190-kDa protein bands in bovine heart, spleen, and the liver, while in the trachea and in the lung only the 190-kDa protein band was detectable. Neither the 190-kDa nor the 49-kDa band was detectable in bovine aorta (Fig. 3). In order to further characterize these proteins, we performed chromatography using a Sepharose-4B-linked human recombinant UG affinity column (see “Experimental Procedures” for details). The solubilized bovine spleen extracts were chromatographed, and the bound proteins were eluted from the column by lowering the pH of the eluting buffer. When the affinity-purified proteins were resolved by SDS-PAGE followed by silver staining, two proteins, each with an apparent molecular mass of 40 and 180 kDa, respectively, were clearly visualized (Fig. 4). Binding and affinity cross-linking experiments showed that both binding proteins interact with 125I-UHG (data not shown). It is not clear at this point whether the two protein bands that bound reduced 125I-human UG are subunits of the UG receptor protein, or if the lower molecular weight form (49 kDa) is a degradation product in which the UG-binding epitope remained intact. The presence of both 190- and 49-kDa bands in the heart, spleen, and the liver and the absence of the 49-kDa band in the trachea suggest that the lower molecular weight form is a degradation product of the 190-kDa protein. If it was a degradation product, under the identical conditions of sample preparation and electrophoresis, we would have detected the 49-kDa band in the trachea and lung as well. Protein microsequencing, cDNA cloning, and characterization will establish which of the two alternatives is correct. In addition to

![Fig. 1. Scatchard plot of specific binding of 125I-UHG (reduced) on NIH 3T3 cells.](Image 370x618 to 492x729)
the two protein bands discussed above, we also noticed a faint band with an apparent molecular mass of 30 kDa. However, this band did not bind $^{125}$I-UG (data not shown), suggesting that this may be an artifact or a degradation product of the 49- or 190-kDa proteins that does not contain the epitope required for UG binding. Taken together, these results suggest that UG binds to several cell types with high affinity and specificity, and this binding is mediated by either one protein with two subunits (49 and 190 kDa) or there are two binding proteins.

The stoichiometry of UG monomers to dimers is not known, although both of these forms have been observed when lung and uterine lavage fluids are resolved by SDS-PAGE under nondenaturing conditions.\footnote{G. C. Kundu, A. K. Mandal, Z. Zhang, G. Mantile-Selvaggi, and A. B. Mukherjee, unpublished results.} UG is a homodimer and theoretically both the reduced and oxidized UG should interact with both the 190- and 49-kDa binding proteins. However, using human trophoblast cells we have previously demonstrated that in these cells only the 190-kDa protein band was cross-linked to radiolabeled dimeric UG (55). In our present study, we find that in some tissues (e.g. heart, spleen, and liver) radiolabeled 190-kDa proteins may be preferentially expressed in a tissue-specific manner. The ongoing efforts to clone and characterize the cDNA(s) and the gene(s) encoding UG-binding proteins are expected to answer these questions.

To determine whether the expression of the UG-binding proteins is inducible and therefore, regulated, we treated the NIH 3T3 cells with various agents that included growth factors, interleukins, and lipopolysaccharide (LPS). Interestingly, when these cells were treated with LPS or interleukin-6 and interleukins, and lipopolysaccharide (LPS). Interestingly, when these cells were treated with LPS or interleukin-6 and affinity cross-linking with $^{125}$I-UG was performed, the intensity of the cross-linked radioactive bands, representing UG-binding proteins, increased considerably compared with the nontreated controls (Fig. 5). Both LPS and interleukin-6 are proinflammatory agents, and since UG is known to possess antiinflammatory properties, the apparent increase of $^{125}$I-UG binding induced by these proinflammatory agents may suggest the existence of a homeostatic mechanism to control the inflammatory response, the dynamics of which is regulated by the interaction of UG with its binding protein(s). This mechanism may be understood more clearly when the UG receptor-mediated cellular signal transduction pathways are studied in detail.

UG possesses potent antiinflammatory and antichemotactic properties (21, 57, 58), and we have previously demonstrated that it binds to a protein with a molecular mass of 190 kDa present on human trophoblast cells (55). We also showed that the ECM invasion by NIH 3T3 and human trophoblast cells was suppressed by UG pretreatment of these cells (55). In the present study we sought to determine (a) whether reduced UG also had the same effect on the cells and if so, (b) whether such effect was dependent upon the presence of the putative UG receptor(s).

The results show that in addition to the nonreduced form, the reduced UG (1 $\mu$m) also suppresses the ECM invasion by NIH 3T3 and human trophoblast cells.
The difference is not apparent when the cells are treated with phorbol and interleukin-6, respectively, compared with the control. However, the UG-binding protein(s) following treatment of the cells with LPS showed a considerable enhancement in intensity of the radioactive bands representing the expression of UG-binding proteins by NIH 3T3 cells.

Hyperplasia is not yet clear. One intriguing possibility is that nephritis, an inflammatory disease (69), and renal parenchymal antiinflammatory protein (58), the development of glomerulonephritis, an inflammatory disease (69), and renal parenchymal fibrosis, a sequelae of the inflammatory process, is understandable. However, the mechanism(s) of distal tubular hyperplasia is not yet clear. One intriguing possibility is that via its binding protein(s), UG may exert an antiproliferative effect on these cell types. This notion is supported by preliminary observations that UG inhibits rat arterial smooth muscle cell proliferation in vitro. All in all, it now appears likely that UG may have some of the properties that are very similar to the proteins of the cytokine/chemokine family, although there are no structural similarities between these proteins. This assumption is supported, at least in part, by the following: (i) UG is a multifunctional secretory protein with a low molecular mass (15.8 kDa), (ii) it circulates in the blood, (iii) it has binding-protein(s) (putative receptor) on several cell types, and (iv) via this putative receptor(s) UG appears to regulate vital cellular functions, such as motility and ECM invasion.

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