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Integrin αvβ3 Promotes Vitronectin Gene Expression in Human Ovarian Cancer Cells by Implicating Rel Transcription Factors

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ABSTRACT

We previously showed that integrin αvβ3 expression upon engagement by its major ligand vitronectin (VN) correlated with enhanced human ovarian cancer cell adhesion, motility, and proliferation, by triggering intracellular signaling events, ultimately leading to altered gene expression. In the present study, we characterized cellular VN expression as a function of αvβ3 and noticed significant upregulation of VN protein which was reflected by elevated VN gene transcription. In order to identify specific transcription factors involved in the αvβ3-regulatory effect on VN, we generated different VN promoter mutants. We noticed that disruption of the DNA consensus motif for Rel proteins did not only prominently reduce VN promoter activity but, moreover, led to a loss of responsiveness to αvβ3, suggesting a crucial role of Rel proteins in αvβ3-provoked VN induction. In cell migration studies, we confirmed increased cell motility as a function of αvβ3/VN which was further enhanced by raising cellular Rel transcription factor levels. Thus, the data of the present study elucidated a positive feedback regulatory loop on VN expression by αvβ3 implicating transcription factors of the Rel family. Hence by altering the composition of the extracellular matrix upon additional VN synthesis and deposition, tumor cells might be enabled to modulate their surrounding reactive microenvironment towards enhanced αvβ3/VN-interactions and, consequently, intrinsic intracellular signaling events affecting cancer progression. J. Cell. Biochem. 112: 1909–1919, 2011.

KEY WORDS: OVARIAN CANCER; INTEGRIN αvβ3; VITRONECTIN; VITRONECTIN PROMOTER; GENE EXPRESSION; REL TRANSCRIPTION FACTORS

Human ovarian cancer cell progression depends on adhesion-mediated tumor cell dissemination on the mesothelial lining and subsequent invasion of the organs of the small pelvis resulting in numerous tumor foci within the peritoneal cavity. Thus, mesothelial cells as well as the surrounding extracellular matrix (ECM), constituting the implantation site for ovarian tumor cells, act as a favorable microenvironment for tumor growth (Ozols et al., 2004). Frequently, these tumor biological events are associated with development of malignant ascites containing floating multicellular carcinoma cell aggregates (Kellouche et al., 2010). Thus, alterations of cell/cell and cell/ECM adhesive properties play an important part in this characteristic behavior majorly implicating adhesive receptors of the integrin superfamily and their respective ECM ligands (Gardner et al., 1995).

Integrins are non-covalently linked heterodimers composed of an α- and a β-subunit. Binding to ECM ligands initiates bidirectional signaling events across the cell membrane (*outside-in* and *inside-out*) which are directly connected to the cellular gene expression machinery. Consequently, during tumor progression, expression, and localization of integrins, their ECM ligands as well as integrin-related signaling molecules may be modulated in human ovarian cancer cells when compared to their normal cellular counterparts (Felding-Habermann, 2003; Lössner et al., 2008, 2009). The importance of αvβ3 and its presence in normal ovarian epithelium as well as in human ovarian cancer tissues had been well documented (Carreiras et al., 1995, 1996, 1999a; Liapis et al., 1997; Cruet et al., 1999; Goldberg et al., 2001; Hapke et al., 2003; Heyman et al., 2008). The αv-subunit was found in normal epithelium and in highly differentiated tumors as well as in the majority of moderately and poorly differentiated carcinomas. The β3-subunit was also detected in normal ovarian tissue and in highly differentiated carcinomas, but not in most of less differentiated tumors (Liapis et al., 1997; Goldberg et al., 2001). Also, vitronectin (VN) as the major ECM ligand of αvβ3 was identified as primary adhesion substrate in differentiated human ovarian tumors as well as established ovarian cancer cell lines (Carreiras et al., 1996; Liapis et al., 1997). We previously showed that ovarian cancer cell adhesive, migratory, and proliferative properties significantly

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correlated with αvβ3 expression and its engagement by VN, making this adhesive system a crucial parameter for ovarian cancer cell biology (Hapke et al., 2003).

The cell adhesive glycoprotein VN is found in abundance in blood where it circulates as a single-chain (75 kDa) and two-chain (10 and 65 kDa) form. Moreover, VN is detectable as an ECM-deposited form (Tomasini and Mosher, 1991; Nakashima et al., 1992; Preissner and Seiffert, 1998; Schvartz et al., 1999). VN encompasses several functional domains which bind a plethora of different ligands, thereby implicating it in a series of biological activities (Casslen et al., 1994; Preissner and Seiffert, 1998; Reuning et al., 1998). Upon binding to integrins, such as αvβ3, αvβ5, or αIIbβ3, via its Arg-Gly-Asp (RGD)-motif (residues 45–47), VN mediates cell adhesion and thus majorly contributes to the control of, for example, hemostasis, vascular remodeling, and proteolytic ECM degradation during embryogenesis, tissue remodeling, wound repair, and cancer (Cheresh et al., 1989; Preissner, 1991; Preissner and Seiffert, 1998; Reuning et al., 1998). The different sites of VN biosynthesis in vivo are still not completely clear. Although the liver represents the primary production site, meanwhile, evidence has been provided for ubiquitous occurrence of VN mRNA in diverse tissues. This raises the possibility that tissue-associated VN is, at least in part, derived from local synthesis. Finally, it was demonstrated that several different human ovarian cancer cell types were indeed able to synthesize VN. Still, only limited information is available with respect to conditions and/or agents that regulate VN biosynthesis. The VN gene had been mapped to the centromeric region of the long arm of chromosome 17 (Fink et al., 1992). Both the human and mouse VN promoter were cloned and the regulatory sequences 1.8 kb pair upstream of the translational initiation site determined (Jenne and Stanley, 1987; Seiffert et al., 1993, 1996; Miyamoto et al., 1998). Besides still unresolved issues concerning transcriptional initiation, also transcription factors contributing to VN promoter activation have so far not been characterized in great detail (Seiffert et al., 1993, 1996). In the present investigation, we observed VN upregulation as a function of αvβ3, implicating Rel transcription factors. In most normal cells, inactive Rel dimers are retained in the cytoplasm bound to IκB inhibitors. Various signals lead to phosphorylation and subsequent proteasomal degradation of IκB, followed by nuclear translocation of Rel dimers. Here, they bind to κB sites in the promoters of specific target genes thereby profoundly affecting gene expression (Lin et al., 2010). Thus, a positive feedback loop on VN expression is constituted, which in favor of tumor progression might enable tumor cells to modulate their "reactive microenvironment" with respect to αvβ3-mediated cell adhesion and intracellular signaling events arising thereof (Fig. 7).

MATERIALS AND METHODS

MATERIALS

The human ovarian cancer cell line OVCAR-3 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). Lipofectin™ transfection reagent, Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), HEPES, Alexa-488-labeled goat-anti-mouse and goat-anti-rabbit IgG, and geneticin (G418), were from Invitrogen (Carlsbad, CA). The dual luciferase reporter gene assay kit and the renilla luciferase reporter gene vector pRL-SV40 were purchased from Promega (Madison, WI). Fibronectin (FN) and VN were obtained from Becton–Dickinson Biosciences (Franklin Lakes, NJ). The monoclonal antibody (mAb) to VN (#VIT-2) and poly-D-lysine (PL) were purchased from Sigma–Aldrich (St. Louis, MO). Monoclonal Ab to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Millipore (Schwalbach, Germany). ECL™ Western blotting detection reagents and Hyperfilm™ were obtained from Amersham Biosciences (Uppsala, Sweden). The protease inhibitor cocktail Complete™ and the GC-RICH PCR solution™ were from Roche Diagnostics GmbH (Mannheim, Germany). Monoclonal Ab directed to αvβ3 (#23C6), mAb raised against the integrin chains αv and β3, respectively, were purchased from Merck (Schwalbach, Germany). The kit for site-directed mutagenesis QuikChange™ was obtained from Stratagene (La Jolla, CA). The NucleoSpin Plasmid™ kit and the NucleoBond AX™ kit for plasmid DNA purification were from Macherey-Nagel (Easton, PA). The expression plasmids encoding p50, p65, c-Rel, and IκB-α, respectively, were kindly provided by Dr. Luisa Guerrini, Department of Biomolecular Sciences and Biotechnology, University of Milan, Milan, Italy. The plasmid containing three κB consensus sequences for Rel proteins following the sequence of the thymidine kinase promoter in front of the reporter gene luciferase was a generous gift of K. Brand (Medizinische Hochschule, Hannover, Germany).

CELL CULTURE

Origin and cultivation of human ovarian cancer cells as well as its αvβ3-mediated adhesive, migratory, and proliferative properties were previously described by us (Hapke et al., 2003). Stable human ovarian OV-MZ-6 and OVCAR-3 cancer cell transfectants overexpressing αvβ3 were generated and isolated upon geneticin selection as described earlier (Hapke et al., 2001).

IMMUNOCYTOCHEMICAL STAINING OF VITRONECTIN AND CONFOCAL LASER SCANNING MICROSCOPY

Immunocytochemical staining of αvβ3. Human OV-MZ-6 and OVCAR-3 cells, respectively, were grown on cell culture microchamber slides, followed by immunocytochemical staining of αvβ3 using mAb #23C6 as previously described (Lössner et al., 2008). *Immunocytochemical staining of VN.* Vector- or αvβ3-transfected cells were passed to microchamber cell culture slides and cultivated for 24 h. Immunofluorescent VN staining was conducted after cell fixation in methanol for 10 min and then in acetone for 10 min at room temperature (RT). Thereafter, cells were washed and blocked in PBS, 2% (w/v) bovine serum albumin (BSA). VN antigen was detected by adding the mAb #VIT-2 for 2 h at RT followed by incubation with the secondary Alexa-488-conjugated goat-anti-mouse IgG for 45 min at RT. For confocal laser scanning microscopy (CLSM), slides were mounted in PBS and fluorescence signal intensity determined. In order to convert fluorescence staining intensity into colors of a glow scale, the look-up table "*glowOv/Un LUT*" provided with the CLSM scanning software was applied: low intensity (red), medium intensity (yellow), and high intensity (white) (Lössner et al., 2008). Staining procedures in

the presence of the secondary Alexa-488-labeled secondary Ab alone, served as controls and resulted in negligible fluorescence signals.

FLOW CYTOFLUOROMETRY

Detection of αvβ3. Stably transfected OV-MZ-6 and OVCAR-3 cells were grown in six-well cell culture plates and harvested after 24 h of cultivation. Immunostaining was performed on viable cells using the mAb directed to αvβ3 (#23C6) for 1 h at RT, followed by detection with secondary Alexa 488-conjugated goat-anti-mouse IgG for 45 min at RT. FACS analysis was performed by using the instrument FACS-Calibur (Becton–Dickinson Biosciences).

DETECTION OF VITRONECTIN BY WESTERN BLOT ANALYSIS

Integrin αvβ3- or vector-transfected OV-MZ-6 and OVCAR-3 cells, respectively, were cultivated for 24 h, lysed on ice in the presence of a protease inhibitor cocktail and processed for Western blot analysis under reducing conditions as previously reported (Lössner et al., 2008, 2009). Staining of membranes was performed by using the mAb #VIT-2 (Sigma–Aldrich) directed to VN. Reactive proteins were visualized using the ECL™ chemiluminescent substrate according to the manufacturers' recommendations. In order to normalize variations of protein concentrations and blotting efficiency, Western blot membranes were thereafter stripped as described and reprobed with mAb directed to GAPDH (Lössner et al., 2008, 2009).

GENERATION OF HUMAN OVARIAN CANCER CELL TRANSFECTANTS OVEREXPRESSING REL PROTEINS

In order to study the effects of Rel transcription factors on VN promoter activity, we generated ovarian cancer cell transfectants overexpressing the Rel proteins p50, p65, and c-Rel in different combinations, in order to promote formation of the transactivating Rel dimers p65/p50 (NF-κB) and p65/c-Rel, respectively. Stably transfected cell clones were isolated as described earlier (Hapke et al., 2003).

CLONING OF THE HUMAN VITRONECTIN PROMOTER

The human VN promoter sequence was generated by PCR using genomic DNA isolated from human ovarian cancer tissue as template. Based on the sequence published by Jenne and Stanley (1987), the following PCR-primers were designed: VN-5′: 5′-AACAAGCTAGCGGTACCAGGCAAGGGTGCCGG-3′ and VN-3′: 5′-AACAAAAGCTTGGCAGGGCTTCTAGCTCAGTGC-3′. In order to overcome problems during amplification of the human VN promoter sequence due to its GC richness, we included the GC-RICH PCR solution™ into PCR reactions. The VN promoter sequence of a size of 1,700 bp was then directionally subcloned into the luciferase reporter gene vector pGL2-Basic. Correctness of the human VN promoter sequence was confirmed by DNA sequencing.

GENERATION OF VITRONECTIN PROMOTER MUTANTS BY IN VITRO SITE-DIRECTED MUTAGENESIS

Mutants of the VN promoter carrying disrupted DNA consensus motifs for several transcription factors were generated by in vitro site-directed mutagenesis (Lössner et al., 2008). Oligodesoxynucleotide primers for mutagenesis were as follows: Rel mut sense: 5′-CAGGTGTTTCATGCCAGTGGCCATGGTCTAGGTAGAGGGATC-3′ and Rel mut antisense: 5′-GATCCCTCTACCTAGACCATGGCCACTGGCATGAAACACCTG-3′; p53 mut sense: 5′-GTTTCTTAGCGAAACCTCACAAGCTTCCAATGGGGTGGGAATTG-3′ and p53 mut antisense: 5′-CAATTCCCACCCCATTGGAAGCTTGTGAGGTTTCGCTAAGAAAC-3′; STAT mut sense: 5′-CCAGGCCCAGTGTGCTAGCAATGTGACCTTTGCTGCAGC-3′ and STAT mut antisense: 5′-GCTGCAGCAAAGGTCACATTGCTAGCACACTGGGCCTGG-3′. PCR was conducted according to the manufacturers' recommendations. Success of mutagenesis was verified by DNA sequencing.

TRANSIENT TRANSFECTIONS AND DUAL LUCIFERASE REPORTER GENE ASSAYS

Human ovarian cancer cells were transiently transfected with the VN promoter reporter gene constructs as previously described (Lössner et al., 2008). Cells were lysed and luciferase activity as measure of VN promoter transactivation recorded by luminometry using the dual firefly/renilla-luciferase reporter gene assay kit according to the manufacturers' recommendations. In order to normalize for varying transfection efficiency, cells were co-transfected with the constitutively active renilla luciferase reporter gene vector pRL-SV40. Relative VN promoter activity is given as quotient between relative light units (RLU) for firefly versus renilla luciferase (Lössner et al., 2008).

DETERMINATION OF CELL MIGRATION BY WOUND SCRATCH ASSAY

For wound scratch cell migration assays, wild-type and stably αvβ3-transfected cells, respectively, were passed to 12-well cell culture dishes. After cell monolayers reached a confluency of approximately 70%, cells were transiently transfected with an expression vector encoding p65 in combination with that coding for p50 or, as control, with empty vector alone. After another 12 h of cell cultivation, a homogeneous wound scratch was set into approximately 90% confluent cell monolayers by using a sterile pipet tip. Cells which were detached during the wounding procedure were immediately removed by washes in PBS. Microscopical images of cell monolayers were taken directly after wounding as well as after 24 h of further cell incubation.

STATISTICAL ANALYSES

Data are expressed as mean ± SD. Significance of differences when compared to the respective vector-transfected cells and the *P*-values were calculated by employing the Mann–Whitney test. A *P*-value <0.05 was considered statistically significant.

RESULTS

GENERATION OF INTEGRIN αvβ3-OVEREXPRESSING HUMAN OVARIAN CANCER CELLS

In order to monitor dose-dependency of αvβ3-mediated effects on VN expression, we isolated different individual OV-MZ-6 cell clones displaying gradually increasing αvβ3 levels (αvβ3 #1–4) as well as αvβ3-overexpressing OVCAR-3 cells. Successful elevation of αvβ3 cell surface expression was confirmed by immunocytochemical staining and subsequent evaluation by CLSM (Fig. 1A). In addition,

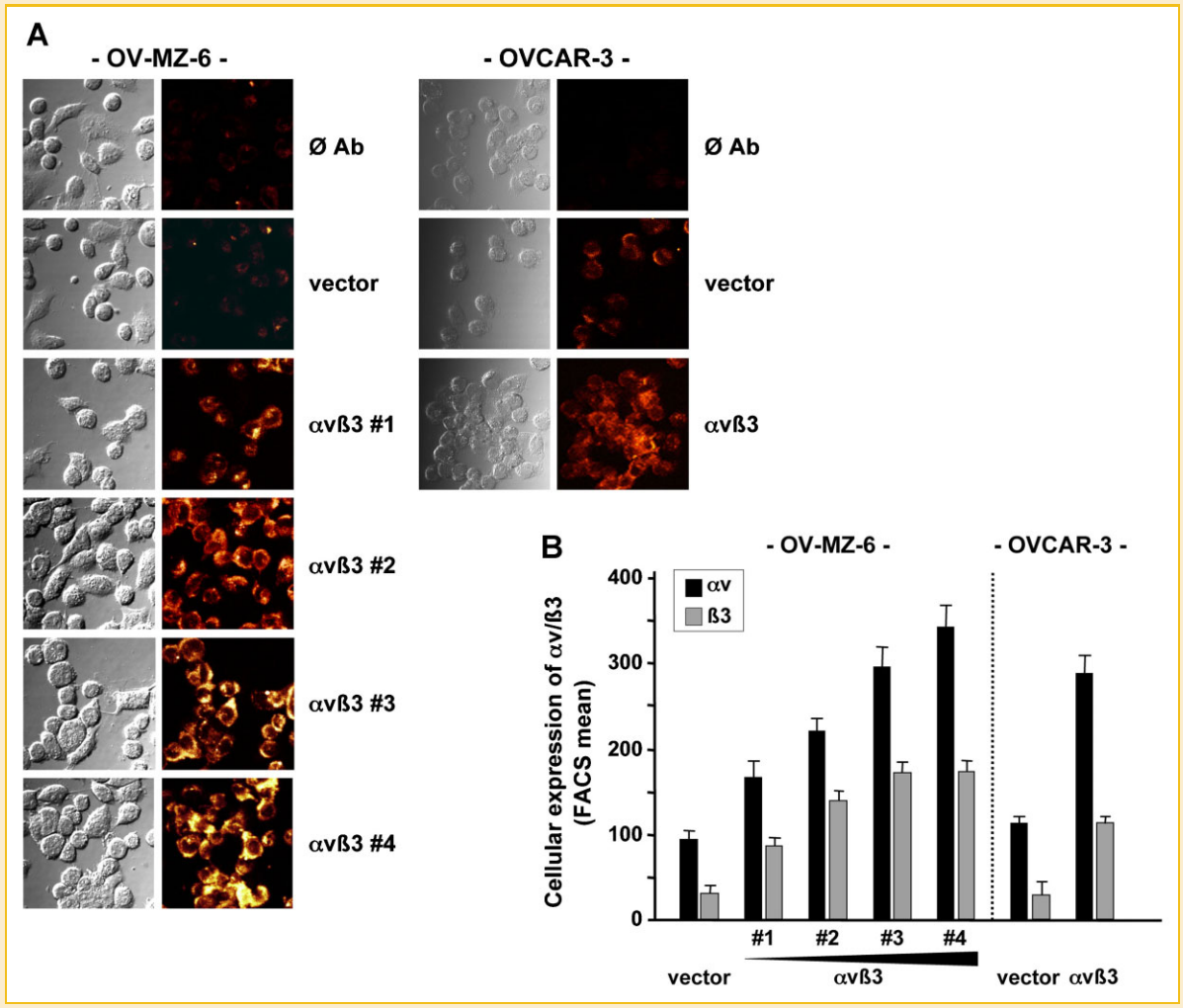

Fig. 1. Stable transfection of human ovarian OV-MZ-6 and OVCAR-3 cancer cells with integrin αvβ3. A: Detection of integrin αvβ3 by immunocytochemical staining. Integrin αvβ3 expression in stable OV-MZ-6 and OVCAR-3 cell transfectants was determined by immunocytochemical staining as described. Depicted are individual OV-MZ-6 cell clones (αvβ3 #1–4) exhibiting gradually increasing αvβ3 expression levels as well as stably αvβ3-transfected OVCAR-3 cells. Also, vector-transfected cells are shown displaying low endogenous αvβ3 expression levels. Stainings in the absence of primary antibodies (Ø Ab) served as controls. Representative fluorescence images together with the corresponding differential interference contrast images are depicted. B: FACS analysis of integrin αv/β3 expression. Four individual OV-MZ-6 cell clones overexpressing αvβ3 at increasing concentrations (αvβ3 #1–4) as well as αvβ3-overexpressing OVCAR-3 cells were processed for FACS analysis as described. Respective vector-transfected cells served as control. Data are given as FACS mean values ± SD.

we determined the cellular content of αv/β3 by FACS analysis and proved up to 5-fold (αv) and 8-fold (β3) increased expression over low endogenous expression levels in vector-transfected OV-MZ-6 cells; stably transfected OVCAR-3 cells displayed up to 3.5-fold elevated αv/β3 expression (Fig. 1B). Enhanced αvβ3 expression corresponded well with enhanced cell adhesive capacity onto VN, with almost unchanged cellular attachment onto FN- or uncoated cell culture dishes (data not shown; see also Hapke et al., 2003).

DETECTION OF VITRONECTIN IN HUMAN OVARIAN CANCER CELLS AS A FUNCTION OF INTEGRIN αvβ3

Detection by immunocytochemistry. Next, we investigated whether changes in αvβ3 levels affected VN protein expression. For this, cells were cultivated in microchamber cell culture slides and immunocytochemical VN staining performed after 24 h. For both human ovarian cancer cell lines, we showed that human VN protein expression was drastically raised over wild-type expression levels in vector-transfectants. VN was localized at the cell periphery as well as within cell/cell-contacts (Fig. 2A). *Detection by Western blot analysis.* In addition, we detected human VN expression in human ovarian cancer cells as a function of αvβ3 by applying cell lysates from vector- (Fig. 2B, lane 3: OV-MZ-6 and lane 5: OVCAR-3) as well as αvβ3-transfected cells (Fig. 2B, lane 4: OV-MZ-6 and lane 6: OVCAR-3) to Western blot analysis. Equal protein loading and blotting efficiency was checked by reprobing the membranes with a mAb raised against GAPDH. In both human ovarian cancer cell lines, this analysis confirmed the findings obtained by immunocytochemistry, revealing increased VN levels as a function of αvβ3 expression. As expected, VN was detected in cellular lysates under reducing conditions as a major protein band of approximately

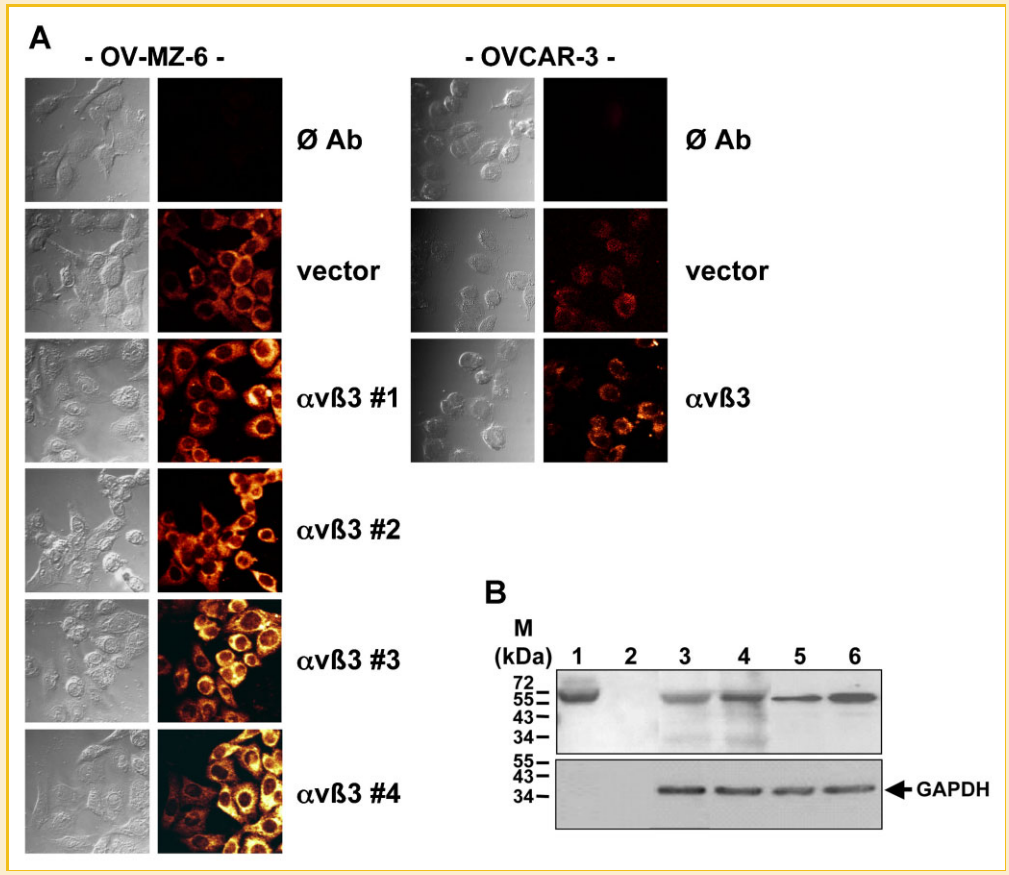

Fig. 2. Vitronectin protein expression in human ovarian cancer cells as a function of integrin αvβ3. A: Detection of VN by immunocytochemical staining. Integrin αvβ3-transfected OV-MZ-6 cell clones exhibiting increasing αvβ3 expression levels (αvβ3 #1–4) as well as αvβ3-overexpressing OVCAR-3 cells were passed to microchamber cell culture slides and cultivated for 24 h prior to immunocytochemical staining of VN. As control served vector-transfected cells displaying low endogenous αvβ3 levels as seen in the respective wild-type cells. Representative fluorescence images together with the corresponding differential interference contrast images are depicted. Signal intensity was evaluated by CLSM as described. B: Detection of cellular VN expression by Western blot analysis. Vector-transfected human ovarian cancer cells (lane 3: OV-MZ-6 and lane 5: OVCAR-3) and αvβ3-transfected cells (lane 4: OV-MZ-6 and lane 6: OVCAR-3), respectively, were harvested and processed for Western blot analysis under reducing conditions as described. Immunostaining was performed by using the mAb raised against human VN (#VIT-2). Reactive proteins were visualized by using the ECL chemiluminescence kit. Equal protein loading and blotting efficiency of cell lysates was confirmed by reprobing the membranes with a mAb raised against GAPDH. In order to prove species specificity of the mAb directed to human VN and to exclude cross-reactivity with bovine VN originating from FCS contained within cell culture media, we applied purified human VN (lane 1, 20 ng) as well as corresponding concentrations of bovine VN in FCS (lane 2) to SDS gels. A representative image of a typical Western blot is depicted.

65 kDa in accordance with that obtained for purified VN from human placenta (Fig. 2B, lane 1). Moreover, in order to control species specificity of the used mAb #VIT-2 directed to human VN and to rule out cross-reactivity with residual bovine VN originating from FCS, we applied corresponding concentrations of bovine VN contained within FCS (Fig. 2B, lane 2) to SDS gels and proved that the used mAb directed to VN recognized human but not bovine VN.

VITRONECTIN PROMOTER ACTIVITY AS A FUNCTION OF INTEGRIN αvβ3 EXPRESSION

Integrin αvβ3-dependent VN protein upregulation was traced back to the level of VN gene transcription. For this, we amplified the human VN promoter sequence by PCR using genomic DNA from human ovarian cancer tissue as template and cloned it into the luciferase reporter gene vector pGL2-Basic. Identity to the human VN promoter sequence published by Jenne and Stanley (1987) was confirmed by DNA sequencing (Fig. 3A). Upon transient transfection of cells with the luciferase reporter vector encompassing the human VN promoter, we demonstrated that αvβ3-induced VN protein was reflected by concomitantly up to 8.5-fold increased VN promoter activity, which corresponded well with the height of αvβ3 expression levels (Fig. 3B). Similarly, also OVCAR-3 cells responded to αvβ3 elevation with increased VN promoter activity, however, not as prominent as noticed for OV-MZ-6 cells (see Fig. 4). In addition, we measured VN promoter activity after transient cell transfections with αv/β3-expression vectors and confirmed the results obtained in stable cell transfectants (Fig. 3C). In parallel, specificity of the αvβ3 effect was controlled by transient co-transfection of cells with expression plasmids encoding either the integrin subunits β1 or αL. Neither elevated expression of β1 nor αL altered VN promoter activity (Fig. 3C). In case αvβ3 was engaged

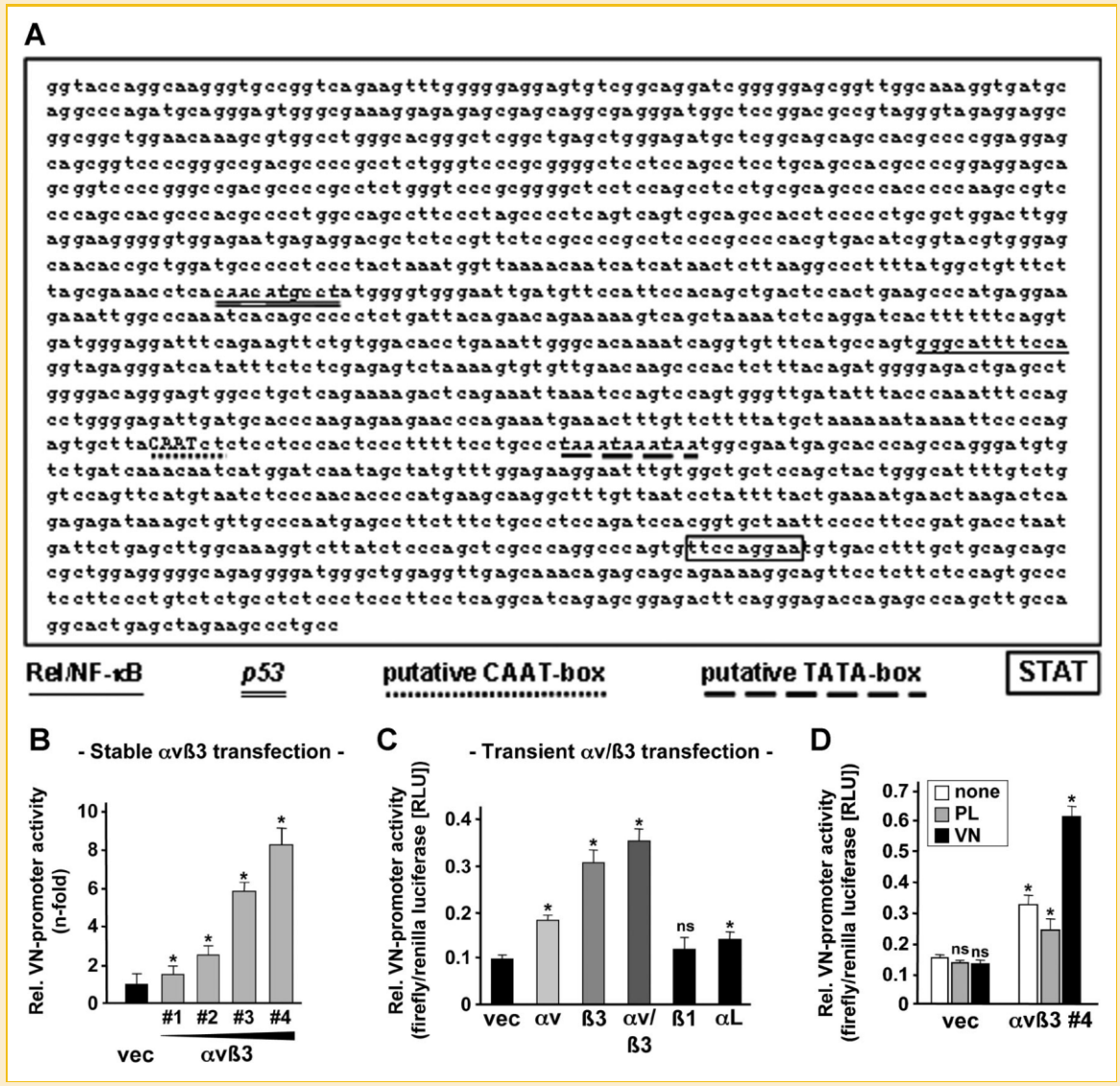

Fig. 3. Vitronectin promoter activity as a function of integrin αvβ3. A: Cloning of the human VN promoter sequence. The human VN promoter sequence was generated by PCR as described using genomic DNA from human ovarian cancer tissue as template. The PCR product (1,700 bp) was cloned into the luciferase reporter gene vector pGL2-Basic. DNA consensus binding motifs for transcription factors Rel/NF-κB (—), p53 (=), and STAT (boxed) are indicated. The putative CAAT- (dotted line) and TATA box (dashed line) are depicted. B: Vitronectin promoter activity as a function of stable integrin αvβ3-transfection. Vector-transfected (vec) as well as four individual αvβ3-overexpressing OV-MZ-6 cell clones (αvβ3 #1–4), displaying up to sixfold elevated αvβ3 levels over wild-type expression were transiently transfected with the VN promoter reporter gene construct and firefly luciferase activity as measure of VN promoter activity determined as described. Relative VN promoter activity was calculated as quotient of relative light units (RLU) for firefly versus constitutively expressed renilla luciferase. Data are given as "n-fold"-induction by setting the relative VN promoter activity in vector-transfected cells to "1." Statistically significant differences ($P < 0.05$) of VN promoter activity when compared to vector-transfected control cells are indicated by an asterisk. C: Vitronectin promoter activity upon transient overexpression of integrin αv/β3. Wild-type ovarian cancer cells were transiently co-transfected with αv, β3, αv plus β3, or, as control for integrin αvβ3-specificity of the effect, with the two irrelevant integrin subunits β1 and αL, respectively. As controls served transfections with the empty expression vector alone (vec). Vitronectin promoter activity was determined as described. Data for relative VN promoter activity are given as quotient of RLU obtained for firefly versus constitutively expressed renilla luciferase. Statistically significant differences ($P < 0.05$) of VN promoter activity when compared to vector-transfected control cells are indicated by an asterisk (ns = not significant). D: Vitronectin promoter activity as a function of elevated αvβ3 in the presence of its ligand VN. Vector-transfected cells (vec) as well as the OV-MZ-6 cell transfectant displaying approximately sixfold elevated αvβ3 levels over wild-type expression (αvβ3 #4) were passed to cell culture dishes which had been pretreated with VN, PL, or left uncoated (none). Transient transfections were performed and VN promoter activity determined as described. Data for relative VN promoter activity are given as quotient of RLU obtained for firefly versus constitutively expressed renilla luciferase. Statistically significant differences ($P < 0.05$) of VN promoter activity when compared to vector-transfected control cells are indicated by an asterisk (ns = not significant).

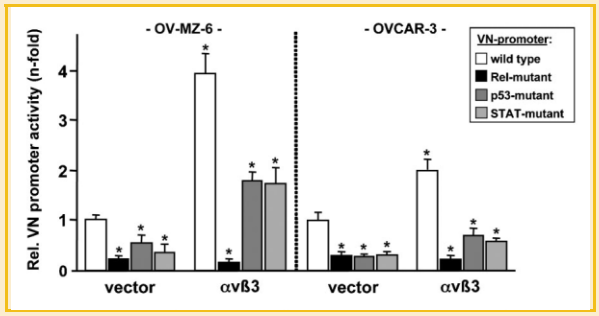

Fig. 4. Effect of integrin αvβ3 on the activity of vitronectin promoter mutants. Vector- and αvβ3-transfected OV-MZ-6 and OVCAR-3 cells, respectively, were transiently transfected with luciferase reporter gene constructs encompasing the human VN wild-type promoter or the VN promoter mutants carrying either a disrupted Rel-, p53-, or STAT-DNA binding motif (see Fig. 3A). Dual luciferase reporter gene assays were conducted as described. Relative VN promoter activity is given as quotient of RLU obtained for firefly versus constitutively expressed renilla luciferase by setting the activity of the respective vector-transfectants to "1." Statistically significant differences ($P < 0.05$) when compared to vector-transfected control cells are indicated by an asterisk.

and activated by its ligand VN, VN promoter induction was even more pronounced (Fig. 3D).

IDENTIFICATION OF TRANSCRIPTION FACTORS INVOLVED IN INTEGRIN αvβ3-MEDIATED VITRONECTIN PROMOTER INDUCTION

We sought to identify DNA elements contained within the human VN promoter, contributing to αvβ3-induced regulation of VN gene transcription. For this, we mutated prominent DNA-binding motifs for transcription factors of the Rel family, p53, and signal transducer and activator of transcription (STAT), respectively (see Fig. 3A). In vector-transfected OV-MZ-6 cells which like wild-type cells express only minor αvβ3 levels, mutation of the p53- or the STAT-motif, resulted in considerably lower VN promoter activity. In both cases the inducibility by αvβ3 was, however, retained. In contrast, the VN promoter mutant encompassing a disrupted Rel binding site, while also displaying drastically up to 75% reduced VN promoter activity, was no longer inducible by αvβ3, indicating the importance of Rel protein binding to the κB-motif of the human VN promoter for αvβ3-dependent VN induction (Fig. 4). In order to prove that the effect was not only true for one selected ovarian cancer cell line, we tested in parallel the activity of the VN promoter and the respective mutants in the widely used human ovarian cancer cell line OVCAR-3. In OVCAR-3 cells we found similar induction of VN by its major receptor αvβ3, even the extent was not as pronounced as in OV-MZ-6 cells (Fig. 4).

EFFECT OF REL PROTEINS ON HUMAN VITRONECTIN PROMOTER ACTIVITY

Based on the latter findings, we studied whether VN promoter activity in human ovarian cancer cells could be raised by enhancing Rel protein expression. For this, we conducted stable cell transfections and proved successful expression of active Rel proteins by luciferase reporter gene assays using a reporter gene plasmid encompassing as promoter three consecutive κB consensus motifs. p50-transfected cells exerted an up to twofold enhanced κB-promoter activity; elevation of p65 and c-Rel, respectively, led to an up to threefold increase. In case cells were transfected with Rel proteins in combination in order to promote formation of Rel dimers (p65/p50 [NF-κB]; p65/c-Rel), an up to fivefold increased κB promoter induction was noticable (Fig. 5A). In stable Rel- and αvβ3-transfectants, respectively, we then determined either the activity of the wild-type VN promoter or its Rel binding-deficient mutant. Stable overexpression of p50 and c-Rel, respectively, resulted in an approximately threefold induction; raising p65 levels provoked an up to sixfold upregulation. Strongest increases were observed upon combined Rel protein overexpression (p65/p50: eightfold; p65/c-Rel: up to sevenfold) and exceeded the induction by αvβ3 alone. As expected, none of the different Rel transfections altered the activity of the Rel binding-deficient VN promoter mutant (Fig. 5B). In addition, stable αvβ3- or vector-transfected cells were transiently transfected with p65/p50 (NF-κB) or p65/c-Rel and the activity of the human wild-type VN promoter and its Rel binding-deficient mutant measured. VN promoter upregulation by additional transient transfection with p65/p50 and p65/c-Rel, respectively, was strongest in stable αvβ3 cell transfectants, the extent of induction even exceeding the enhancing effect of stable αvβ3 transfection alone (Fig. 5C). As control, we transiently overexpressed the Rel inhibitor IκB-α which resulted, as expected, in an up to 70% (vector) and 80% (αvβ3) reduction of wild-type VN promoter activity. Neither expression of Rel proteins nor the inhibitor IκB-α exerted an effect on the activity of the Rel binding-deficient VN promoter mutant (Fig. 5C).

EFFECT OF VITRONECTIN INDUCTION ON INTEGRIN αvβ3-MEDIATED CELL MOTILITY

The impact of elevated VN expression on cell migratory activity was determined by wound scratch assays. For this, wild-type cells and stably αvβ3-transfectants, respectively, were transiently transfected with an expression vector encoding the Rel protein p65 in combination with that coding for p50 or, as control, with empty vectors alone. Microscopical images were taken directly after wounding as well as after 24 h of further cell incubation. As reported earlier, αvβ3-transfected cells showed a significantly increased migratory activity towards the wound gap when compared to wild-type cells with low endogenous αvβ3 expression. Here we demonstrate that this increased cell motility in αvβ3-transfectants was further enhanced upon transient elevation of p65/p50 in those cells, whereas transfection with empty expression vectors had no effect. Also, wild-type cells cells displayed increased closure of the wound gap upon transient transfection with p65/p50 (Fig. 6).

DISCUSSION

A variety of tumor cells express αvβ3 during their growth and metastatic phases and are more aggressive than those expressing inactive αvβ3 (Felding-Habermann et al., 1992, 2001; Takano et al., 2000). During ovarian cancer progression there is substantial evidence that αvβ3 in concert with VN crucially contributes to each

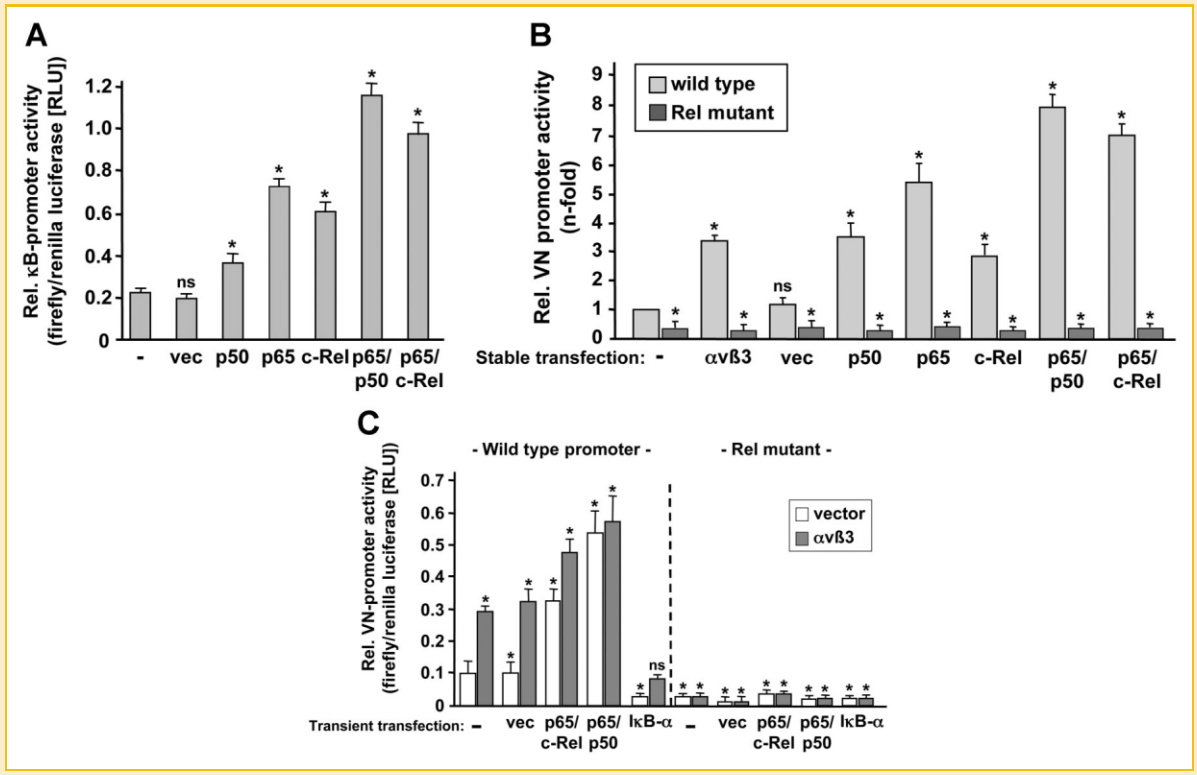

Fig. 5. Effect of Rel transcription factors on vitronectin promoter activity. A: Rel activity in stably Rel-transfected human ovarian cancer cells. In order to investigate the impact of Rel transcription factors on VN promoter activity, we generated stable human ovarian cancer cell transfectants expressing elevated levels of the Rel proteins p50, p65, and c-Rel, respectively, or in combination: p65/p50 (NF-κB) or p65/c-Rel. As controls served vector- (vec) as well as untransfected cells (−). In order to characterize the activity status of transfected Rel proteins, cells were transiently transfected with a luciferase reporter gene vector encompassing as promoter three consecutive κB-sites preceding the thymidine kinase promoter. Data are given as quotient of RLU obtained for firefly versus constitutively expressed renilla luciferase. Statistically significant differences ($P < 0.05$) in Rel κB-promoter activity when compared to untransfected control cells are indicated by an asterisk (ns = not significant). B: Effect of Rel proteins on vitronectin promoter activity. Stably Rel protein-overexpressing cells were transiently transfected with the human VN wild-type promoter or its Rel binding-deficient mutant. VN promoter activity was recorded as described. Data are calculated as quotient of RLU obtained for firefly versus renilla luciferase and given as "n-fold"-induction by setting the relative VN wild-type promoter activity in untransfected cells (−) to "1." Statistically significant differences ($P < 0.05$) when compared to wild-type VN promoter activity in untransfected control cells are indicated by an asterisk (ns = not significant). C: Effect of Rel proteins on vitronectin promoter induction by integrin αvβ3. Stably vector- (vec) or αvβ3-transfected human ovarian cancer cells, were transiently co-transfected with expression vectors encoding p65/p50 (NF-κB), p65/c-Rel, or the empty vector or left untransfected (−). As additional control, also the Rel inhibitor IκB-α was co-transfected. Dual luciferase reporter gene assays were conducted as described determining the activity of the human VN wild-type promoter versus that of its Rel binding-deficient mutant. Relative VN promoter activity is given as quotient of RLU obtained for firefly versus constitutively expressed renilla luciferase. Statistically significant differences ($P < 0.05$) in relative VN promoter activity when compared to that in vector-transfected control cells without further transient transfection are indicated by an asterisk (ns = not significant).

adhesion-dependent step. Our previous findings of an important tumor biological role of the αvβ3/VN system in human ovarian cancer cell adhesion, proliferation, and migration (Hapke et al., 2003) are in good agreement with data obtained by others. Also human ovarian IGROV1 adenocarcinoma cells migrated towards VN in an αvβ3-dependent fashion (Carreiras et al., 1999b). Moreover, in wound healing assays, restitution of the wounded area by human ovarian cancer cells was induced by the exogenous addition of VN in the presence of αvβ3 (Heyman et al., 2008).

Despite the importance of this integrin for the metastatic process, only few analyses of its occurrence in clinical tumor specimens have been published to date. Regarding ovarian cancer, the prognostic significance of αvβ3 had so far been investigated in a study with a relatively small patient cohort. Here, no correlation between αvβ3 and disease outcome was reported although lower αvβ3 levels were detectable in tumors of low malignant potential when compared to invasive carcinomas (Liapis et al., 1997). Goldberg et al. (2001) demonstrated that αv mRNA expression correlated with poor patient survival and αv was then suggested as a novel prognostic marker in advanced cancer stages. Carreiras et al. (1996) evaluated β3 expression in a small number of human ovarian tumors and found higher expression in well-differentiated cancers than in high-grade cancers. These findings were later confirmed in a larger patient cohort (Maubant et al., 2005). In contrast, more recently, β3 was reported to significantly suppress ovarian cancer cell growth and metastasis (Kaur et al., 2009). In order to clarify these existing discrepancies and to evaluate the impact of αvβ3 on tumor differentiation and aggressiveness, further investigations of larger patient cohorts are certainly needed.

The major αvβ3 ligand VN was also found to be associated with a variety of tumors by influencing cancer cell adhesion and motility through αvβ3-mediated mechanisms (Kellouche et al., 2010; Hurt

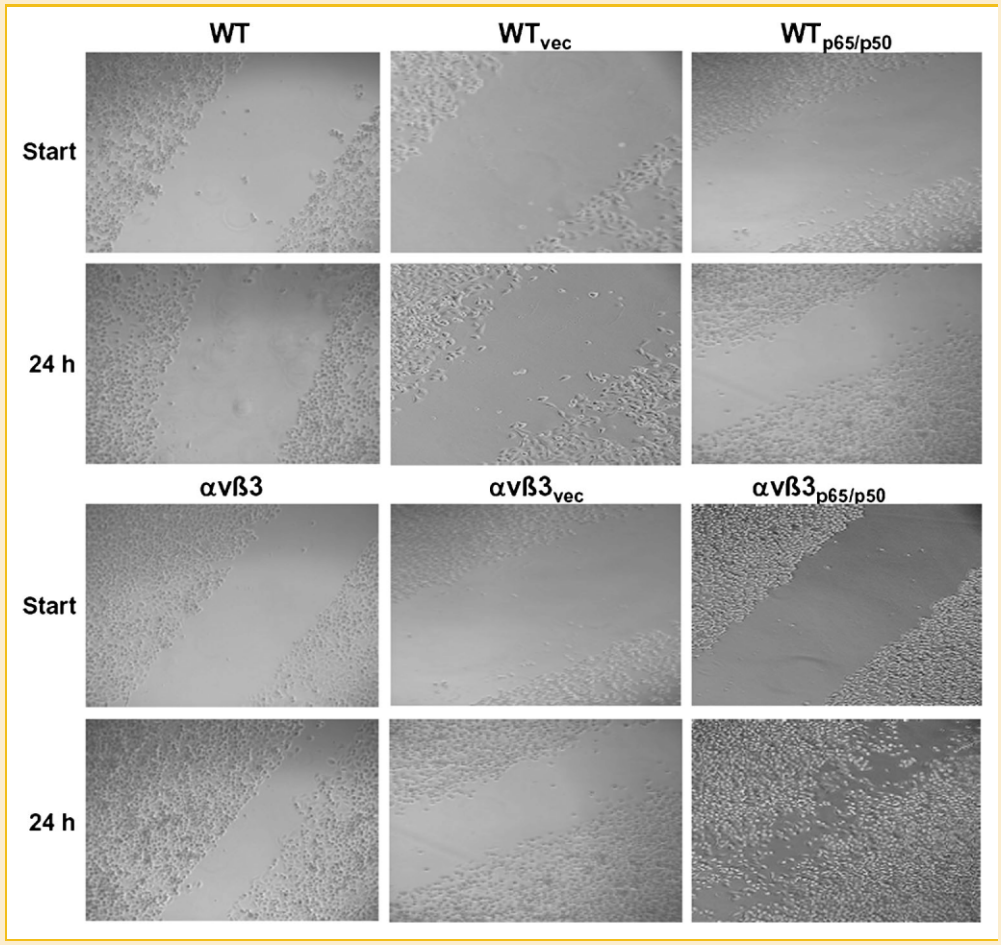

Fig. 6. Cell migratory activity as a function of integrin αvβ3-mediated vitronectin induction. Wound scratch assays were performed as described. After monolayers of wild-type (WT) and αvβ3-transfected cells (αvβ3) reached a confluency of approximately 70%, cells were transiently transfected with expression vectors encoding p65 in combination with that coding for p50. As control, cells were transfected with empty expression vectors alone. After another 12 h of cell cultivation, a homogeneous wound scratch was set. Microscopical images of monolayers were taken immediately after wounding (start) and after 24 h of further cell incubation.

et al., 2010). Still, little is known about the cellular origin and endogenous synthesis of VN. The predominant production site for VN is the liver. However, meanwhile, evidence had been provided for ubiquitous occurrence of VN mRNA in diverse other cells and tissues, including stromal cells derived from colorectal adenocarcinomas or cancer cell lines derived from cervix, lung, or pancreas (Yasumitsu et al., 1993; Tomasini-Johansson et al., 1994; Carreiras et al., 1999a). Also, in a series of human ovarian cancer cell lines and differentiated ovarian tumors, the presence of VN had been demonstrated which raised the possibility that tissue-associated VN is, at least in part, derived from local synthesis (Carreiras et al., 1996, 1999b; Liapis et al., 1997; Heyman et al., 2008). However, for a long time, it still remained a matter of debate whether cellular VN originated from direct synthesis or from internalization of VN-enriched extracellular fluids. Finally, direct synthesis of VN by human ovarian cancer cells was confirmed. In the present study, we not only provided evidence for VN expression in cultured human ovarian cancer cells but also documented VN protein upregulation as a function of αvβ3 which we were able to trace back to enhanced VN gene transcription. The fact that a VN promoter mutant which displayed a disrupted binding site for Rel transcription factors was no longer responsive to changes in αvβ3 pointed at least to a participation of Rel proteins in αvβ3-provoked VN induction. Indeed, Rel proteins are well known to affect cardinal features of neoplastic transformation by inducing genes involved in clonal cell expansion, adhesion, extravasation, angiogenesis, and ECM degradation (Pahl, 1999). Thus, it does not come as a surprise that cancer cells acquire constitutive nuclear activation and enhanced expression of Rel proteins in advanced ovarian cancer stages correlating with poor progression-free patient survival (Guo et al., 2008; Kleinberg et al., 2009). In line with this, also low IκB-α expression was observed in human ovarian cancer cell lines (Bours et al., 1994; Gilmore et al., 1996; Nakshatri et al., 1997; Dejardin et al., 1999; Ravi and Bedi, 2004). Indeed, in our study, ovarian cancer cell transfectants expressing increased Rel protein levels displayed significantly enhanced VN promoter activity. In agreement with our observations, also in glioblastoma cells, VN gene transcription and integrin expression was stimulated by the potent NF-κB-inducing phorbolester PMA or by NF-κB overexpression (Ritchie et al., 2000). Moreover, in cell migration studies, we

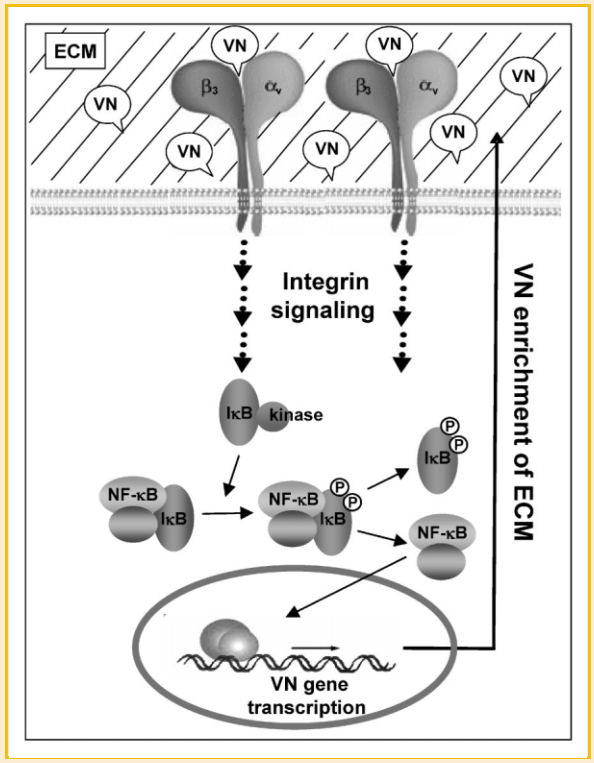

Fig. 7. Scheme of integrin αvβ3-mediated vitronectin induction. In the present study, we documented VN protein upregulation as a function of its major integrin receptor αvβ3. The effect was traced back to enhanced VN gene transcription. The fact that a VN promoter mutant displaying a disrupted binding site for Rel transcription factors was no longer responsive to αvβ3 indicated at least a contribution of Rel proteins to αvβ3-provoked VN induction. Thus, in an upregulated state, αvβ3 induces within a positive feedback regulatory loop VN expression in human ovarian cancer cells, resulting in VN enrichment of the surrounding cellular ECM microenvironment. This confers enhanced adhesive/migratory and cell signaling capacity to the cells leading to the modulation of the "reactive tumor microenvironment" in favor of tumor progression.

confirmed our previous data regarding increased cellular motility upon raising αvβ3 expression levels (Hapke et al., 2003). Here, in addition, we showed that cell migration was even further enhanced in αvβ3-transfectants by raising cellular Rel transcription factor p65/p50 levels.

Taken together, in the present study, we provided substantial evidence for VN protein upregulation as a function of its major integrin receptor αvβ3. The effect was mirrored by increases of VN gene transcription. The fact that a VN promoter mutant displaying a disrupted binding site for Rel transcription factors was no longer responsive to αvβ3 indicated at least a contribution of Rel proteins to αvβ3-provoked VN induction. Thus, in human ovarian cancer cells, in an upregulated state, αvβ3 induces within a positive feedback regulatory loop the expression of its own major ligand, resulting in VN enrichment of the surrounding cellular ECM microenvironment. This in turn endows the cells with enhanced adhesive/migratory and cell signaling capacity, modulating the "reactive tumor microenvironment" in favor of tumor progression (Fig. 7).

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