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Integrin receptors for anosmin

Cell Adhesion to Anosmin via α5β1, α4β1, and α9β1 Integrins

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ABSTRACT

Anosmin is an extracellular matrix protein, and genetic defects in anosmin result in human Kallmann syndrome. It functions in neural crest formation, cell adhesion, and neuronal migration. Anosmin consists of multiple domains, and it has been reported to bind heparan sulfate, FGF receptor, and UPA. In this study, we establish cell adhesion/spreading assays for anosmin and use them for antibody inhibition analyses to search for an integrin adhesion receptor. We find that α5β1, α4β1, and α9β1 integrins are needed for effective adhesive receptor function in cell adhesion and cell spreading on anosmin; adhesion is inhibited by both RGD and α4β1 CS1-based peptides. This identification of anosmin-integrin adhesion receptors should facilitate studies of anosmin function in cell and developmental biology.

Keywords

α5β1 integrin, α4β1 integrin, α9β1 integrin, anosmin, extracellular matrix, cell spreading

Introduction

Anosmin is an extracellular matrix protein that has multiple roles in cell adhesion and migration, as well as in diseases. Genetic defects in the human anosmin gene can cause Kallmann syndrome.[1-6] Anosmin consists of multiple domains, including a cysteine-rich region, a whey protein domain, and four repeated fibronectin-type III (FnIII) domains. The cell surface receptor that binds anosmin to mediate cell adhesion has not yet been identified. However, anosmin is known to interact with heparan sulfate and urokinase-type plasminogen activator (UPA). Anosmin has been shown to activate the FGF receptor by direct binding[7] and by enhancing dimerization of FGF receptor with FGF8.[8] Since anosmin has four FnIII domains, and some peptides with mutations in FnIII domains could enhance cell adhesion,[9] it is possible that an integrin might serve as a receptor. Recently, Choy et al.[10] reported that human anosmin could induce intracellular signaling by interacting with β1 integrins in human cancer cells, as established by immunoprecipitation and co-immunostaining analyses. Anosmin triggered integrin receptor-mediated signaling activation involving FAK and AKT, but it has not yet been established which β1 integrin(s) could be involved, nor whether one or more integrin receptor actually mediates cell adhesion to anosmin.

Integrins are receptors for a number of other extracellular matrix (ECM) molecules, and they can be crucial for cell survival, proliferation, differentiation, and migration of many cell types. Integrins consist of heterodimers with 24 α and β subunits in humans. They play multiple roles, including mediating cell adhesion and spreading with numerous matrix proteins via specific heterodimers, such as the well-characterized processes of adhesion and downstream signaling via α5β1 binding to fibronectin.[11-23]

Anti-integrin function-blocking antibodies against each subunit have proven to be valuable tools for identifying integrin functional interactions with specific ECM proteins.[24]

In this study, we develop cell adhesion/spreading assays for anosmin and establish α5β1, α4β1, α9β1 integrins as adhesion receptors for anosmin.

Results and Discussion

In order to determine whether integrins serve as adhesion receptors for anosmin, we established a cell adhesion/spreading assay using human cell lines with well-characterized anti-human integrin function-blocking antibodies to identify potential anosmin adhesion receptors. Since the poly-L-lysine substrate used in previous work to study anosmin signaling[25] could by itself induce cell attachment and spreading, we first attempted to apply a standard approach of ligand adsorption to glass or plastic substrates.[26-28]

Recombinant anosmin failed to coat glass and various plastic substrates according to ELISA and SDS-PAGE/western blotting assays. In contrast, GST-tagged anosmin and GST as a control were effectively captured onto anti-GST antibody pre-coated glass chambers or plastic wells (see Experimental Procedures).

Using this assay, we established that anosmin has cell adhesion/spreading activity for both primary human cells (HFF) and fibrosarcoma HT-1080 cells (Figure 1B and E) compared with fibronectin (Figure 1C and F), while control GST had no activity (Figure 1A and D). Cell morphology and phalloidin-stained actin cytoskeleton in cells adhering to anosmin were compared with those on fibronectin. As shown in Figure 1G and H, whereas primary HFF showed classical long, prominent stress fibers of F-actin on fibronectin, on anosmin they showed few intracellular stress fibers and instead

displayed multiple lamellipodia with short filopodial spikes of F-actin (Figure 1G and H), indicating that the cell adhesive response to anosmin is distinct from that of fibronectin. These differences suggest that the cells may be using different migration modes, which would be interesting to examine in the future. Similar comparisons of malignant HT-1080 cells on anosmin versus fibronectin reveal differences that include loss of F-actin stress fibers and more filopodia on anosmin compared to fibronectin (Figure 1I and J).

Next, we searched for co-localization of β1 integrin and paxillin on anosmin by using immunofluorescence staining. Co-localization of β1 integrin and paxillin was observed in HFF on anosmin (Figure 1K, L and M), although the co-localization pattern was not identical on fibronectin (Figure 1N, O, and P). These results indicate that β1 integrin is involved in the cell spreading process, as described previously.[10]

Next, we modified the adhesion assay to enhance its efficiency for multi-well screening. This second-generation assay was based on immobilization of GST chimeras using glutathione-derivatized plates. Another feature of integrin-based cell adhesion involves its requirement for divalent cations. As shown in Fig. 1Q and R, cell adhesion and spreading on anosmin was completely blocked by 5 mM EDTA, consistent with involvement of integrins such as β1 integrin.

First- and second-generation assays provided similar results (compare Figure 2A and B with Figure 1A and B). After this validation of the new assay, we systematically searched for a requirement for one or more integrins by use of well-studied integrin function-blocking antibodies against each of multiple integrin subunits. As shown in Figures 2A and B, we found that α4, α5, and β1 integrin function-blocking antibodies significantly reduced the numbers of cells undergoing full spreading (white), versus

partial spreading (gray), and then versus attached but not spread (black) cells on anosmin (Figure 2B). We additionally found that α9β1 integrin function-blocking antibody significantly reduced the number of cells undergoing partial spreading or full spreading in HFF (Figure 2C) and LN-229 (Figure 2D). Thus, we conclude that α4β1, α5β1, α9β1 integrins are crucial for cell spreading on anosmin, and that these integrins can function as adhesion receptors for anosmin.

Because α5β1 and α4β1 integrins recognize RGD or the CS1 peptide, respectively, we searched for such sequences or related variants in anosmin (Table 1). No matches to the canonical RGD or LDV/CS1[29] sequences were found, though regions of sequence similarity were observed. We could not find an exact match to the RGD sequence anywhere in anosmin. Interestingly, there is good precedent for α5β1 binding with excellent affinity to non- RGD sequences, such as to the peptide CRRETAWAC (Table 2) or to VEGF receptor-1. [30-32] Consistent with this previous work, even though no RGD or homologous sequence was present, the RGD-binding region in this integrin could be involved in such non-RGD recognition.[31]

Consequently, in order to evaluate whether the RGD-binding site of this integrin could be involved in the process of cell spreading on anosmin, we tested inhibition by an RGD peptide. As shown in Figure 3A and B, we found that RGD peptide could significantly inhibit full cell spreading (70% inhibition compared to 88% inhibition by anti-α5 integrin in Fig. 2B*)* on anosmin when compared with the control RGE peptide (Figure 3A, B). These results are quantitatively similar to the inhibitory effects of anti-α5 subunit antibody and provide further support for the role of the α5β1 integrin as a receptor for anosmin. Similarly, treatment with the EILDVPST peptide that is known to inhibit

fibronectin interaction with α4β1 was also inhibitory, mimicking effects of anti-α4 subunit antibody inhibition (Figure 3C and D). We speculate that the RGD and EILDVPST simply occupy the cell-adhesive peptide recognition regions of the α5β1 and α4β1 integrins, respectively, occluding receptor access to anosmin.

Since anosmin has four fibronectin type III (FnIII) domains known to bind to integrins,[33] integrins have been suggested as potential receptors for anosmin. Recently, Choy et al.[10] reported that His- or GFP-tagged human anosmin could interact with the β1 class of integrin according to co-immunoprecipitation and co-immunostaining in the human glioblastoma cell line LN-229. However, whether integrins can function as direct adhesion/spreading receptors for anosmin had not been addressed, as well as which specific integrin heterodimer(s) could be involved in adhesion. We find that the α5β1, α4β1, and α9β1 integrins contribute to cell adhesion to anosmin, whereas the α1, α2, α3, αV, αVβ3, β2, β3, and β4 integrins do not.

We had previously shown that anosmin can enhance BMP5 protein activity and its gene expression during cranial neural crest formation in chicken embryos.[8] Interestingly, we also found subsequently that *BMP5* gene expression can be enhanced by overexpression of chicken α5β1 integrin in the neural ectoderm of chicken embryos.[28] Combining this information with the identification of α5β1 as an anosmin adhesion receptor in this paper, we speculate that anosmin might regulate *BMP5* gene expression through its adhesive interactions with the α5β1 integrin during cranial neural crest formation in chicken embryos – a potential new pathway that would be interesting to examine in future studies.

Materials and Methods

Construction of anosmin-GST and control GST expression vectors

As described previously, chicken anosmin in the pEF6/V5-His vector was digested with *BamH I* and *Pme I*, and anosmin-V5-His was inserted by blunt-end ligation into the *Xho I* site of the pCAGGS vector[34] (pCAGGS-anosmin-V5-His,[8]). To generate a recombinant anosmin protein that could be reliably anchored to a cell culture substrate for adhesion assays, GST was inserted into the *Not I* site located at the C terminus anosmin in the pCAGGS-anosmin-V5-His vector to generate pCAGGS-anosmin-GST-V5-His. For secretion of control GST proteins in conditioned media, a signal peptide sequence (MVSERAPGASLALLLWVTAVSG) derived from chicken anosmin was first cloned in the pEF6/V5-His vector, then GST was inserted into a *Not I* site in the vector (pEF6-S-GST-V5-His). Then, the S-GST in pEF6/V5-His was digested with *BamH I* and *Pme I*, and S-GST-V5-His was inserted by blunt-end ligation into the *Xho I* site of the pCAGGS vector (pCAGGS-S-GST-V5-His). Purification of anosmin-GST or control GST protein was performed exactly as previously described in the Supplemental Experimental Procedures for Endo et al.[8] for CW1-GST protein purification. Briefly, 48 hr-conditioned media from B16-F10 cells (ATCC) transfected with these plasmids using Lipofectamine 2000 (Life Technologies) were loaded onto Glutathione Sepharose 4 Fast Flow columns (GE Healthcare). The columns were washed sequentially with wash buffer 1 (PBS, pH 7.4), wash buffer 2 (PBS, 2 M NaCl, pH 7.4) and wash buffer 3 (PBS, pH 8.0). GST recombinant anosmin or control GST proteins were then eluted with elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, Sigma-Aldrich, pH 8.0). After concentration by Centriprep filtration (Millipore), the purified proteins were dialyzed against PBS, pH

7.4 for two 2-hour periods at 4^{o}C and stored at -80^{o}C after snap-freezing in dry ice. Quality of the purified protein was confirmed by SDS-PAGE.

Cell lines

Primary human foreskin fibroblasts (HFF), human HT-1080 cells, human glioblastoma cells (LN-229, ATCC) and mouse melanoma cells (B16-F10, ATCC) were cultured in DMEM containing 10% FBS, or in 5% FBS for the LN-229 cells. All maintenance cultures also contained 100 U/ml penicillin and 100 μg/ml streptomycin. One day before plasmid transfection, B16-F10 cells were seeded on plastic tissue culture 15 cm-dishes in DMEM containing 10% fetal bovine serum (FBS) without antibiotics. During transfection, these cells were cultured in serum/antibiotic-free DMEM for 5-7 hours. Then, the medium was changed to fresh FBS/antibiotic-free DMEM for 48 hours.

Cell adhesion/spreading assay

Cell adhesion/spreading was assayed as described previously.[26-28] Briefly, Lab-Tek II glass chamber slides (Nalge Nunc International, #154941) were coated with anti-GST monoclonal antibody (2 μg/ml, Sigma) in DPBS at 4^{o}C overnight. After blocking non-specific adsorption sites with 1% heated-denatured BSA in DMEM at room temperature for 1 hr, the chamber slides were coated with 20-50 μg/ml anosmin-GST or control GST protein in 0.1% heated-BSA in DMEM at room temperature for 2 hr, followed by re-blocking with 1% heated-BSA in DMEM at room temperature for 1 hr. Alternatively, the chamber slides were coated with 5-20 μg/ml human plasma fibronectin in Dulbecco's PBS without calcium or magnesium (DPBS) overnight at 4^{o}C . After washing with DPBS, non-specific sites on the substrate were blocked with 1% heat-denatured-BSA in DMEM at 37^{o}C for 1 hour. Human HFF, HT-1080, or LN229 cells were suspended using

trypsin-EDTA and allowed to recover from the enzymatic treatment by incubation for 40 min in 10% FBS / DMEM at 37^{o}C. The cells were then washed three times with serum-free 0.1% heated-denatured BSA in DMEM, and 1x10^{5} cells in 0.1% BSA / DMEM were seeded in the anosmin-GST, control GST, or fibronectin pre-coated glass chamber wells. After incubation for 2 hr at 37^{o}C, the chambers were gently washed twice with DPBS, and adherent cells were fixed in 4% PFA / DPBS at room temperature for 20 min.

For our second-generation cell adhesion/spreading assay, Pierce Glutathione Coated Plates (Thermo Scientific) were coated with 20-50 μg/ml anosmin-GST or control GST in DPBS overnight at 4^{o}C. After washing with DPBS, non-specific sites on the substrate were blocked with 1% heat-denatured BSA in DMEM at 37^{o}C for 1 hour. Human HFF cells were suspended using trypsin-EDTA and allowed to recover from the enzymatic treatment by incubation for 40 min in 10% FBS / DMEM at 37^{o}C. The cells were then washed three times with serum-free DMEM containing 0.1% heat-denatured BSA, and cell concentrations were adjusted to 1x10^{5} cells / ml in 0.1% heated-BSA / DMEM. Depending on the experiments, they were incubated with 5 mM EDTA, 20 μg/ml integrin antibodies, or 2 mg/ml of RGD (H-Gly-Arg-Gly-Asp-Ser-OH) or RGE (H-Gly-Arg-Gly-Glu-Ser-OH) peptides (BACHEM), or 2 mg/ml EILDVPST peptide (BACHEM) derived from the fibronectin CS-1 sequence at 37^{o}C for 20 min or 1 hr, respectively. 2x10^{4} cells were seeded in the anosmin-GST or control GST-coated wells. After incubation for 2 hour at 37^{o}C, the chambers were gently washed twice with DPBS, and adherent cells were fixed in 4% PFA / DPBS at room temperature for 20 min. Adherent cells were counted in randomly selected 0.77 mm^{2} regions for integrin function-blocking

assays (n = 2-3 fields) or 0.57 mm^2 regions for the RGE/RGD peptides (n=3 for RGE, 3 for RGD) or for the EILDVPST peptide (n = 3).

Integrin antibodies

Anti-integrin antibodies, α1 (MAB1973), α2 (MAB1950), α3 (MAB1952), α4 (MAB16983), β2 (MAB1962), β4 (MAB2060), β6 (MAB 2076), and αVβ3 (MAB1976) were purchased from Millipore, α9β1 (Y9A2, ab27947) was from Abcam, and α5 (mAb16) and β1 (mAb13) were from BD Pharmingen; αV was isolated from supernatants of the L230 hybridoma[35,36] obtained from ATCC. For fluorescence immunostaining, anti-β1 integrin and anti-paxillin antibodies were purchased from Abcam.

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Table 1. Comparison of CS1 sequence (DELPQLVTLPHPNLHGPEILDVPST, 25 amino acids) with chicken anosmin Table 1. Regions of homology between the fibronectin CS1 sequence[29] and anosmin.[37] The upper lines indicate the full CS1 sequence with a matching region of weak homology in anosmin that lacks the key functional LDV sequence of fibronectin. The short middle and lower pairs of sequence indicate sequences elsewhere in anosmin with weak homology to short regions of the full-length CS1 sequence.

```
      CS1: DELPQLVTLPHPNLHGPEILDVP
  Anosmin: DLPPFLPHRPHLKQHHPHHYKPP
           *  * *   **   * *     *

      CS1: PEILDVP
  Anosmin: PEEPDIP
           **  * *

      CS1: LPH-PNL
  Anosmin: LPHRPHL
           *** * *
```

Table 2. Comparison of CRRETAWAC with chicken anosmin Table 2. Region of homology between the CRRETAWAC peptide and anosmin.

```
      CS1: CRR--ETAWAC
  Anosmin: ARRQDE-AFST
            **  * *
```

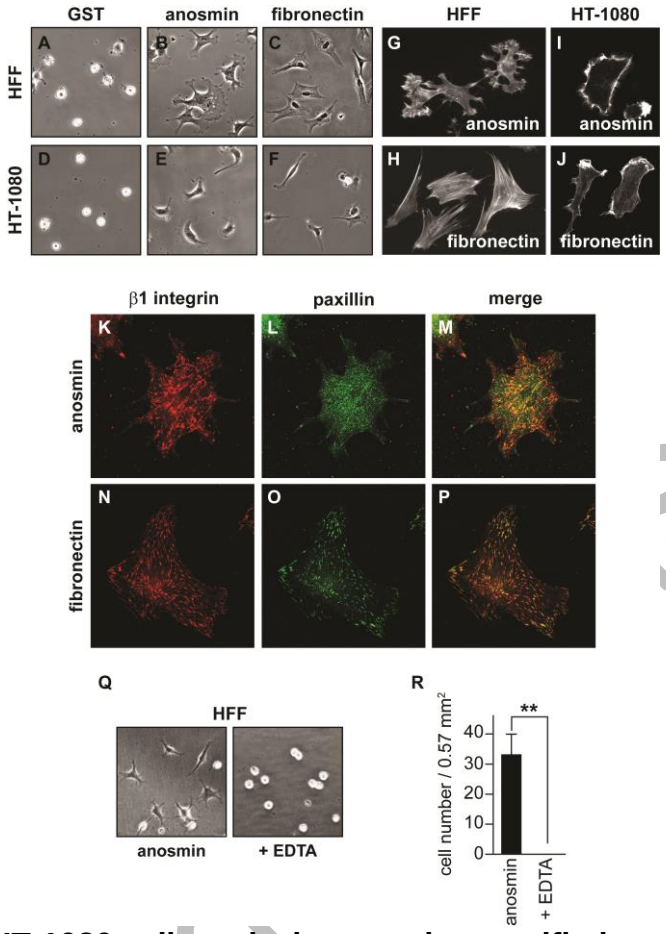

Figure 1. HFF and HT-1080 cells actively spread on purified anosmin

(A-F) HFF or HT-1080 cells were seeded on purified control GST, anosmin, or fibronectin pre-coated glass chamber slides (A and D, B and E, or C and F, respectively) to evaluate cell spreading. Effective cell spreading of both HFF and HT-1080 cells on anosmin are shown in B and E, respectively.

(G, H) HFF cells were seeded on purified anosmin or fibronectin pre-coated glass chamber slides and filamentous (F)-actin was stained by rhodamine phalloidin. HFFs on anosmin had multiple lamellipodia with prominent microspikes or filopodia-like extensions, whereas they displayed prominent stress fibers of F-actin on fibronectin (G and H, respectively). (I, J) HT-1080 cells seeded on purified anosmin or fibronectin

stained by rhodamine phalloidin. HT-1080 cells on anosmin had moderately increased microspikes and fewer F-actin stress fibers. (K-P) Immunofluorescence staining of cell-matrix adhesions of HFFs on anosmin (K-M) versus fibronectin (N-P). Note the partial co-localization (yellow) of β1 integrin (red) and paxillin (green) in focal adhesion structures of both proteins that differ in patterns of adhesion organization.

(Q, R) HFF cells were seeded on purified anosmin and examined for dependence of cell spreading on divalent cations by chelation with 5 mM EDTA. EDTA completely blocked cell spreading on anosmin. **, $p < 0.01$.

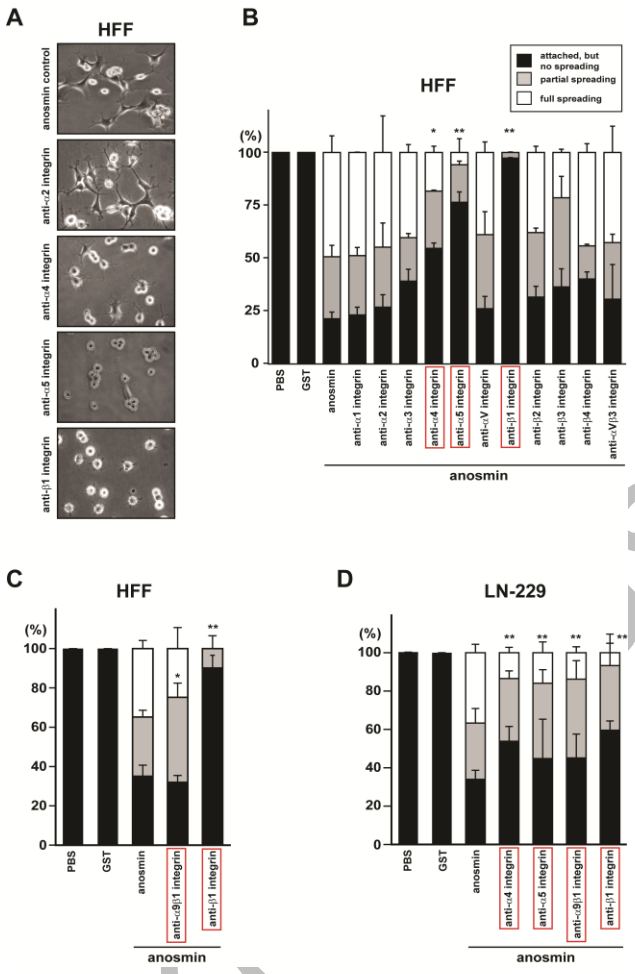

Figure 2. α5β1, α4β1, α9β1 integrins are candidate receptors for anosmin

(A) Images are shown from HFF cell spreading assays on anosmin using anti-α2, α4, α5 and β1 integrin function-blocking antibodies compared to the untreated control. (B) Quantification of cell spreading assays using a series of antibodies against specific integrin subunits based on percentages of total cells counted for each treatment. Shown in black: attached, but with no cell spreading; gray: partial cell spreading; white: full spreading. α4, α5 and β1 integrin antibodies significantly blocked full spreading on anosmin. *, $p < 0.05$, **, $p < 0.01$. (C) Weak but significant effect of anti-functional α9β1 antibodies on cell spreading of HFFs. *, $p < 0.05$, **, $p < 0.01$. (D) Comparable partial inhibitory effects of anti-α4, anti-α5, and α9β1 antibodies on cell spreading of LN-229

cells, with slightly larger effects of anti-functional antibodies against the β1 subunit that is shared by all three of these integrins (*, $p < 0.05$, **, $p < 0.01$).

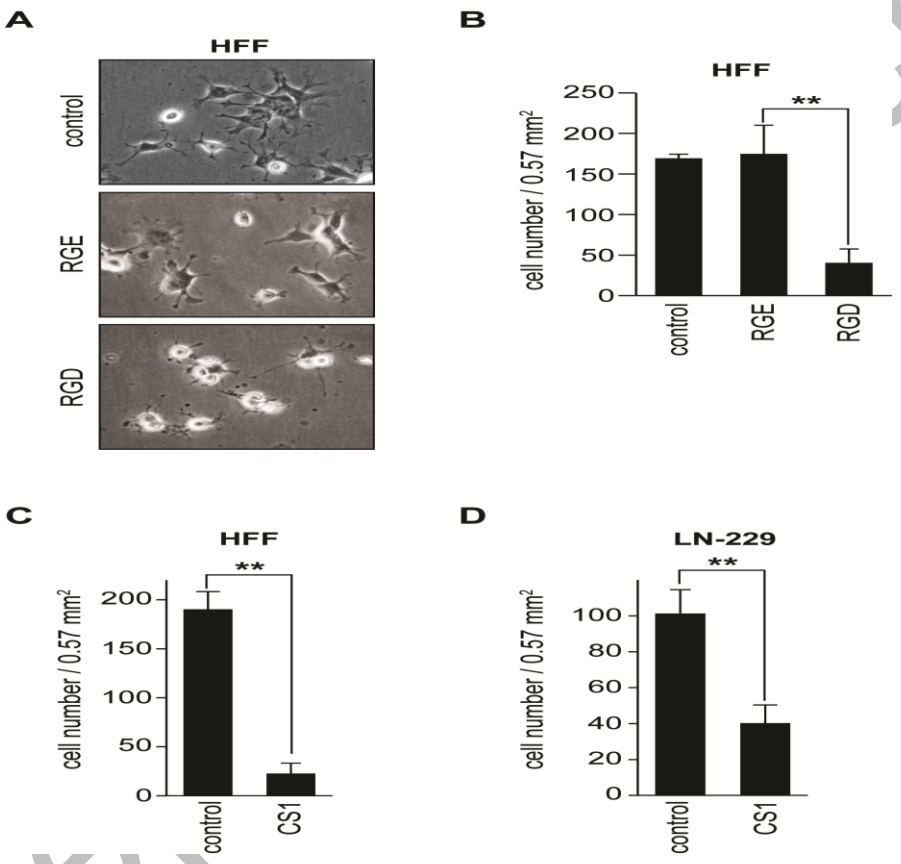

Figure 3. RGD and CS1 function-blocking peptides inhibit cell spreading on anosmin

(A) Images of HFF cell spreading on anosmin pre-coated wells in the presence of the control RGE or function-blocking RGD peptides using the 2nd generation assay. (B) Quantification of numbers of spread cells in the presence of either control RGE or RGD

peptide. RGD peptide inhibited cell spreading on anosmin-coated wells compared with control RGE peptide, **, $p < 0.01$.

(C, D) Inhibition of HFF or LN-229 cell spreading by the EILDVPST octapeptide from the CS1 region of fibronectin, which binds to α4β1. **, $p < 0.01$.