Adhesion GPCRs CD97 and GPR56: From structural regulation to cellular function
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GPS autoproteolysis is required for CD97 to up-regulate the expression of N-cadherin that promotes homotypic cell–cell aggregation

GPS autoproteolysis is required for CD97 to up-regulate the expression of N-cadherin that promotes homotypic cell–cell aggregation

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1. Introduction

As the second largest G protein-coupled receptor (GPCR) subfamily in the human proteome, the adhesion-GPCRs [1] have been shown to play an important role in the recognition and uptake of apoptotic cells [2], metastasis of melanoma cells [3], the normal development of brain cortex [4], the control of tissue polarity and morphogenesis [5,6], the normal auditory and visual function [7], male fertility [8], and the immune regulation [9].

Adhesion-GPCRs are characterized by the chimeric composition of a long ectodomain (ECD) and a seven-span transmembrane (7TM) region [10]. A unique post-translational modification by self-catalytic cleavage at the GPCR proteolysis site (GPS) dissects the receptor into the ECD- and 7TM-subunits [11]. The two resultant subunits are thought to associate non-covalently on the cell surface as a receptor complex. However, recent results suggested that it is also possible for the two subunits to behave independently as separate protein entities [12,13]. GPS auto-proteolysis has been shown as a prerequisite for efficient surface trafficking of some but not all adhesion-GPCRs [11,14,15]. Furthermore, mutations that impair GPS cleavage are linked to human genetic disorders [4,16]. Thus, GPS auto-proteolysis is considered as an essential step for the functional maturation of adhesion-GPCRs. However, a link between the GPS proteolysis and the cellular functions of adhesion-GPCRs has not been systematically investigated.

In this report, we aim to evaluate the role of GPS proteolysis in the functional maturation of adhesion-GPCRs. CD97 was first identified as an early activation marker of lymphocytes [17–19]. Later, it was found to be expressed restrictedly in cells of myeloid and lymphoid lineages and smooth muscle cells. Aberrant expression of CD97 was also often identified in various carcinoma cells and tissues [20]. CD97 belongs to the epidermal growth factor module-containing seven-transmembrane receptor sub-group of adhesion-GPCR family and contains a total of 5 epidermal growth factor (EGF)-like motifs in the N-terminal ECD [21,22]. Alternative mRNA splicing generated three major CD97 isoforms that contain different combinations of EGF-like motifs, including CD97(125), CD97(1235), and CD97(1-5) [18]. Recent studies have identified several specific cellular ligand(s) that interact with CD97 either in an isoform-dependent or -independent fashion. Thus, CD55 binds strongly to CD97(125) but weakly to CD97(1235) and CD97(1-5) [23]; chondroitin sulfate binds only to CD97(1-5) [24]; α5β1 and αvβ3 integrins bind to all CD97 isoforms via a RGD motif in the stalk region [25].

Abstract

Most adhesion-class G protein-coupled receptors (adhesion-GPCRs) undergo a novel self-catalytic cleavage at the GPCR proteolysis site (GPS) to form a hetero-dimeric complex containing the extra-cellular and seven-span transmembrane subunits. However, little is known about the role of GPS auto-proteolysis in the function of adhesion-GPCRs. Here we show that GPS cleavage is essential for the homotypic cell aggregation promoted by CD97 receptor, a leukocyte-restricted adhesion-GPCR often aberrantly expressed in carcinomas. We find that CD97 does not mediate cell aggregation directly. Instead, expression of the wild type – but not the GPS cleavage-deficient CD97 up-regulates the expression of N-cadherin, leading to Ca²⁺-dependent cell–cell aggregation. Our results provide a clear evidence for the role of GPS proteolytic modification in the cellular function of adhesion-GPCRs.

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References


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Keywords: CD97
Homotypic cell–cell aggregation
N-cadherin
Epidermal growth factor module-containing seven-transmembrane receptor
GPS cleavage
G protein-coupled receptor

Preface

CD97 promotes homotypic cell–cell aggregation

Abbreviations: 7TM, seven-transmembrane; ECD, ectodomain; EDTA, 2,2′-2″-ethylene-bis(2-aminoethane-1,2-diyldinitrilo)tetraacetic acid; EGF-TM7, epidermal growth factor module-containing seven-transmembrane receptor; EGF, glycol-bis(2-aminoethyl)ether)-(N,N,N,N′-tetraacetic acid; Fc, fragment crystallizable; GPCR, G protein-coupled receptor; GPS, GPCR proteolysis site; WT, wild-type

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1. Introduction

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Adhesion-GPCRs are characterized by the chimeric composition of a long ectodomain (ECD) and a seven-span transmembrane (7TM) region [10]. A unique post-translational modification by self-catalytic cleavage at the GPCR proteolysis site (GPS) dissects the receptor into the ECD- and 7TM-subunits [11]. The two resultant subunits are thought to associate non-covalently on the cell surface as a receptor complex. However, recent results suggested that it is also possible for the two subunits to behave independently as separate protein entities [12,13]. GPS auto-proteolysis has been shown as a prerequisite for efficient surface trafficking of some but not all adhesion-GPCRs [11,14,15]. Furthermore, mutations that impair GPS cleavage are linked to human genetic disorders [4,16]. Thus, GPS auto-proteolysis is considered as an essential step for the functional maturation of adhesion-GPCRs. However, a link between the GPS proteolysis and the cellular functions of adhesion-GPCRs has not been systematically investigated.

In this report, we aim to evaluate the role of GPS proteolysis in the functional maturation of adhesion-GPCRs. CD97 was first identified as an early activation marker of lymphocytes [17–19]. Later, it was found to be expressed restrictedly in cells of myeloid and lymphoid lineages and smooth muscle cells. Aberrant expression of CD97 was also often identified in various carcinoma cells and tissues [20]. CD97 belongs to the epidermal growth factor module-containing seven-transmembrane receptor sub-group of adhesion-GPCR family and contains a total of 5 epidermal growth factor (EGF)-like motifs in the N-terminal ECD [21,22]. Alternative mRNA splicing generated three major CD97 isoforms that contain different combinations of EGF-like motifs, including CD97(125), CD97(1235), and CD97(1-5) [18]. Recent studies have identified several specific cellular ligand(s) that interact with CD97 either in an isoform-dependent or -independent fashion. Thus, CD55 binds strongly to CD97(125) but weakly to CD97(1235) and CD97(1-5) [23]; chondroitin sulfate binds only to CD97(1-5) [24]; α5β1 and αvβ3 integrins bind to all CD97 isoforms via a RGD motif in the stalk region [25].
Functional studies using specific Abs, recombinant CD97 pro-
tiens, and knock-out animals have revealed a role for CD97 in leu-
ocyte migration, recruitment and activation [26–29]. In addition, CD97 is known to be involved in angiogenesis and the migration and invasion of tumor cells [20,25,30]. In this report, we show that GPS auto-proteolysis is required for CD97 to promote cell aggrega-
tion in HT1080 cells. The expression of a functional CD97 receptor up-regulates the expression of N-cadherin and its associated intra-
cellular adaptors, leading to Ca++-dependent homotypic cell–cell
aggregation. This is one of the first reports linking the GPS proteo-
ytic modification and the cellular function of an adhesion-GPCR.

2. Materials and methods

2.1. Reagents and cell culture

General chemicals were of analytical grade and obtained from Sigma, unless otherwise stated. CLB/CD97-1 mAb (mouse IgG2a) was purchased from AbD Serotec (Kidlington, UK). CLB/CD97-3 mAb and 2A1 and 2B1 mAbs were gifts from Dr. Jörg Hamann (University of Amsterdam, The Netherlands). Function-blocking anti-integrin mAbs for β1 integrin (Teramec, CA) are anti-s2 (clone P1E5), anti-sV (clone LM609), anti-sV5 (clone P1F6), and anti-j1 (clone P4C10). Blocking (clone IIa1) and non-blocking (clone VCS) anti-CD95 and Abs to N-terminal N-cadherin (clone 32/N-cad-
herin) are Abs from BD Biosciences (San Jose, CA). Mouse isotype
control Ab and β-catenin (clone 7F7.2) mAb were purchased from R&D system (Minneapolis, MN) and Millipore, respectively. Anti-
γ-catenin (clone 1G5) and anti-p120 catenin (H-90) were obtained from Santa Cruz (Delaware Avenue, CA). Anti N-terminal N-cad-
herin mAb (clone GC-4) and Horseradish peroxidase (HRP)-conju-
gated secondary Ab were acquired from Sigma. All culture media
were from Invitrogen and were supplemented with 10% heat inac-
tivated fetal calf serum (FCS), 2 mM L-glutamine, 50 IU/ml penicil-
in and 50 μg/ml streptomycin. HEK-293T was cultured in
Minimum Essential Medium (MEM) containing non-essential ami-
no acids and 1 mM sodium pyruvate. HEK-293T was cultured in
Duibecco’s Modified Eagle Medium (DMEM). Purified CD97 (1-5)-
miC, CD97 (125)-miC, EMR2 (125)-miC and miC have been de-
scribed previously [31].

2.2. Construction of expression vectors

The retroviral expression constructs were generated using the
pFB-Neo vector (Stratagene, La Jolla, CA). The wild type (WT) and
SSS1A mutant of CD97 (125) and CD97(1-5) isoforms were ampli-
ﬁed by PCR using respective templates. Primers (CD97-Sal I-5’:
5’-ata aag tcc tgc gac gac ctg acg tgc-3’) and (CD97-Not I-3’:
5’-tgg gcc gtc cag aag ccc ccg cct tca tat ggc-3’) were used to
generate the CDNA inserts to clone into the pFB-Neo vector via
the Sal I and Not I sites.

2.3. Retroviral infection and selection of stable cell lines

HEK-293T packaging cells in 100-mm dishes were transfected with 3 μg each of the pFB-Neo expression construct, pFB-PACK-
VSV-G and pV-Pack-GP vectors (Stratagene) plus 50 μl of Lipofect-
amine™ in Opti-MEM medium, as recommended by the supplier. Virus-containing supernatants were harvested 48 hr post-trans-
fection, to which a final concentration of 5 μg/ml polyethylene solution was added. HT1080 cells (~40–50% confluence) in 6-well plates were infected with 1 ml of viral supernatants. Cells were spun for
90 min at 25°C and 600 g, followed by the addition of 2 ml of fresh
complete medium 3 h later. The infected cells were then incubated for an additional 24 h at 37°C before selection in medium contain-
ing 1 mg/ml G418. G418-resistant cells were collected after
~2 weeks of selection and confirmed by appropriate analysis. Cell
proliferation assays were performed using the CellTiter 96 Aque-
ous One solution assay (Promega, WI, USA) according to the man-
ufacturer’s instructions. Cells (1 × 10^3 cells/well) were seeded in
a 96-well plate at day 0 and cell growth measured everyday for
6 days with medium replenishment at day 3.

2.4. Cell aggregation assay

Cells were serum-starved for 20 h before harvesting in PBS/2 mM
2,2’,2’’-(ethane-1,2-diyl)diminio)tetracetic acid (EDTA) and
resuspended at 5 × 10^5 cells/ml in serum-free medium/5% BSA. Cells (100 μl/well) were incubated in 96-well plates for 1 h at 37°C, fixed
with 1% formaldehyde and the number of single cells was counted
with a hemocytometer. The extent of cell aggregation was deter-
mined using the equation: (1 – N1/N0) × 100, where N0 is the
number of single cells at the start of the experiment and N1 is the
number of single cells after 1 h incubation. When necessary, various
reagents such as cation chelators (EDTA and glycol-bis-2-aminoeth-
ylene)-tetraacetic acid (EGTA, 5 mM), antibodies (5–
150 μg/ml), and soluble proteins (5–10 μg/ml) were pre-incubated
with cells for 15 min on ice before cell aggregation assay.

2.5. RT-PCR analysis and quantitative real time PCR

To identify N-cadherin, β-catenin and E-cadherin RNA tran-
scripts, semi-quantitative RT-PCR and quantitative real time PCR
(qRT-PCR) were performed using the i cycle system (Bio-Red, CA,
USA). In brief, total RNA was isolated using Trizol reagents (Invitro-
gen). 1 μg of DNA-free total RNAs were reverse transcribed using
oligo-dT12-18, and MMLV reverse transcriptase as described previ-
ously [9]. For the semi-quantitative RT-PCR assay, cDNA products
amplified by the specific primers (Supplementary Table 1) were
sequenced by electrophoresis in 1% agarose gels. For the qRT-PCR
assay, 25 μl reaction containing 2 μl cDNA, 0.3 μM of each primer
and 12.5 μl Maxima™ SYBR Green qPCR Master Mix (Fermentas,
Canada) was used to monitor double-strand DNA synthesis. qRT-
PCR was carried out following the recommended thermal profile:
95°C for 10 min (initial denaturation), followed by 40 cycles of
95°C for 15 s (denaturation) and 60°C for 1 min (annealing/elong-
ation). Fluorescence intensity of the amplified products was mea-
sured at the end of each PCR cycle. Results of qRT-PCR were
collected and analyzed by iQ™Optical System Software (Bio-
Red, CA, USA). The end results of N-cadherin mRNA expression
were normalized to internal control GAPDH mRNA.

2.6. Western blotting and flow cytometry analysis

For Western blotting, total cell lysates were separated in 8% or
10% SDS-PAGE gels, blotted and probed with specific primary Abs
at optimal concentrations. Following extensive wash, blots were
sequentially incubated with HRP-conjugated secondary Abs and
ECL for detection. Flow cytometric analyses were carried out using
standard procedures as described previously [14]. Briefly, cells were
harvested and fixed with 4% paraformaldehyde at room temperature
for 15 min. Cells were blocked in PBS containing 4% normal goat ser-
um and 1% BSA (blocking buffer) for 30 min and then stained with
appropriate 1st and 2nd Abs in blocking buffer, all at 4°C. Cells were
collected and analyzed on FACSscan using the Flowjo software.

2.7. Statistical analysis

All experiments were performed at least three times. Results are
shown as the mean ± S.E.M., and comparisons were made using
Student’s t-test. A probability (P) of ≤0.05 is considered significant.
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3. Results and discussion

3.1. Generation of HT1080 cell lines stably expressing wild-type and GPS cleavage-deficient CD97 isoforms

To investigate the role of GPS auto-proteolysis in CD97-mediated cellular function, we established stable HT1080 cell lines expressing CD97(1-5)WT, CD97(1-5)S531A, CD97(125)WT, and CD97(1-5)S531A isoforms using a retroviral expression system (Fig. 1A). The CD97(125)WT and CD97(1-5)WT isoforms represented two major alternatively-spliced CD97 gene products containing the EGF-like domains 1-2-5 and 1-2-3-4-5, respectively. The S531A mutant was chosen because the single Ser to Ala mutation at the GPS site renders the receptor deficient in cleavage, but has no adverse effect on receptor expression (unpublished results). As revealed by the flow cytometry analysis, pFB-Neo vector-transduced HT1080 cells show a basal expression of CD97, while all stable cell lines display upregulated CD97 expression at a comparable level (~10-fold over the basal) (Fig. 1B). 2A1 mAb that recognizes a related adhesion-GPCR, EMR2, shows no expressional changes in all cell lines, confirming the specific over-expression of CD97. Western blot analysis using CD97/1 mAb that recognizes the first EGF-like domain identifies specific bands corresponding to the respective isoforms (1-5WT, ~85 kDa; 125WT, ~65 kDa; 1-5S531A, ~110 kDa; 125S531A, ~90 kDa) (Fig. 1C). A faint band of ~80-85 kDa representing endogenous CD97 molecules was detected in the parental and vector-transfected HT1080 cells (Fig. 1C). Thus, we confirm CD97(125)WT and CD97(1-5)WT are fully cleaved, while CD97(125)S531A and CD97(1-5)S531A are not proteolytically processed. All stable cell lines are found to be similar to the parental cells in the growth rate and cell morphology (Fig. 1C and Supplementary Fig. S1).

3.2. A role for GPS auto-proteolysis in regulating homotypic cell aggregation by CD97

Although these cell lines are characteristically similar in normal culture conditions, we found that serum-starved CD97(125)WT and CD97(1-5)WT stable cells tended to aggregate faster and form much larger aggregates in comparison to the control and CD97S531A-expressing cells (Fig. 2A). Quantitative analysis showed that CD97WT stable cells display a ~91% degree of aggregation, while control cells and CD97S531A-expressing cells only reach ~50-60% degree of aggregation (Fig. 2B). These results suggest that GPS proteolytic modification is absolutely required for the expression of a functional CD97 receptor to promote homotypic cell–cell aggregation.

3.3. CD97 upregulates the expression of N-cadherin, which promote HT1080 cell aggregation

We next investigate the mechanism whereby the expression of WT CD97 in HT1080 cells promotes homotypic cell aggregation. As an adhesion-GPCR, many cellular ligands including CD55, chondroitin sulfate, and 95/1 and α5β3 integrins, have been identified for CD97 [23–25]. It is possible that interaction of CD97 with some of these ligand(s) leads to enhanced cell aggregation. Alternatively, cell aggregation could be induced secondarily as a result of WT CD97 expression in HT1080.

We first evaluate whether CD97 itself can mediate cell aggregation directly. Interestingly, addition of anti-CD97 Abs, soluble CD97-mFc proteins and various anti-integrin Abs all fail to inhibit cell aggregation even at high concentrations (Fig. 3). These data suggest that CD97 is not involved directly in mediating cell aggregation. This result prompts us to consider whether the expression
of WT CD97 in HT1080 somehow modulates cellular phenotypes to promote cell aggregation.

The cell aggregation seen here is reminiscent of the homotypic cell–cell adhesion mediated by members of the cadherin-superfamily such as E-cadherin and N-cadherin [32]. RT-PCR analysis shows that while no E-cadherin transcripts are ever detected in these cells, N-cadherin is indeed expressed in parental HT1080 cells. (Fig. 4A). Importantly, both semi-quantitative RT-PCR and real-time qRT-PCR analysis reveal a ~1.5-fold increase of N-cadherin transcript in cells expressing CD97 WT isoforms in comparison to the vector control cells and CD97S531A-expressing cells (Fig. 4A and Supplementary Fig. S2).

Next, the level of cell surface N-cadherin is determined by flow cytometry. Again, CD97 WT stable cells are found to display ~two-fold more N-cadherin than do control cells and CD97S531A-expressing cells in serum-starved and, to a less extent, in normal culture conditions (Fig. 4B and Supplementary Fig. S3). Finally, Western blot analysis revealed an increased expression of N-cadherin as well as β-catenin and α-catenin, two downstream adapters of N-cadherin, in CD97 WT cells (Fig. 4C) [32]. Interestingly, the expression of p120-catenin, another N-cadherin adapter, seems to be compatible in all cell lines. These results indicate that expression of CD97 WT, but not CD97S531A isoforms increases specifically the expression of N-cadherin and some of its adapter proteins.

N-cadherin mediates homotypic cell–cell adhesion in a Ca++-dependent manner [32]. We therefore examine HT1080 cell aggregation in the presence of divalent cation chelators (EDTA and

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**Fig. 2.** GPS auto-proteolysis is essential for CD97 to promote HT1080 cell aggregation. (A and B) Serum-starved single cell suspension was incubated for 1 h at 37 °C. Cell aggregation was represented either by the microscopy observation (A) or by the measurement of the degree of aggregation (B) as described in “Section 2.” The numbers 1–6 represent control and stable HT1080 cell lines as indicated in Fig. 1C. Large cell aggregates (arrowhead) were readily observed in cells over-expressing CD97 WT isoforms (panels 3 and 5), while much smaller aggregates (arrow) were found in control cells (panels 1 and 2) and cells expressing GPS cleavage-defective CD97 isoforms (panels 4 and 6). Data in B are means ± S.E.M. of 4 independent experiments performed in triplicate. **⁎⁎⁎** P < 0.005 compared to the control.

**Fig. 3.** CD97 does not mediate cell aggregation directly. Cell aggregation was analyzed on control and two CD97 WT stable HT1080 cells in the presence of (A) isotype control and anti-CD97 mAbs; (B) soluble CD97 protein isoforms and control proteins; (C) isotype control and anti-integrin mAbs all at 10 μg/ml as indicated. Data are means ± S.E.M. of 3 independent experiments performed in triplicate.

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EGTA). As shown in Fig. 4D, both EDTA and EGTA strongly inhibit cell aggregation in all cell lines examined. EDTA plus Mg ++ partially restores cell aggregation in CD97WT stable cells, but not in other cells. In contrast, EGTA plus Ca ++ restores cell aggregation fully in all cell lines. This result indicates that the cell aggregation observed herein is indeed Ca ++ -dependent. Finally, the involvement of N-cadherin in the cell–cell adhesion is confirmed using a blocking anti N-cadherin Ab, which significantly reduces cell aggregation in a dose-dependent manner (Fig. 4D and Supplementary Fig. S4).

In the present report, we have provided strong evidence for a link between the GPS auto-proteolysis and the cellular function of CD97. The establishment of HT1080 stable cell lines expressing CD97 WT and cleavage-deficient (S531A) receptors allows for the systematic evaluation of the functional role of GPS auto-proteolysis (Fig. 1). The comparable expression levels of WT and cleavage-deficient CD97 proteins indicates that GPS cleavage per se is not a prerequisite for CD97 receptor trafficking and expression. Instead, our results indicate that GPS cleavage is essential for the formation of a functional CD97 receptor.

The observation that the expression of CD97WT but not CD97S531A isoforms enhanced cell aggregation in HT1080 cells is surprising at first (Fig. 2). As stable cell lines express CD97WT and CD97S531A proteins at a comparable level, it is unlikely the aggregation is mediated directly by CD97 itself. Indeed, our data show that anti-CD97, anti-integrin Abs, and soluble CD97 proteins all fail to inhibit cell aggregation (Fig. 3). Taken together, these data suggest that the cell aggregation phenotype is induced as a result of the expression of a functional CD97 receptor. The fact that enhanced cell aggregation only occurred when CD97 was properly modified by GPS cleavage indicates its important role in the formation of a functional receptor complex. It is possible GPS auto-proteolysis leads to a conformational alteration of the receptor that activates intracellular signaling events.

Although the nature of intracellular signaling is currently unknown, our data indicate clearly that N-cadherin is one of the target genes induced by CD97 WT proteins (Fig. 4). In addition, some of the adapter proteins of N-cadherin such as β-catenin and α-catenin are also up-regulated. The resulting effect is the enhanced homotypic cell–cell aggregation. Interestingly, in a recent study using CD97 transgenic mice, specific over-expression of mouse CD97 in enterocytes was shown to up-regulate the expression of β-catenin, α-catenin, and p120-catenin, which in turn stabilize the structural integrity of E-cadherin-based adherens junctions [33]. As a result, the CD97-transgenic mice are more resistant than WT mice to dextran sodium sulfate-induced colitis.

In conclusion, we demonstrate that expression of CD97 WT isoforms in HT1080 cells up-regulates the expression of N-cadherin and its intracellular adaptor proteins (β-catenin, α-catenin), which in turn promote Ca ++ -dependent homotypic cell–cell aggregation. This cellular phenotype requires the expression of a functional
CD97 receptor generated by proper GPS auto-proteolytic modification. As the GPS motif is present in almost all adhesion-GPCRs, our data suggest the GPS auto-proteolysis plays an essential role in the function of adhesion-GPCRs.

Acknowledgements

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Appendix A. Supplementary data


References


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Appendix A. Supplementary data


References

Supplementary data

Fig. S1. Morphological examination of HT1080 stable cell lines. The cell morphology of parental HT1080 and G418-resistant stable cells as indicated was examined by light microscopy at 600× magnification. Scale bar, 20 μm.

Fig. S2. Quantitative analysis of N-cadherin mRNA in HT1080 stable cells. qRT-PCR analysis of mRNA expression of N-cadherin was performed on RNA samples isolated from parental HT1080 and different stable cells. Results were normalized to internal control GAPDH mRNA. Data are the means ± SEM (n = 3). *, P < 0.05 versus parental HT-1080.
Fig. S4. Function-blocking N-cadherin Ab inhibits homotypic cell-cell aggregation. (A and B) Serum-starved single cell suspension was pre-treated with different concentrations (10–150 μg/ml) of N-cadherin blocking antibody (clone GC-4) for 15 min on ice, followed by 1 h incubation at 37 °C. Cell aggregation was represented either by the microscopy observation at 400× magnification (A) or by the measurement of the degree of aggregation (B) as described in "Materials and methods". Data are means ± SEM of 3 independent experiments performed in triplicate. Scale bar, 20 μm.

Fig. S3. Differential surface N-cadherin expression in HT1080 stable cells. Flow cytometry analysis of the cell surface N-cadherin expression in various HT1080 stable cells cultured in serum-containing complete medium. MFI: mean fluorescence intensity.
<table>
<thead>
<tr>
<th>Gene</th>
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