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# Influence of integrin αvβ3 activation on anoikis resistance of anchorage-dependent human ovarian cancer cells

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For Opaoma

# Abstract

Ovarian cancer is one of the most aggressive and prognostic inauspicious tumors. Especially after the formation of metastases, overall patient survival is significantly reduced. The metastatic process begins with the detachment of cancer cells from the primary tumor, followed by their survival under anchorage-independent conditions in ascites as single tumor cells or as spheroids till they eventually adhere again and colonize at different locations within the peritoneal cavity.

Upon loss of adherence to the ECM and/or neighboring cells within the primary tumor, per se anchorage-dependent epithelial-type cancer cells may gain the capacity to escape apoptosis/anoikis and survive anchorage-independently in the peritoneal fluid. During these processes, cell adhesion and signaling receptors of the integrin superfamily, including integrin αvβ3, are shown to be majorly involved.

In the present work, anoikis resistance, resistance to chemotherapy, and pro-survival signaling of human ovarian cancer cells suspended in ascites in dependence of the conformational transmembrane activation state of αvβ3 in a transfected cell model were investigated. The results indicated that a fully active αvβ3 displaying dissociated transmembrane domains (TMD) disclosing high ligand binding affinity and constitutive signaling competence favors delayed anoikis and increased cisplatin resistance. Indeed, this activated αvβ3 variant led to a pro-survival signaling due to an initiation of integrin-related intracellular signaling pathways including the focal adhesion kinase (FAK), src, and the protein kinase B (PKB/Akt). Moreover, in comparison to an inactive αvβ3 variant with associated TMD harboring low ligand binding affinity and lack of signaling capacity, constitutively active αvβ3 fostered epidermal growth factor receptor (EGF-R) expression and activation known to functionally cooperate with αvβ3. Furthermore, this ovarian cancer cell transfectant showed reduced cell proliferation in ascites, involving the upregulation of the cyclin-dependent kinase inhibitor $p27^{Kip-1}$. This also points to anoikis avoidance under inappropriate growth conditions via slowing down cell proliferation and thus the response to chemotherapeutic drugs.

Even more, constitutive αvβ3 activation led to the increased induction of anti-apoptotic factors, like Bcl-2 and survivin.

In conclusion, these findings demonstrate that anchorage-independent survival and, consequently, peritoneal metastatic colonization of ovarian carcinoma cells is dependent on the integrin αvβ3 TMD conformational activation state, underscoring the importance of targeting this receptor within cancer therapy.

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# List of abbreviations

| | |
|---|---|
| °C | Degree Celsius |
| Ab | Antibody |
| Apaf-1 | Apoptosis protease activating factor-1 |
| APS | Ammonium persulphate |
| BCA | Bicinchoninic acid |
| BRCA | BReast CAncer |
| BSA | Bovine serum albumin |
| CAM | Cell adhesion molecules |
| CDK | Cyclin-dependent kinase |
| CHO | Chinese hamster ovary |
| CLSM | Confocal laser scanning microscope |
| Cu | Copper |
| dest | Distilled |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | Dimethylsulfoxide |
| DNA | Deoxyribonucleic acid |
| DPBS | Dulbecco's Phosphate-buffered Saline |
| ECM | Extracellular matrix |
| EDTA | Ethylene diamine tetraacetic acid |
| EGF(-R) | Epidermal growth factor (-receptor) |
| EOC | Epithelial ovarian cancer |
| ErbB | Erythroblastosis oncogene B |
| FACS | Fluorescence activated cell sorting |
| FADD | Fas-associated protein with death domain |
| FAK | Focal adhesion kinase |
| FBS | Fetal bovine Serum |
| FIGO | *Fédération Internationale de Gynécologie et d'Obstétrique* |
| FITC | Fluorescein isothiocyanate |
| FN | Fibronectin |
| FSC | Forward scatter |
| g | Gravity |

| | |
|---|---|
| g/l | Gram/liter |
| GAPDH | Glyceraldehyde-3-Phosphate Dehydrogenase |
| GpA | Glycophorin A |
| h | Hour |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HRP | Horseradish peroxidase |
| HRP | Horseradish peroxidase |
| IAP | Inhibitor of apoptosis |
| IB | Incubation buffer |
| ICC | Immunocytochemistry |
| IgG | Immunoglobulin G |
| ILK | Integrin-linked kinase |
| l | Liter |
| M | Molar mass |
| mA | Milliamperes |
| mAb | Monoclonal antibody |
| MDCK | Madin-Darby canine kidney |
| min | Minutes |
| ml | Milliliters |
| mM | Millimolar |
| nm | Nanometers |
| NMR | Nuclear magnetic resonance |
| OD | Optical density |
| OV-MZ-6 | Human ovarian cancer cell line |
| PARP | Poly ADP-ribose polymerase |
| PI | Propidium iodide |
| PI3K | Phosphoinositide 3-kinase |
| PKB | Protein kinase B |
| PS | Phosphatidylserine |
| PSI | Plexin-semaphorin-integrin |
| PVDF | Polyvinylidene fluoride membrane |
| qcm | Square centimetres |
| Ras-ERK/MAPK | Rat sarcoma- extracellular-signal regulated kinase/mitogen activated protein kinase |

| | |
|---|---|
| RGD | Arginine-glycine-aspartic acid |
| RT | Room temperature |
| SD | Standard deviation |
| SDS | Sodium dodecyl sulfate |
| SDS-PAGE | SDS-polyacrylamide-gel-electrophoresis |
| SFKs | Src family kinases |
| SSC | Side scatter |
| TBS | Tris Buffered Saline |
| TEMED | Tetramethylethylendiamine |
| TMD | Transmembrane domain |
| TNF | Tumor necrosis factor |
| TNM | Tumor (T), nodes (N), and metastases (M) |
| Tris | Trishydroxymethylaminomethan |
| V | Volts |
| v/v | Volume/volume |
| VEGF(R) | Vascular endothelial growth factor (receptor) |
| VN | Vitronectin |
| w/v | Weight/volume |
| WT | Wild type |
| µl | Microlitres |
| µm | Micrometers |

# 1 Introduction

## 1.1 Ovarian Cancer – a clinical overview

Overall, ovarian cancer is located at the eighth position of the most common tumors of women and the third most frequent gynecological tumor type, after breast and endometrium cancer in Germany (Robert Koch-Institut, 2022). Data of the Robert Koch-Institute show that in 2019 7319 women fell ill with ovarian cancer. The prognosis with a 5-year survival rate of 42% in 2019 is unsatisfactory (Robert Koch-Institut, 2022). One reason is the often delayed diagnosis in a later stage (FIGO III/IV) (reviewed in Stewart et al., 2019), due to atypical symptoms. The majority of women affected consults doctors when they recognize an increase of the abdominal girth (Weyerstahl T., 2013). Further symptoms could be voiding disorders, disorders in defecation or in the gastrointestinal tract, and pains in the lumbar region (Goff et al., 2004). A typical appearance of sufferers is a raised belly because of the formation of ascites and peritoneal carcinomatosis in combination with a sunken face in form of cancer cachexia, the so called 'Facies ovarica' (Weyerstahl T., 2013). The probability to become diseased at the ovaries increases until the age of 85, the median age of ovarian cancer onset is 69 years (Robert Koch-Institut, 2022). Besides the age, risk factors include childlessness and no intake of oral contraceptives (Weyerstahl T., 2013). These factors let assume the impact of the number of ovulations in progression of ovarian cancer. The influence of environmental and dietary factors is not totally clarified. Further important facts in getting ovarian cancer are genetical reasons, whereas mainly BRCA 1 and BRCA 2 mutations are critical determinants (Doubeni et al., 2016).

There are different types of ovarian carcinoma, with the epithelial type being the most common (Roett & Evans, 2009).

The staging is classified via TNM- and International Federation of Gynecology and Obstetrics (FIGO)-categories as published in Berek et al. (2018): T1/FIGO I indicates a stage where tumor occurrence is restricted to one or both ovaries, whereas T2/FIGO II describes already tumor spread to the small pelvis. In stage 3/III, the tumor invades the peritoneum beyond the small pelvis and/or the occurrence of retroperitoneal lymph node metastases. In stage M1/IV, the tumor has spread to distant organs to form metastases. (Berek et al., 2018).

The most relevant therapy (accompanying with the staging) for ovarian cancer, independent of its grade of malignancy, is still the radical tumor debulking surgery by laparotomy including an inspection and palpation of the whole abdominal cavity with an irrigation cytology, a hysterectomy with adnexectomy, removal of the omentum majus, a pelvic and para-aortic

lymph node resection, an appendectomy (in case of mucinous and unclear type), and resection/ biopsy of each invaded (peritoneal) structure (Leitlinienprogramm Onkologie (Deutsche Krebsgesellschaft), 2022). If a macroscopic absence of tumor can be achieved and clinically inconspicuous lymph nodes are present, a pelvic and para-aortic lymph node resection should be avoided in advanced ovarian carcinomas, because of the lack of benefit in reduced postoperative morbidity and mortality (Harter et al., 2017).

The patient prognosis strongly depends on the success and the radicality of this staging-surgery, being best upon tumor-free resection (R0). Basically, the majority of patients (exception stage IA grade 1 after complete surgical staging) receive an adjuvant chemotherapy consisting of Carboplatin, in advanced stages (FIGO II-IV) in combination with Paclitaxel (Roett & Evans, 2009). At the stage of FIGO IIIB, IIIC and IV (according to the 'old' FIGO classification of 2009: corresponds to stages FIGO IIIA1 and IIIB-IV in the current FIGO classification), in addition Bevacizumab, a humanized monoclonal antibody directed to the vascular endothelial growth factor (VEGF), is recommended (Leitlinienprogramm Onkologie (Deutsche Krebsgesellschaft), 2022; Oza et al., 2015; Perren et al., 2011). Patients afflicted with high-grade epithelial ovarian cancer at stage III/IV and also confirmed BRCA-mutation, who showed response to the platin based-first line therapy are further treated with poly ADP-ribose polymerase (PARP) inhibitors, for instance Olaparib, as maintenance treatment (Lheureux et al., 2019). Niraparib is recommended as monotherapy for maintenance treatment of advanced epithelial (FIGO stages III and IV) high-grade ovarian carcinoma after sensitive first-line platinum-based chemotherapy (Gonzalez-Martin et al., 2019). In special cases, radiotherapy is indicated for the treatment of ovarian cancer recurrence to control symptoms (Yang et al., 2020). Prognostic factors are besides the postoperative tumor rest and the therapy in accordance with the regulatory guidelines, the tumor stage, the age of the woman, the grading, the histological type of the tumor, and also the general condition of the patient (Leitlinienprogramm Onkologie (Deutsche Krebsgesellschaft), 2022). Although the success of the therapy of ovarian cancer and therefore the survival rate increased during the last years, the overall survival is still not satisfactory. Especially the late diagnosis makes a curative therapy most difficult and often not any more achievable. In 2019 5291 women died of ovarian cancer in Germany (Robert Koch-Institut, 2022). Hence, the metastasis process and the involved mechanisms and factors, which are known so far, are discussed below.

## 1.2 Ovarian cancer progression at the cellular level

Ovarian cancer cells spread mostly by two different ways: on one hand, metastasis occurs after disruption of the ovarian capsule, followed by the shedding of primary tumor cells into the peritoneal fluid or the ascites. Eventually, the tumor cells reattach then to adjacent organs, for instance the uterus or the fallopian tubes, and to structures like the peritoneum. This special form of metastasis is called peritoneal carcinomatosis. Moreover, floating tumor cells may be distributed to other pelvic and abdominal organs and may even build tumor knots on the diaphragm. Another frequent localization to build metastases is the omentum. On the other hand, ovarian cancer cells spread via lymphatic vessels followed by a formation of lymph node metastases, especially paraaortic and pelvine. Hematogenous metastasis of ovarian carcinoma cells is less frequent and occurs very late compared to other cancer types, however possible regions are the liver, the pleura, and also the lungs. (reviewed in Lengyel, 2010; Lisio et al., 2019).

The process of cancer cell shedding from the primary tumor, the anchorage-independent survival in ascites, and the invasion of different metastasis sites require various mechanisms on the cellular level, which are not yet entirely understood (reviewed in Zeeshan & Mutahir, 2017). Whereas a normal epithelial-type cell, which is detached from the underlying extracellular matrix (ECM) undergoes a special form of apoptosis which is called anoikis (Frisch & Francis, 1994; Meredith et al., 1993), tumor cells may gain the ability to resist the apoptotic process and to survive under anchorage-independent conditions (Chiarugi & Giannoni, 2008; Frisch & Screaton, 2001). In the case of ovarian carcinoma cells, shed primary tumor cells float as single cells, but also as multicellular aggregates, the so called spheroids, within the peritoneal fluid, or ascites (Shield et al., 2009). The formation of spheroids fosters epithelial tumor cells to survive under suspension by enhancing cell-cell contacts (reviewed in Zhong & Rescorla, 2012), on the other, it protects them from chemotherapeutic agents. Thus spheroid formation represent a major challenge in the context of chemoresistance (Shield et al., 2009).

In all of these events, cell-ECM and cell-cell adhesion processes play crucial roles.

The key players are cell adhesion molecules (CAM). For detachment of a single cancer cell from its main tumor, the expression of the adhesion molecule epithelial (E)-cadherin is downregulated and switched to an upregulation of neural (N)-cadherins or platelet (P)-cadherins (reviewed in van Baal et al., 2018). During flotation in the peritoneal fluid and especially for reattachment in different locations, cell adhesion and signaling receptors of the integrin superfamily take over major roles (Shield et al., 2009). Integrins are heterodimeric transmembrane receptors, which are (over-)expressed in various malignant cancer types, also

in ovarian carcinoma (Nieberler et al., 2017). Due to their ability to recognize proteins like vitronectin, fibronectin, or laminin they mediate the formation of cell adhesion to the extracellular matrix (ECM) (Nieberler et al., 2017). This is followed by the initiation of an integrin-related bi-directional signaling cascade and tumor biological processes arising thereof, like cell proliferation, migration, and invasion (Takada et al., 2007).

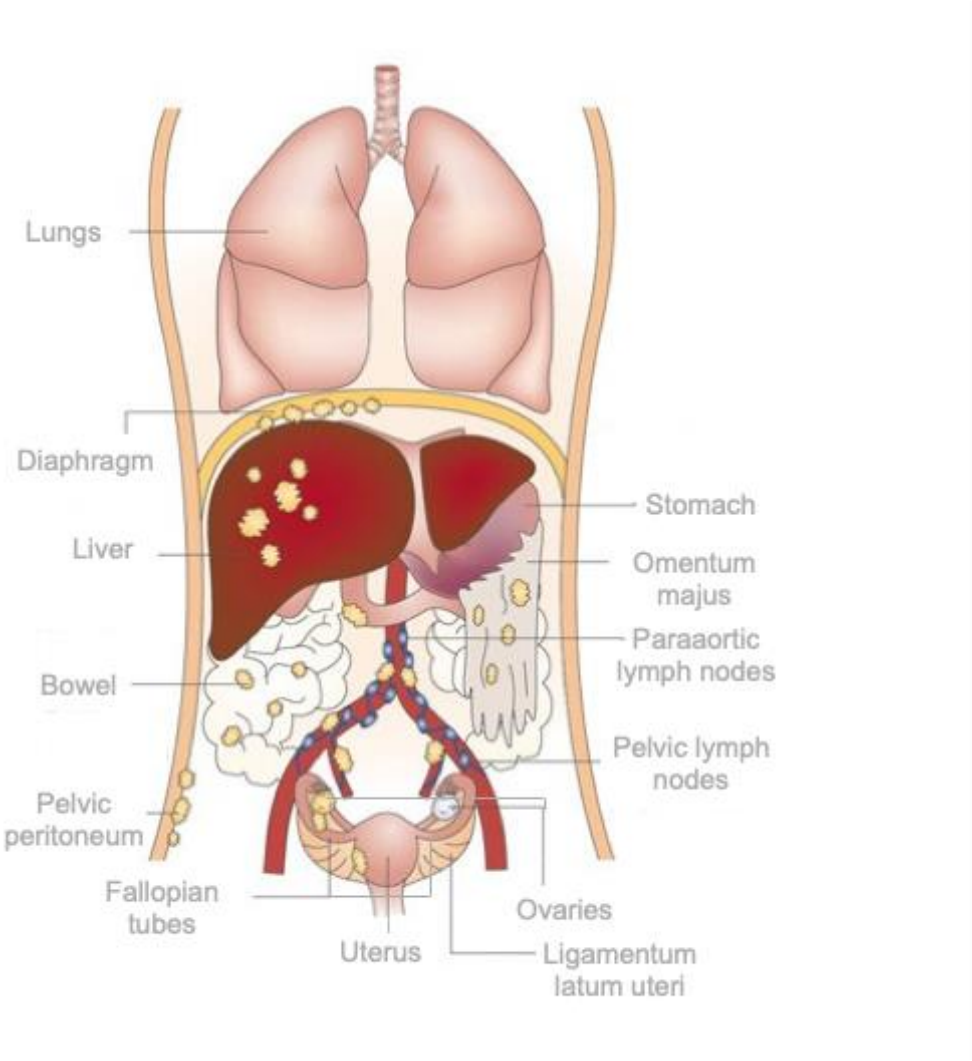


**Figure 1 Depiction of the metastatic process in ovarian cancer**
Naora and Montell (2005) illustrated the metastatic lesions originating by a primary ovarian carcinoma. After the detachment of a cancer cell from the primary ovarian tumor, it floats in the peritoneal fluid or in ascites and reattach on secondary structures. First metastatic stations are organs and structures in the small pelvis, like the uterus or the fallopian tubes. With increasing malignancy, the suspended ovarian cancer cells invade structures beyond the small pelvis and also the peritoneum. After attachment to these structures in different locations, ovarian cancer cells build secondary tumor knots. Another mechanism in building ovarian cancer metastasis is by lymphogenous spread. Major metastatic regions are the pelvic and paraaortic lymph nodes. The hematogenous ovarian cancer cell spread is rather rare but may affect the lungs. (Figure from Naora & Montell, 2005).

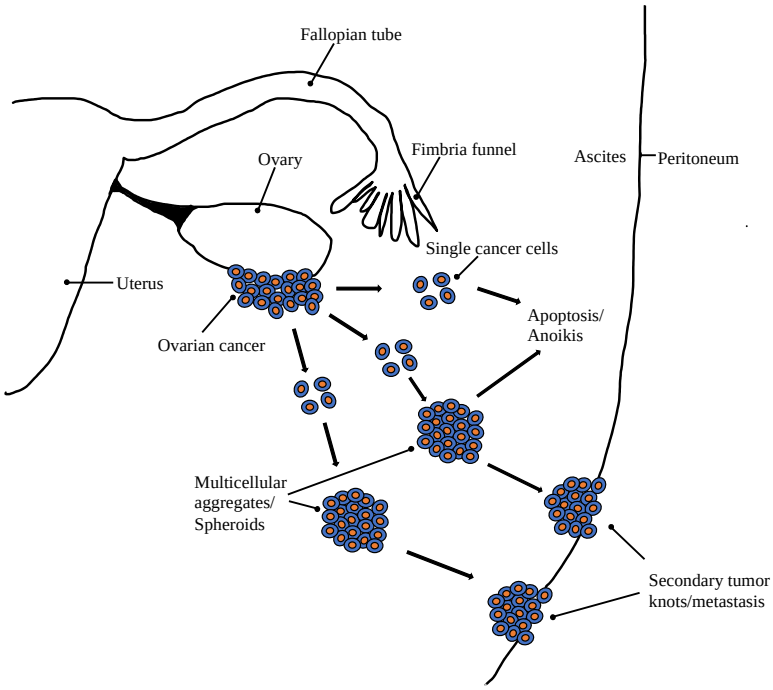


**Figure 2 Ovarian cancer progression**
Early steps of ovarian cancer progression involve the disruption of the primary tumor capsule, followed by the detachment of ovarian cancer cells into the peritoneal fluid (or ascites). Whereas physiologically, a detached epithelial cell without attachment to the underlying ECM undergoes a special form of apoptosis which is called anoikis, cancer cells may develop the ability to evade this process and survive under anchorage independence. Shed ovarian cancer cells float as single cells, but also build multicellular spheroids, which fosters the survival due to cell-cell contacts, but also provides a protection against chemotherapeutic agents. This cell survival enables further tumor spread through metastasis and thus represents a major problem in the context of tumor control. (Fig. modified according to Ahmed et al., 2007).

## 1.3 The superfamily and the structure of integrins

Integrins in common are expressed in various different cell types, e.g., in platelets, leukocytes, endothelial and epithelial cells but also in cancer cells, in the latter frequently at elevated expression levels (Dejana, 1993; Hynes, 1992). Besides their involvement in many physiological processes, such as hemostasis, tissue repair and organ development, they take over major roles in tumorigenesis, like tumor growth, invasiveness, cancer aggressiveness and metastasis (reviewed in Nieberler et al., 2017).

Their main function as a cell membrane receptor is to mediate communication to the ECM, but some integrin subtypes do also promote cell-cell interactions. They are also involved in cell migration, proliferation, differentiation, and the regulation of cell survival and death through the so-called apoptosis.

Integrins consist of one of 18 α and one of 8 β subunits, which are non-covalently linked (Hynes, 1987, 2002). Different combinations of these two subunits result in a total of 24 known human integrins (Hynes, 1987, 1992; van der Flier & Sonnenberg, 2001).

The integrin subunits are composed of a large extracellular ligand binding domain (N-terminus), a hydrophobic transmembrane region (TMD) and a relatively small cytoplasmic domain (C-terminus) (Hynes, 2002). Xiong et al. published the crystal structure of the extracellular tail by using the example of the integrin αvβ3 and determined a head which is linked to two legs (Xiong et al., 2001). The head is built out of a seven-bladed β-propeller from αV and β3 tail, which is linked to an immunoglobulin (Ig)-like 'hybrid' domain in β3. The regions on the top of the two extracellular domains constitute the ECM ligand binding site.

The αv tail is further formed of three β-sandwich regions: an Ig-like upper thigh and two similar modules that constitutes the lower thigh, the so-called calf-1 and calf-2. In the extracellular region of the β3 chain a plexin-semaphorin-integrin (PSI)-, four epidermal growth factor (EGF) domains and a β-tail are attached to the Ig-like hybrid domain. (Hynes, 2002; Xiong et al., 2001).

One integrin subtype may bind different ECM ligands like e.g., vitronectin and fibronectin, the recognition motif RGD (arginine-glycine-aspartic acid) and, vice versa, one distinct ligand may be bound by various integrin subtypes (Hynes, 1992; Ruoslahti, 1996; van der Flier & Sonnenberg, 2001).

Due to the non-covalently linkage of the α and the β subunit, integrins own a very flexible structure, which enables a series of conformational changes during their function as adhesion receptors, but also in their role as transmitters of signals across the cell membrane.

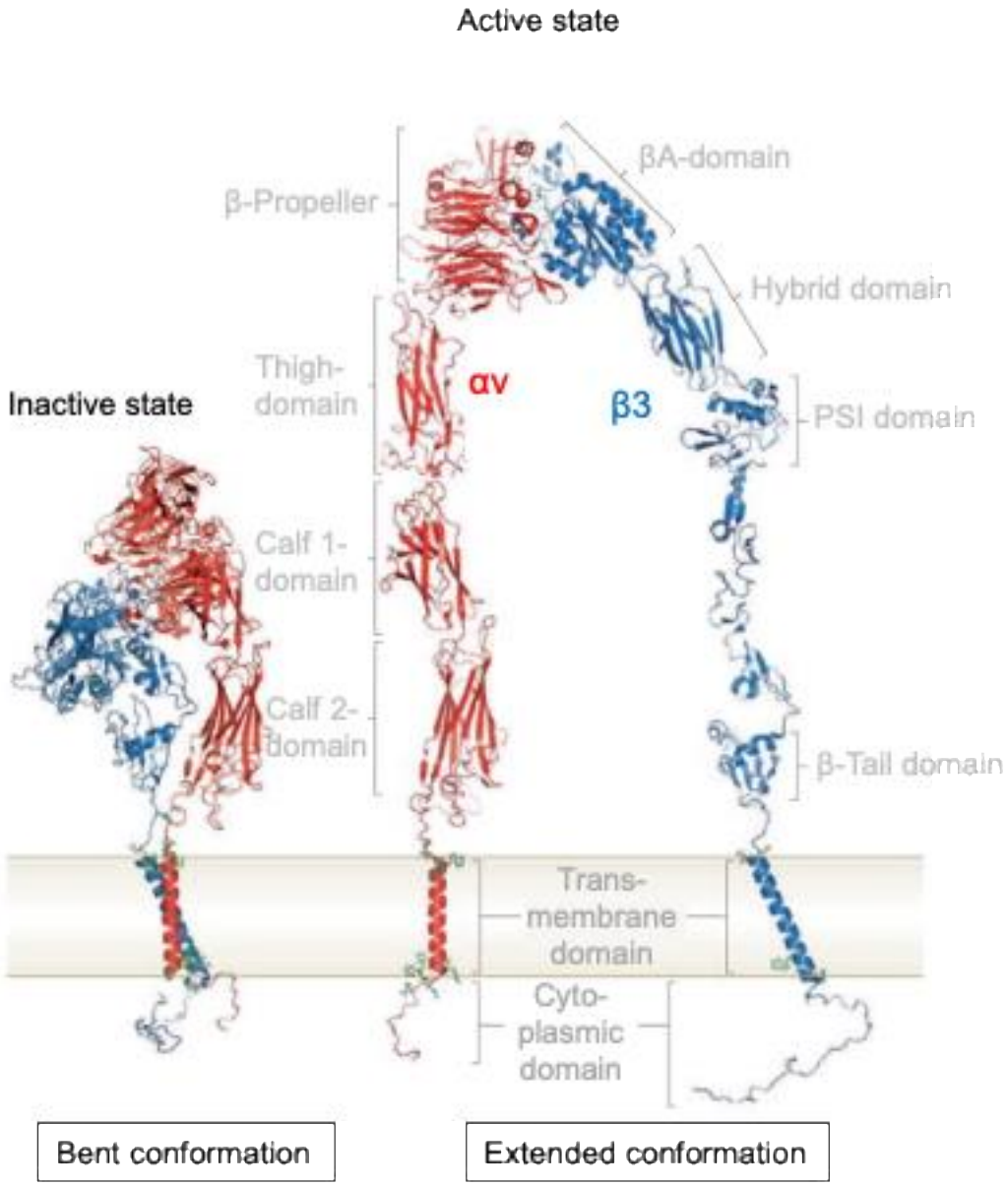


**Figure 3 The integrin structure by the example of the integrin *αv*β3 and its conformation in an active and inactive state**

Integrins are heterodimers, which consist of one α and one β subunit. Every subunit is separated in an extracellular domain, a transmembrane domain, and a short cytoplasmic tail. The figure depictures on the left side the inactive, bent integrin. On the right side the active and erected integrin is presented, where the individual subunits are better distinguishable. The extracellular area of the α subunit is structured in a β propeller unit and a protein tail, which is separated in a thigh and two calf domains. The extracellular region of the β subunit a βA-domain is linked to an Ig-like hybrid domain and a PSI domain, closed with a short β-tail domain. The two head regions of the both subunits build the extracellular ligand binding site. (Fig. modified according to Shattil et al., 2010).

## 1.4 The integrin αvβ3 and its role in cancer

In the investigations to the present doctoral thesis, the main scientific focus was laid on the integrin αvβ3. Beside other integrins, αvβ3 is expressed in various cell types, amongst others in cancer cells. Its overexpression in tumor cells of various cancer entities promotes tumor invasion and metastasis and, consequently, poor patient prognosis (reviewed in Nieberler et al., 2017). As such, both integrin subunits (αv and β3) are expressed in normal ovarian epithelium and also in highly differentiated ovarian carcinomas (Carreiras et al., 1996). Furthermore, it could have been shown, that levels of the integrin αvβ3 are higher in invasive than in low malignant ovarian cancer cells (Liapis et al., 1997). These detections already indicate the importance of this integrin family member in tumor progression. It also fosters proliferation and migration of an ovarian cancer cell (Carreiras et al., 1999; Hapke et al., 2003). So, these integrin αvβ3-mediated contacts contribute to the characteristic peritoneal carcinomatosis occurring in advanced ovarian carcinoma stages (reviewed in van Baal et al., 2018). Also, αvβ3 expression in invasive melanoma cells is accompanied by a higher metastatic potential (Gehlsen et al., 1992). In malignant breast cancer cells, higher levels of integrin αvβ3 and also vitronectin were observed, which makes them promising biomarkers for detection of early stage breast cancer (Radwan et al., 2019).

The integrin αvβ3 was formerly named vitronectin (VN) receptor, because of its preferred ECM ligand VN (Pytela et al., 1985a). However, αvβ3 is a highly promiscuous integrin since it interacts with a whole panel of different ECM ligands.

The first publication of the integrin αvβ3 occurred in 1985 by Pytela and co-workers. They described a VN and a fibronectin (FN) receptor, which recognizes the proteins via their RGD-motif and initiate consequently cell adhesion to the ECM (Pytela et al., 1985a, 1985b). However, further studies showed, that this peptide sequence also exists in other proteins (D'Souza et al., 1991). Hynes et al. published in 1992 that the denotation 'VN-receptor' for integrin αvβ3 is too limited, because integrins bind other proteins besides vitronectin and vitronectin can be recognized by other receptors (Hynes, 1992).

Moreover, integrin αvβ3 plays a crucial role in angiogenesis. Due to its expression on angiogenetic endothelial cells, it is recognized as a predominant marker for tumor angiogenesis, empowering tumor cells to enhance growth and metastasis (Brooks et al., 1994). Studies showed, that the interaction of the integrin αvβ3 with the vascular endothelial growth factor receptor 2 (VEGFR-2), a receptor tyrosine kinase, contributes to neovascularization (Bazzazi et al., 2018). More precisely, the phosphorylation of VEGFR-2, induced after binding of the vascular endothelial growth factor-A (VEGF-A), is increased when endothelial cells bind via

integrin αvβ3 to ECM proteins, such as VN, leading to enhanced angiogenesis in physiological processes like wound healing, but also in cancer progression (Soldi et al., 1999; Somanath et al., 2009).

Not only the interaction with VEGFR, but also the important cooperation with the epidermal growth factor receptor (EGFR) leads to enhanced tumor cell proliferation and survival as well as to increased migratory activity (Guha et al., 2019).

Besides their function as cell adhesion receptors, integrins are able to mediate bidirectional signaling (*outside-in* and *inside-out*) across the cellular membrane. For this process, integrins must be activated upon binding to an ECM ligand as well as to intracellular proteins.

However, the exact mechanism of the integrin activation and the associated changes in the conformation, as well as the bidirectional transmission of signals are not yet fully understood.

## 1.5 Conformational changes of integrins during the activation process and integrin-mediated signal transduction

For high binding affinity of integrins towards ECM ligands and their fully active signaling competence, several conformational changes in both integrin subunits have been proposed in recent years. This does not only involve the extracellular domain but also structural alterations in the transmembrane and short cytoplasmic integrin domains.

So far, three different integrin activation states have been proposed: 1.) a bent, inactive status 2.) an extended state of the extracellular domains with closed headpieces and an associated TMD, which displays an intermediate status, and 3.) an erected, activated state with dissociated headpieces and a separated TMD resulting in an activated integrin with full signaling capacity (Campbell & Humphries, 2011; Gottschalk, Gunther, et al., 2002; Takagi et al., 2002).

Xiong et al revealed in 2001 the crystal structure of the extracellular domain of the integrin αvβ3 (2001). For the extracellular tail, a bent inactive state with low affinity has been described. During the integrin activation process, the extracellular domains erect and the headpieces open, leading to a higher affinity towards ECM ligands (Takagi et al., 2002). However, for the activation of integrins not only an erection of the two extracellular subunits is necessary, but also the separation of the TMD (Hantgan et al., 1999). This domain was already point of interest in integrin research. Using the platelet integrin αIIbβ3 as an example for the integrin conformation in the low affinity state, two right-handed helices, which build a coiled-coil structure with interactions over the whole length of the two subunits, were disclosed by NMR spectroscopy (Lau et al., 2009). By studying integrin TMD association and separation during the activation process, Senes et al. recognized a GxxxG-like motif in the integrin TMD, which

showed close similarity to the GxxxG-dimerization within the TMD of the strongly dimerizing erythrocyte protein glycophorin A (GpA) (Chasis & Mohandas, 1992; Senes et al., 2004). This GxxxG-motif mediates strong association between the two transmembrane subunits of this protein (Senes et al., 2004). Moreover, various studies suggested, based on diverse experimental methods and molecular modeling (Vinogradova et al., 2002), that in the resting integrin state (Xiong et al., 2001) a firm association of the two integrin TMD does not allow integrin signaling (Luo et al., 2004).

Based on these findings, Prof. Dr. Reuning and colleagues established integrin αvβ3-GpA-chimera in order to study integrin activation and signaling as a function of the integrin TMD conformation (Müller et al 2013). As cell model human ovarian OV-MZ-6 cells were transfected with different αv- and β3-expression plasmids. By molecular engineering, they exchanged in both subunits of αvβ3 the TMD by the complete TMD of GpA (TMD-GpA). This integrin variant displayed in human OV-MZ-6 cells an intermediate integrin activation state exhibiting low binding affinity for ECM proteins as well as lack of signaling competence. For investigation of the function of an integrin with unclasped TMD, a second chimera was generated, in which the TMD-dimerization motif GxxxG was mutated to GxxxI (TMD-GpA-I). This mutation is well known from studies on GpA to no longer mediate TMD dimerization. This led to a high affinity integrin with full and constitutively active signaling capacity. (Muller et al., 2013). (Figure 4) These findings underlined the crucial role of integrin TMD in the context of conformationally deriven integrin activation and signaling, which is the focus in this doctoral thesis.

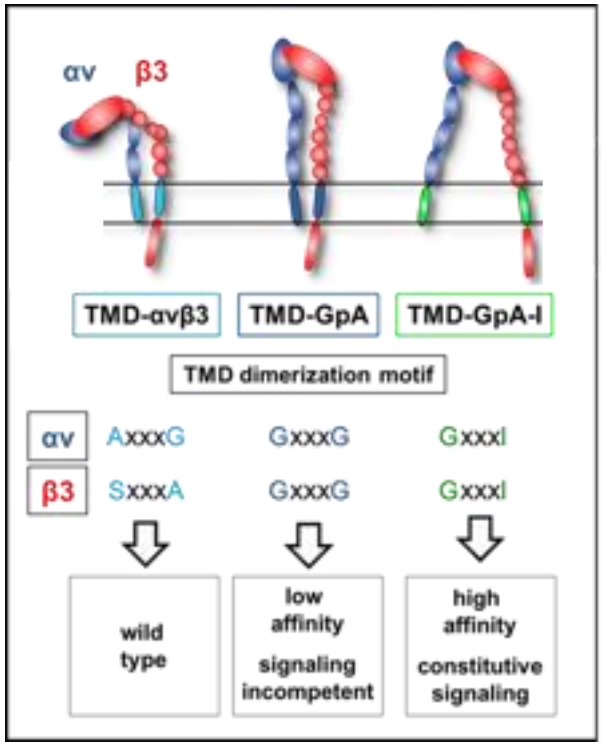


**Figure 4 Epithelial ovarian cancer (EOC) αvβ3 cell transfectant model**
Shown are the cell transfectants used in this dissertation project. In addition to the αvβ3 wild type, other variants were generated in previously performed projects of the working group of Prof. Dr. Reuning. For generation of the TMD-GpA variant, the complete TMDs of the αv and β3 subunits were replaced by the strongly dimerizing TMD of GpA, which is associated with low ligand binding affinity and signaling incompetence.
This was compared with the TMD-GpA-I transfectant, for which the dimerization motif within GpA, GxxxG, was mutated to GxxxI, which is known to abrogate TMD association, resulting in a high affinity αvβ3 receptor with constitutive signaling capability. (Fig. from Dolinschek et al., 2021; published in Muller et al., 2013).

Also, the cytoplasmic tails play a huge role during integrin activation. Hughes et al. postulated for the α- and the β-cytoplasmic tail a specific motif: GFFKR for the α-, and LLv-iHDR for the β-subunit (Hughes et al., 1996). Moreover, various studies described a membrane-proximal, cytoplasmic interchain salt bridge between the α-(R995) and the β-(D723) subunit, which fosters the inactive integrin state with associated, bent subunits (Hughes et al., 1996; Lau et al., 2009; Vinogradova et al., 2002). Müller et al. also investigated the effects of a postulated salt bridge in the cytoplasmic integrin domains on integrin activation and signaling (2014). They found that the disruption of the salt bridge increased the adhesion process to proteins of the ECM, like VN, and moreover, enhanced *outside-in* and *inside-out* signaling (Muller et al., 2014). (Figure 6) Furthermore, studies revealed that physiologically the binding of talin to the β-cytoplasmic tail is the main step in activation of integrins (Tadokoro et al., 2003). After binding, the interchain salt bridge disrupts, leading to a dissociation of the cytoplasmic regions and the TMD resulting in an activated integrin (Anthis & Campbell, 2011). However, besides

talin, also kindlin represents an important integrin activator (Meves et al., 2009). This intracellular activation, followed by conformational changes of the two subunits, enables signal transmission from the cellular inside to the outside, which regulates the binding affinity to ECM ligands and consequently the adhesive capacity (reviewed in Anthis & Campbell, 2011). (Figure 5)

In contrast, however, the binding of extracellular proteins to the integrin subunits mediates triggering of diverse intracellular signaling pathways to the interior of the cell that, among other things, are responsible for initiation of cell proliferation, migration, and survival (Hynes, 2002).

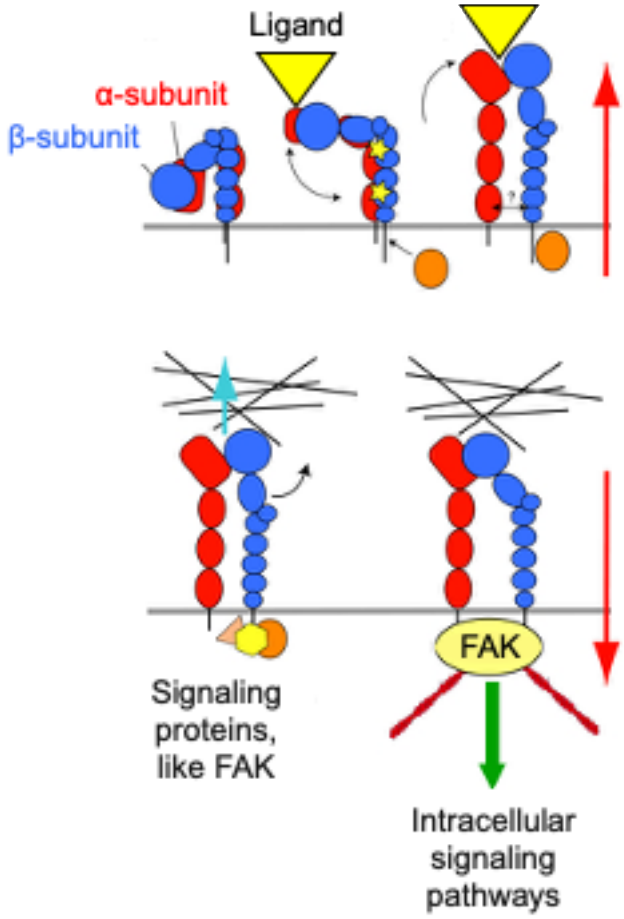


**Figure 5 Proposed conformational changes during integrin activation**
An inactive, resting integrin state is characterized by bent subunits (A). After binding of an intracellular protein like talin to the β subunit, the whole integrin gets activated and erects (B). This activation also leads to an enhanced affinity to extracellular ligands. This process describes the *'inside-out'* signaling (A-C). After connection to the ECM by the integrin head pieces, signals from extracellular are transmitted to the cell interior (D), followed by an activation of integrin-related signaling pathways, for instance via the focal adhesion kinase (FAK) (*'outside-in'* signaling) (E). Due to these mechanisms, integrins are responsible for the cell behavior, like survival, proliferation, or migration. (Fig. modified according to Askari et al., 2009).

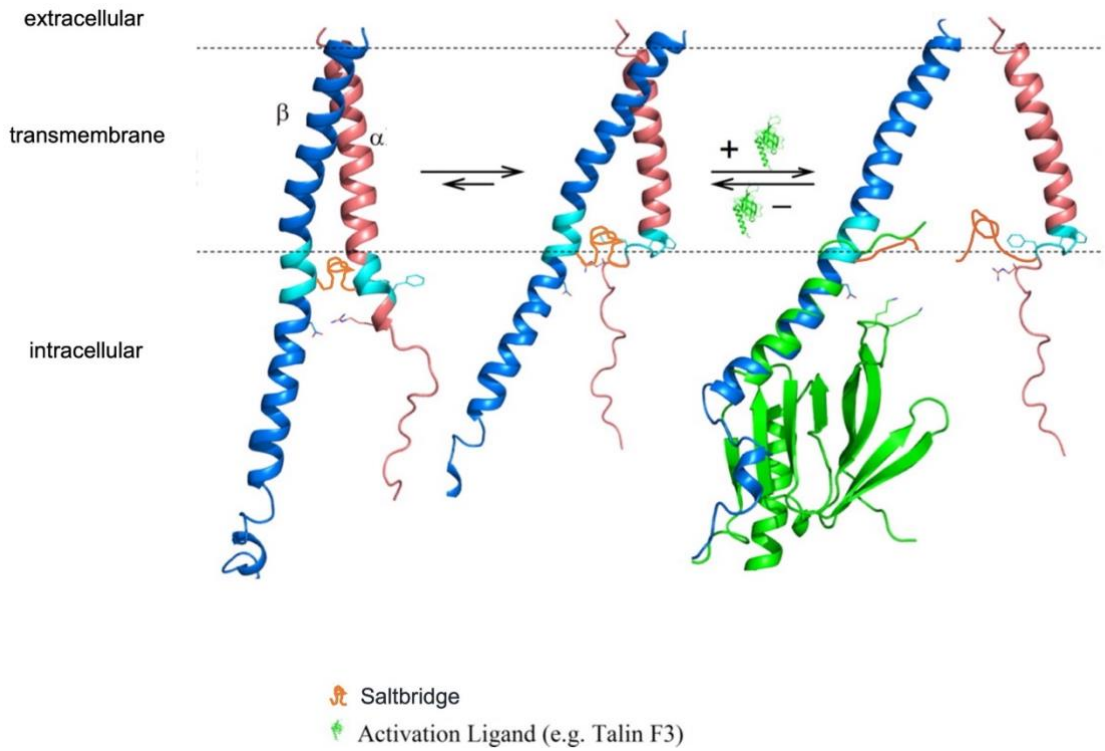


**Figure 6 The integrin TMD and its conformational change during activation**
The TMD has a crucial role in transmitting of signals over the cell membrane (*'inside-out'* and *'outside-in'* signaling). The integrin subunits and their connection via a putative salt bridge are depictured. This salt bridge stabilizes via electrostatic forces the integrin in an inactive resting state. After binding of an intracellular protein, like talin, to the β subunit the two subunits separate. During this process, the salt bridge between the subunits disrupts and the integrin is fully activated. This state leads to an enhanced affinity to extracellular ligands and also to higher competence in signaling (Fig. modified according to Yang et al., 2009).

## 1.6 Integrin-mediated cell signaling pathways

The role of the integrins as transmembrane receptors with their main function as adhesion receptors enables transmission of signals over the cellular membrane. For this they do not have intrinsic kinase activity but collaborate with other integrins by clustering as well as other cell surface receptors in focal adhesions in order to transmit signals *outside-in* and *inside-out* (Shattil et al., 2010).

The extracellular tails of the integrin remain active and bind ligands. Ligand-bound integrins build via talin a linkage to the intracellular actin network, further leading to the activation of FAK and src family kinases (SFKs), resulting in the initiation of intracellular signaling pathways, including the phosphoinositide 3-kinase (PI3K/Akt), and the rat sarcoma-extracellular-signal regulated kinase/mitogen activated protein kinase (Ras-ERK/MAPK) pathway (reviewed in Cooper & Giancotti, 2019; Schwartz et al., 1995). (Figure 7)

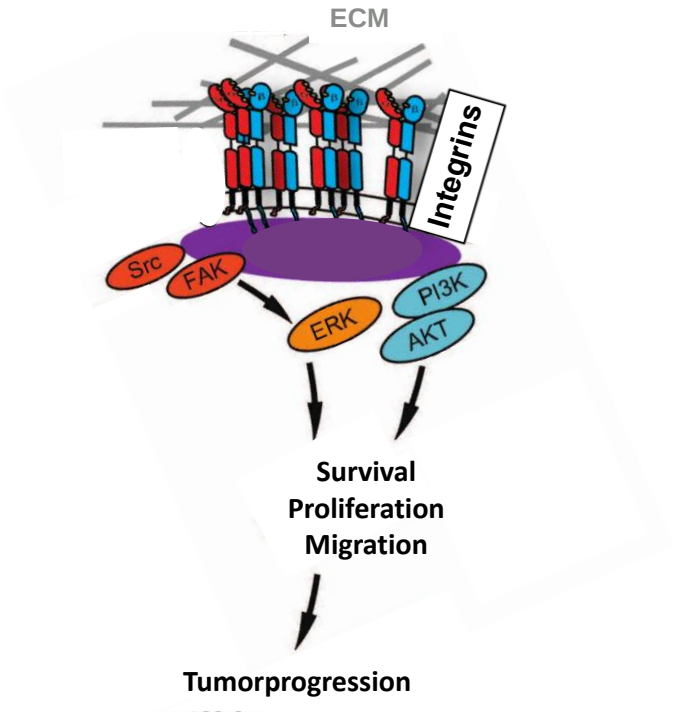


**Figure 7 Overview of integrin-related intracellular signaling cascades**
Signals from the cellular outside lead to further initiation of integrin-mediated intracellular signaling pathways resulting in cell proliferation, migration, or survival. Thus, in cancer cells the activation of the various signaling molecules like FAK, src, and PI3K/Akt leads to tumor progression. (Fig. modified according to Young, 2013).

### 1.6.1 Focal adhesion kinase (FAK)

FAK is a non-receptor protein-tyrosine kinase which is involved in integrin-related signaling pathways. After integrin binding to ECM proteins, like VN or FN, clustering of adjacent integrins is triggered into focal adhesion sites, where signaling takes place. FAK gets activated via autophosphorylation, which then generates SH2-binding sites for members of the src-family kinases (Calalb et al., 1995; Schaller et al., 1994). In turn, these kinases then phosphorylate other binding sites of FAK, which results in a fully activated FAK and an enhancement of the initiation of integrin-mediated intracellular signaling pathways (Schaller et al., 1994; Schlaepfer et al., 1994).

It is well known that FAK expression in cancer cells correlates with increased cell proliferation and migration leading to facilitated cancer invasiveness and metastasis (reviewed in Sulzmaier et al., 2014).

### 1.6.2 Src family kinases

Src kinases are cytoplasmic tyrosine kinases. The src family contains ten proteins and the core member is c-src (Thomas & Brugge, 1997). Each of the members has the same organization: a N-terminal domain, followed by SH3, SH2, linker and tyrosine kinase regions and a short C-terminal segment (citated in Parsons & Parsons, 2004). After mutations of the cellular src in the Rous sarcoma virus, the proto-oncogene v-src was formed (Brown & Cooper, 1996). Over the years research has shown many important src-related roles on the cellular level, such as in cell proliferation, migration, or adhesion (Thomas & Brugge, 1997). Moreover, it was shown that integrin activation by ECM ligands triggers the recruitment of src, which also is capable of activating intracellular signaling molecules, for instance FAK (Playford & Schaller, 2004).
C-src, the product of the human src-gene, was found to participate in the progression of various human cancer types (Irby & Yeatman, 2000).
In platelets, it was demonstrated that src gets activated after binding to the β3 subunit of the αIIbβ3 integrin (Obergfell et al., 2002). Under normal conditions, epithelial-type cells are anchorage-dependent, which is mediated by integrins after their binding to ECM ligands via the activation of src and FAK in the downstream signaling cascades leading to the triggering of the PI3K/Akt, and/or MAPK/Erk pathways (summarised by Shishido et al., 2014). Consequently, abrogation of cell/ECM interactions impacts on cell survival, triggering in most non-transformed cell types a special kind of apoptosis, named ‘anoikis’ (Chiarugi & Giannoni, 2008). Anoikis is an important event during tissue development and homeostasis as well as in several diseases, including cancer. However, in contrast, tumor cells are frequently not sensitive to anoikis, meaning they acquire anchorage-independence which appears as a hallmark of tumor growth, survival of circulating tumor cells, and metastasis (Carduner et al., 2014; Chiarugi & Giannoni, 2008). As such, Desgrosellier et al. (2009) showed in pancreatic cancer cells, which express the integrin αvβ3 that despite of losing contact to the ECM, the non-adherent tumor cells progressed due to c-src activation after binding to the β3 domain. Surprisingly, this process occurred independently of FAK activation (Desgrosellier et al., 2009).

### 1.6.3 The protein kinase B/Akt pathway

Another integrin-mediated member of intracellular signaling proteins is the phosphatidylinositol 3-kinase (PI3-kinase) (King et al., 1997). This kinase is necessary to activate Akt, a serine/threonine kinase (Burgering & Coffer, 1995). To date, three very similar members of these serine/threonine kinases are known: PKBα (Akt1), PKBβ (Akt2), and PKBγ (Akt3), which take a major role in cancer progression (reviewed in Nicholson & Anderson,

2002). One mechanism of Akt, which leads to cellular survival that has also been observed in cancer cells, is the interaction by phosphorylation resulting in inactivation of pro-apoptotic factors like Bad (Datta et al., 1997) or procaspase 9 (Cardone et al., 1998).

## 1.7 The role of the integrin αvβ3 in apoptosis

Integrins recognize ECM proteins via the RGD motif and transmit signals bidirectionally across the cellular membrane resulting in an activation of intracellular signaling pathways. Thus, deprivation of this transcellular connection accompanies with decisive effects on the cellular survival leading to the integrin-mediated cell death via the so-called anoikis (reviewed in Brassard et al., 1999; Stupack et al., 2001; Zhao et al., 2005). However, especially cancer cells may acquire the capacity to overcome anoikis and survive anchorage-independently without pro-survival signals from the ECM.

### 1.7.1 The cellular death

Apoptosis is a physiological mechanism to regulate cell population in form of preventing a proliferation of abnormal cells. Thus, apoptosis plays a crucial role in embryogenesis, in physiological cell turnover, and in maintaining of the immune system. Moreover, failures in the apoptotic process can lead to autoimmune or neurodegenerative diseases, but can also be participated in the development of cancer. (reviewed in Elmore, 2007).

Within this context, studies have demonstrated a role of the ECM as an important survival factor (reviewed in Simpson et al., 2008). As mentioned before, normally when an epithelial-type cell to which most tumor cells belong, including ovarian cancer cells, is detached from its underlying ECM, a form of apoptosis is triggered which is called anoikis (from Greek ‘homelessness’). Since integrins mediate ECM contact, it represents an integrin-mediated cell death (Frisch & Francis, 1994). Cancer cells have developed a mechanism to overcome this anoikis process and still survive anchorage-independently (reviewed in Buchheit et al., 2014). By this way floating tumor cells may reach distant sites in the body allowing their eventual re-attachment to form metastases, also e.g., during ovarian cancer peritoneal carcinomatosis (Shield et al., 2009).

‘Apoptosis’ was first mentioned from Kerr et al. (1972). They described a process, which is characterized by condensation of the nuclear and cytoplasmic structures, the fragmentation of the nucleus, and the shrinkage and disintegration of the cell membrane. The crushed cell structures form themselves to small apoptotic bodies, which are surrounded by a cell membrane and finally are phagocytized by macrophages (Kerr et al., 1972).

Apoptosis can be mediated by two different signaling pathways. The extrinsic pathway is characterized by triggering the cellular death due to ligand binding to the so-called death-receptors. These receptors are transmembrane receptors and are members of the tumor necrosis factor (TNF) receptor superfamily (Locksley et al., 2001). After binding of a ligand, like FasL/FasR, TNF-α/TNFR1, or Apo3L/DR3, death signals are transmitted over the cell membrane and lead to the activation of the adaptor protein Fas-associated protein with death domain (FADD) (Pistritto et al., 2016). FADD cooperates with procaspase 8, which becomes activated after auto-catalyzation (Elmore, 2007; Paoli et al., 2013; Wajant, 2002). Once caspase 8 is active, the executive pathway of the apoptosis process is irreversible (Elmore, 2007). The intrinsic pathway acts receptor-independently. Radiation, toxic substances, infections, or hypoxia lead to a permeabilization of the mitochondria and a disruption of the mitochondrial transmembrane potential (Kroemer et al., 2007), resulting in a release of cytochrome c and Smac/DIABLO (Du et al., 2000). Cytochrome c binds to apoptosis protease activating factor-1 (Apaf-1) (Zou et al., 1997) and procaspase-9, which display the so-called apoptosome (Chinnaiyan, 1999; Zou et al., 1999). This initiates the further activation of caspases followed by the mediation of the executive apoptosis process. During the initiation of the intrinsic pathway, different proteins of the Bcl-2 family play an important role (Cory & Adams, 2002). This family consists of about 25 members. They act on one hand pro-apoptotically, like Bid, Bad, Bim, or Bik and, on the other, anti-apoptotically, like Bcl-2, Bcl-x, or Bcl-XL (Elmore, 2007; Kale et al., 2018).

The common final route of the extrinsic and the intrinsic pathway is the execution pathway. Through activation of the so-called execution caspases, like caspase-3, caspase-6, and caspase-7, various cellular components are shredded and destroyed (Slee et al., 2001). (Figure 8)

During the last decades, different assumptions concerning the cellular survival were published. Nevertheless, the exact process, especially with regard to the anchorage-independent cell survival and the role of the involved integrins is not fully resolved yet.

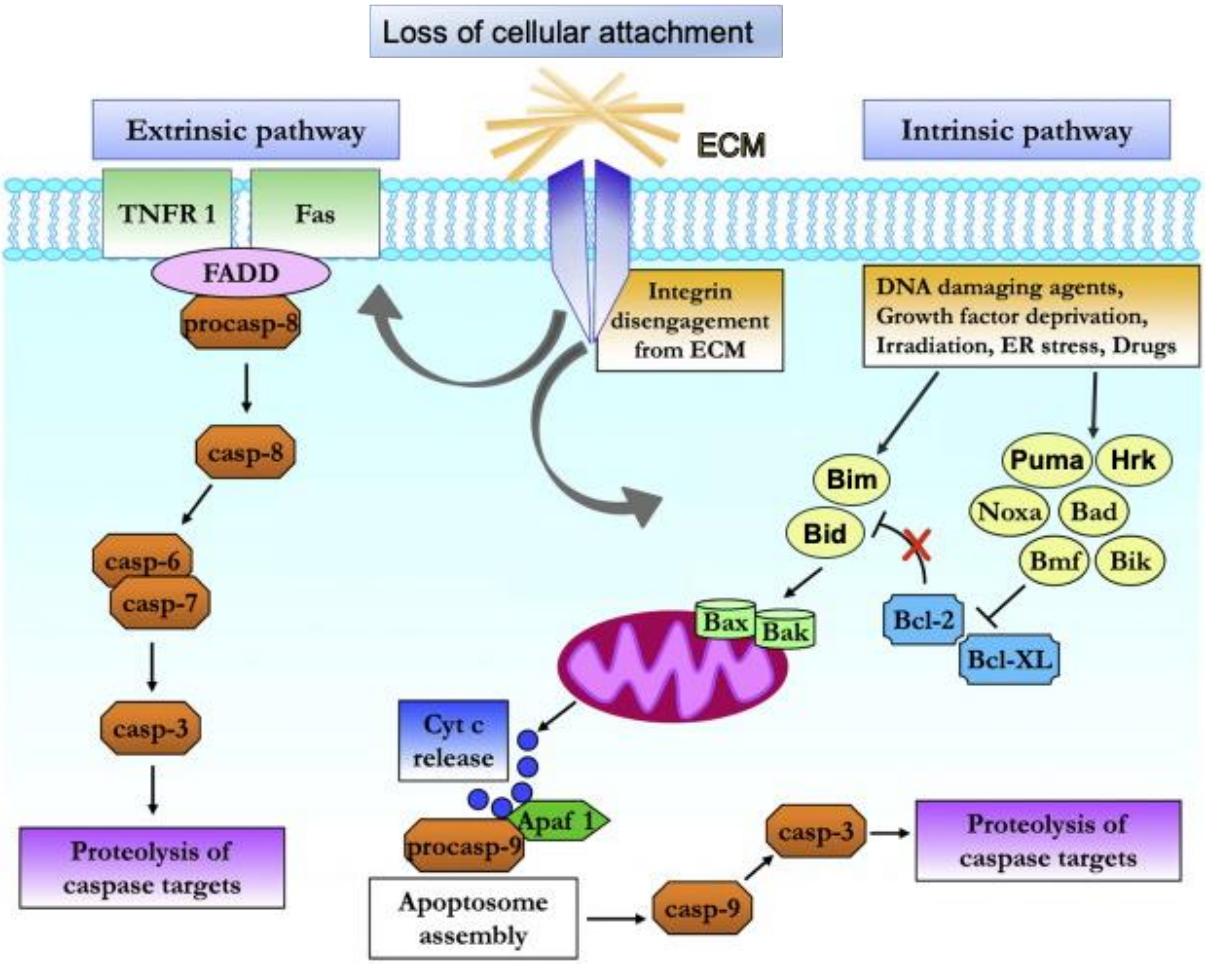


**Figure 8 The extrinsic and intrinsic apoptosis pathway.**
The extrinsic pathway is characterized by the initiation of the cell death via death receptors (TNFR, Fas) leading to the activation of initiator (caspase 8) and effector caspases (caspase 6, 7, 3). The intrinsic pathway is activated by intracellular stress, like toxins or radiation. Based on the interaction of pro- and anti-apoptotic (Bcl-2) proteins with a preponderance of the pro-apoptotic markers (Bid, Bad, Bim) the mitochondrial membrane gets permeabilized, accompanied by a release of cytochrome c, followed by the activation of effector caspases. The subform of the apoptotic process, the so called anoikis is induced after loss of the cellular contact to the ECM and is executed either via the extrinsic or the intrinsic pathway. (Fig. modified according to Paoli et al., 2013).

## 1.8 The role of integrin αvβ3 in cancer therapy

Because of the crucial role of the integrin αvβ3 in cancer progression, the interest concerning the development of a therapeutic target against this transmembrane receptor was already high early on. One of the promising integrin αvβ3 targeting antagonists is Etaracizumab (synonym: Vitaxin, Abegrin, MEDI-522), a humanized monoclonal antibody (mAb) version of the first anti-angiogenic antibody LM609 (Cai et al., 2006; Wu et al., 1998). In 2010 (Hersey et al.), a randomized phase II trial investigated the effects of this antibody with or without dacarbazine in patients with untreated metastatic melanoma. Unfortunately, the patients' benefit and the

reduction of the cancer progression were not as expected (Hersey et al., 2010). Thus, this antibody has not found entry into cancer therapy regimen so far.

Another auspicious integrin αvβ3-targeting antagonist is represented by Cilengitide (cyclo (RGDf(*N*Me)V; EMD 121974). This cyclic pentapeptide acts as a ligand mimetic and binds via its RGD-motif to integrin αvβ3, but also with lower affinity to αvβ5 (Nisato et al., 2003). This small molecule drug was developed in 1995 by Horst Kessler from the Technical University Munich and the company Merck entered the production shortly afterwards (Mas-Moruno et al., 2010). Many preclinical studies showed promising prospects concerning the effects of this antagonist on inhibition of tumor growth (Mitjans et al., 2000) and angiogenesis (Taga et al., 2002). Especially in the treatment of glioblastoma, Cilengitide was tested in phase III of clinical trials (reviewed in Mas-Moruno et al., 2010). However, the registration trial for the therapy of glioblastoma was without success, since no significant survival benefit was achieved (Stupp et al., 2014). Apart from that, an opposite effect could be determined after application of Cilengitide in lower doses. Reynolds et al. published an enhanced tumor growth accompanied by an increased angiogenesis at lower concentrations of Cilengitide instead of an inhibiting effect on endothelial cell sprouting, an observation which in fact could point to an agonisting ligand behavior (2009). Thus, low dose Cilengitide exerts agonistic functions by provoking integrin-mediated signaling and concomitant VEGF-mediated angiogenesis (Li et al., 2019; Reynolds et al., 2009). These findings led to a change of a paradigm in cancer therapy from anti-angiogenic strategies to vascular promotion/normalization therapy (Carmeliet & Jain, 2011; Van der Veldt et al., 2012). In mouse and human cancer models, the administration of Cilengitide in combination with Verapamil, a calcium channel blocker, which increased vessel dilation and blood flow in tumors by relaxing blood vessel muscles, enhanced gemcitabine, a cytostatic drug, delivery to the tumor bed and thus the efficacy of this drug to provoke tumor regression and metastasis suppression (Wong et al., 2015).

Over the years, many other investigations concerning the blocking of the integrin αvβ3 for inhibition of tumor progression were performed. Meena et al. published in 2020 (2020) new approaches for integrin αvβ3 antagonists. They reported five new RGD-containing cyclic peptides as effective inhibitors to integrin αvβ3 (Meena et al., 2020). Moreover, a Chinese research group (Lv et al., 2020) presented initial research results in the production of integrin αvβ3 targeting protein nanoparticles for cancer treatment. They showed that the RGD peptide enhances the cytotoxicity of BAK nanoparticles (a pro-apoptotic protein and member of the Bcl-2 family) to tumor cells, providing a new targeted therapeutic approach for integrin αvβ3-overexpressing cancer cells (Lv et al., 2020).

The successes achieved to date underline the high value of integrins as tumor cell surface targets for cancer therapy and thus integrins, especially their structure and conformational changes during the activation process, still remain an essential topic for ongoing and future cancer research.

## 1.9 Aim of the thesis

The aim of this work was to analyze the influence of the activation of integrin αvβ3 on anchorage-independent survival and anoikis avoidance of human ovarian carcinoma cells in ascites. For this, a transfected human ovarian cancer cell model was used, allowing the investigation and direct comparison of different αvβ3 effects depending on its TMD conformational activation state:

- **Detection of early apoptotic events** by Annexin-V-Fluos/PI apoptosis assays, determining the fraction of viable, apoptotic, and necrotic human ovarian cancer cells upon loss of anchorage as a function of their expression of αvβ3 in its differently active conformational states in order to monitor anoikis avoidance.
- **Analysis of chemotherapy resistance** by determining the response of the different αvβ3-transfected human ovarian cancer cells grown under anchorage-independence in ascites to the chemotherapeutic drug cisplatin by performing Annexin-V-Fluos/PI apoptosis assays.
- **Analysis of proliferation** of floating αvβ3-TMD-transfected human ovarian cancer cells depending on their conformational transmembrane activation state of αvβ3 and investigation of αvβ3-controlled expression of the CDK inhibitor $p27^{Kip-1}$.
- **Contribution of αvβ3-dependent pro-survival signaling** to anoikis resistance by measuring the activation and thus phosphorylation state of the important integrin-related signaling molecules FAK, src, and PKB/Akt by Western blot analysis.
- **Measurement of the expression of the anti-apoptotic factors** Bcl-2 and survivin as a function of the expression of αvβ3 in its differently active conformational states by Western blot analysis.

# 2 Materials and methods

## 2.1 Materials

### 2.1.1 Cell line

For the experiments, stable transfectants of the human ovarian carcinoma cell line OV-MZ-6 were used. This cell line has originally been isolated from ascites of a patient with high grade serous papillary cystadenocarcinoma (Mobus et al., 1992).

Stably transfected OV-MZ-6 cells with different integrin αvβ3 conformational activation states (established in a previous project of the research group of Prof. Dr. Ute Reuning) were kindly provided for this dissertation project (Muller et al., 2013). (Figure 4) The influence of the integrin αvβ3 activation state on different integrin-mediated signaling pathways and cell adhesion to protein ligands of the ECM has already been shown by Müller et al. (Hapke et al., 2003; Muller et al., 2014; Muller et al., 2013).

#### 2.1.1.1 Generation of Integrin αvβ3-TMD-transfectants

OV-MZ-6 cell transfectants expressing the TMD-αvβ3 wildtype, the TMD-GpA and the TMD-GpA-I, generated by Müller et al., were kindly provided for the projects of this doctoral thesis (Muller et al., 2013). (Figure 4)

**Table 2.1 Overview of the integrin αvβ3 cell transfectants used**

| **Overview of the cell-transfectants used** | |
|---|---|
| αv-WT + β3-WT | αvβ3-WT (= TMD- αvβ3)<br>activatable integrin αvβ3 |
| αv-TMD-GpA + β3-TMD-GpA | TMD-GpA<br>Associated TMD; intermediate integrin activation state, low ligand binding affinity, signaling incompetent |
| αv-TMD-GpA-I + β3-TMD-GpA-I | TMD-GpA-I;<br>constitutively active integrin with dissociated TMD; high ligand binding affinity, constitutive signaling capability |

### 2.1.2 Cell culture reagents

**Table 2.2 Cell culture reagents**

| Product | Origin |
|---|---|
| Dulbecco's Modified Eagle Medium (DMEM) + GlutaMAX™ – I | Gibco, Thermo Fisher Scientific, Waltham, MA, USA |
| Dulbecco's Phosphate-buffered Saline (DPBS) | Gibco, Thermo Fisher Scientific |
| Fetal bovine Serum (FBS) | Gibco, Thermo Fisher Scientific |
| Bovine serum albumin (BSA) | Sigma-Aldrich, St. Louis, MO, USA |
| 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES Buffer Solution) | Gibco, Thermo Fisher Scientific |
| L-arginine and L-asparagine | Sigma-Aldrich |
| Ethylene diamine tetraacetic acid (EDTA 1% (w/v)) | Biochrom AG, Berlin, Germany |
| Geniticin® G 418 | Invitrogen, Carlsbad, CA, USA |
| Dimethylsulfoxide (DMSO) | Sigma-Aldrich |
| Trypan blue solution 0.4% (w/v) | Sigma-Aldrich |
| Trypsine | Invitrogen |

### 2.1.3 Cell culture consumables

**Table 2.3 Cell culture consumables**

| Product | Origin |
|---|---|
| Cell culture flask, adherent, 25, 75, and 175 qcm | Cellstar, Greiner Bio-One GmbH, Frickenhausen, Germany |
| Cell culture flask, suspension, 25, 75, and 175 qcm | Sarstedt AG & Co. KG, Nürnbrecht, Germany |
| Pasteur pipettes | Hirschmann, Eberstadt, Germany |

| | |
|---|---|
| Pipettes 2 ml, 5 ml, 10 ml, 25 ml, 50 ml | Greiner Bio-One GmbH, Frickenhausen, Germany |
| Pipette tip 10 µl, 100 µl, 200 µl, 1000 µl, 2000 µl | Sarstedt AG & Co. KG<br>Biozym, Hessisch Oldendorf, Germany |
| Tubes 15 ml and 50 ml | Cellstar, Greiner Bio-One GmbH |
| Minisart® sterile filter | Sartorius, Aubagne, France |
| Gloves | Ansell, Yarra City, Australia |

### 2.1.4 Western blot reagents

**Table 2.4 Western blot reagents**

| **Product** | **Origin** |
|---|---|
| Trishydroxymethylaminomethan (Tris) | Roth, Karlsruhe, Germany |
| Glycine | Sigma-Aldrich, St. Louis, MO, USA |
| Glycerin | Roth |
| Methanol | Roth |
| Ethanol | Roth |
| Skimmed milk powder | Sigma-Aldrich |
| NaCl | Roth |
| Sodium dodecyl sulfate (SDS) | Roth |
| Tween-20 | AppliChem GmbH, Darmstadt, Germany |
| Acrylamide/Bis | Roth |
| Ammonium persulphate (APS) | Roth |
| Tetramethylethylendiamine (TEMED) | Applichem GmbH |
| PVDF gel blotting membrane | Millipore GmbH, Schwalbach, Germany |
| Triton X-100 | Sigma-Aldrich |
| Tween | AppliChem GmbH |

| | |
|---|---|
| Pierce ECL™ chemiluminescent Western blotting substrate | Gibco, Thermo Fisher Scientific, Waltham, MA, USA |
| Page Ruler Plus Prestained Protein Ladder | Thermo Fisher Scientific |
| Biotinylated Protein Ladder | Cell Signaling Technology, Danvers, MA, USA |
| Roti®Load 4x concentrated, reducing | Roth |

### 2.1.5 Gel-production for Western blot

**Table 2.5 Consumables for separating gel production**

| **Separating gel** | 12% | 10% | 7,5% |
|---|---|---|---|
| $H_2O_{dest.}$ | 4.3 ml | 4.8 ml | 5.4 ml |
| 1.5 M Tris-HCl. pH 8.8 | 2.5 ml | 2.5 ml | 2.5 ml |
| 10% (w/v) SDS | 100 µl | 100 µl | 100 µl |
| Acrylamide/Bis 40% (w/v) | 3 ml | 2.5 ml | 1.9 ml |
| 10% (w/v) APS | 50 µl | 50 µl | 50 µl |
| TEMED | 5 µl | 5 µl | 5 µl |
| **Total volume** | 10 ml | 10 ml | 10 ml |

**Table 2.6 Consumables for stacking gel production**

| **Stacking gel** | 4% |
|---|---|
| $H_2O_{dest.}$ | 6.3 ml |
| 0.5 M Tris-HCl. pH 8.8 | 2.5 ml |
| 10% (w/v) SDS | 100 µl |
| Acrylamide/Bis 40% (w/v) | 1 ml |
| 10% (w/v) APS | 50 µl |
| TEMED | 10 µl |
| **Total volume** | 10 ml |

## 2.1.6 Production of cell culture media

**Table 2.7 Reagents for growth medium**

| **Growth medium for OV-MZ-6** | |
|---|---|
| DMEM<br>complemented with: | 500 ml |
| HEPES | 272 mM |
| Arginine | 10 mM |
| Asparagine | 550 mM |
| FBS | 10% (v/v) |
| Maintenance of the selection pressure for the stably transfected OV-MZ-6 cell transfectants by usage of the antibiotic Geniticin G 418 (0.5 g/l) | |

**Table 2.8 Reagents for medium for cell cryoconservation**

| **Medium for cell cryoconservation** | |
|---|---|
| FCS | 90% (v/v) |
| DMSO | 10% (v/v) |

**Table 2.9 Reagents for cell splitting solution**

| **Cell splitting solution** | |
|---|---|
| EDTA | 0.5% (v/v) |
| in DPBS | |

## 2.1.7 Buffer solutions for adhesion assay

**Table 2.10 Reagents for hexoaminidase substrate**

| **Hexoaminidase substrate (pH 5.0)** | |
|---|---|
| *p*-nitrophenyl-*N*- acetyl-β-D-glucosaminide | 15 mM |
| Triton X-100 | 0.5% (v/v) |
| sodium citrate | 100 mM |

**Table 2.11 Reagents for stopp-buffer solution**

| **Stopp-buffer solution** | |
|---|---|
| NaOH | 0.2 M |
| EDTA | 5 mM |

## 2.1.8 Buffer solutions for Western blot

**Table 2.12 Reagents for Tris buffer solution**

| **Tris Buffered Saline (TBS) (10x)** | |
|---|---|
| NaCl | 1.5 M |
| 1 M Tris-HCl (pH 8.0) | 0.5 M |
| $H_2O_{dest.}$ | ad 1 liter |
| For 1 x TBS | 100 ml ad 1000 ml $H_2O_{dest.}$ |
| For TBS-T | 1 x TBS 0.1% (v/v) Tween-20 |

**Table 2.13 Reagents for lysis buffer**

| **Lysis buffer** | |
|---|---|
| HEPES (pH 7.5) | 50 mM |
| NaCl | 150 mM |
| EDTA | 1 mM |
| Glycerin | 10% (v/v) |
| Triton X-100 | 1% (v/v) |
| Protease inhibitor cocktail (EDTA-free) | 0.1% (v/v) |

**Table 2.14 Reagents for TBS stock solution**

| **TBS stock solution for protein determination** | |
|---|---|
| Tris-HCl (pH 8.5) | 0.2 M |
| NaCl | 7.3% (w/v) |
| | 1:10 in $H_2O_{dest.}$ as working solution |

**Table 2.15 Reagents for electrophoresis buffer**

| **10x Electrophoresis buffer** | |
|---|---|
| Tris | 250 mM |
| Glycine | 1.6 M |
| SDS | 40 mM/1% (w/v) |
| $H_2O_{dest.}$ | ad 1 liter |

**Table 2.16 Reagents for transfer buffer**

| **Wet-Blotting (transfer) buffer** | |
|---|---|
| Tris | 25 mM |
| Glycine | 192 mM |
| Methanol | 20% (v/v) |
| $H_2O_{dest.}$ | ad 1 liter |

**Table 2.17 Reagents for blocking buffer**

| **Blocking buffer** | |
|---|---|
| Skimmed milk powder | 2.5% (w/v) |
| 1x TBST 0.1% | 50 ml |

**Table 2.18 Reagents for stripping buffer**

| **Stripping buffer** | |
|---|---|
| Glycine | 3.7 g |
| NaCl | 4.1 g |
| $H_2O_{dest.}$ | 250 ml |
| pH 2.5 | |

### 2.1.9 Antibodies (Ab) for Western blot and Immunocytochemistry (ICC)

**Table 2.19 Used antibodies and their concentration in Western blot and ICC analysis**

| Antibody | Origin | Concentration in Western blot or ICC |
|---|---|---|
| anti-biotin, HRP (horseradish peroxidase)-linked antibody | Cell Signaling Technology, Danvers, MA, USA | 1:1000 in 5% (w/v) skimmed milk/1xTBS-T 0.1% |
| monoclonal mouse IgG Alexa Fluor® 488 | Invitrogen, Carlsbad, California, USA | 1:1000 in 1% (w/v) BSA/1xTBS-T 0.1% |
| polyclonal rabbit IgG directed against Akt (Clone 9272) | Cell Signaling Technology | 1:2000 in 5% (w/v) BSA/1xTBS-T 0.1% |
| monoclonal mouse IgG directed against phospho-Akt (Ser473) (Clone 587F11) | Cell Signaling Technology | 1:1000 in 5% (w/v) skimmed milk/1xTBS-T 0.1% |
| monoclonal mouse IgG directed against Bcl-2 (Clone 124) | Cell Signaling Technology | 1:200 in 5% (w/v) BSA/1xTBS-T 0.1% |
| monoclonal mouse IgG directed against EGF-R (Clone 13) | BD Biosciences, San Jose, CA, USA | 1:2000 in 3% (w/v) BSA/1xTBS-T 0.1% |
| polyclonal rabbit IgG directed against p-EGF-R (Clone Y845) | R&D Systems, Minneapolis, MN, USA | 1:2000 in 3% (w/v) BSA/1xTBS-T 0.1% |
| monoclonal mouse IgG directed against FAK (Clone 77) | BD Biosciences | 1:1000 in 1% (w/v) BSA/1 x TBS-T 0.1% |

| | | |
|---|---|---|
| monoclonal mouse IgG directed against p-FAK (pY397) (Clone 18) | BD Biosciences | 1:400 in 1% (w/v) BSA/1 x TBS-T 0.1% |
| monoclonal mouse IgG directed against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Clone 6C5) | EMD Millipore Corporation, Temecula, CA, USA is now Merck KGaA, Darmstadt, Germany | 1:10000 in 0,1% (w/v) BSA/1 x TBS-T 0.1% |
| monoclonal mouse IgG directed against $p27^{Kip-1}$ (Clone 57) | BD Biosciences | 1:5000 in 3% (w/v) BSA/1xTBS-T 0.1% |
| monoclonal rabbit IgG directed against src (Clone 36D10) | Cell Signaling Technology | 1:1000 in 5% (w/v) BSA/1xTBS-T 0.1% |
| polyclonal rabbit IgG directed against p-src (Tyr416) | Cell Signaling Technology | 1:1000 in 5% (w/v) BSA/1xTBS-T 0.1% |
| monoclonal mouse IgG directed against survivin (Clone 6E4) | Cell Signaling Technology | 1:500 in 3% (w/v) BSA/1xTBS-T 0.1% |
| goat-anti-rabbit IgG, HRP-linked | Life Technologies, Carlsbad, CA, USA | 1:10000 in 5% (w/v) skimmed milk or BSA/1xTBS-T 0.1% |
| goat-anti-mouse IgG, HRP-linked | Life Technologies | 1:10000 in 5% (w/v) skimmed milk or BSA/1xTBS-T 0.1% |

### 2.1.10 Kits

**Table 2.20 Used kits**

| **Product** | **Origin** |
|---|---|
| Annexin-V-FLUOS Staining Kit | Roche Diagnostics, Mannheim, Germany |
| PierceTM BCA Protein Assay Kit | Thermo Fisher Scientific, Waltham, MA, USA |

### 2.1.11 Other reagents

**Table 2.21 Other used reagents**

| **Product** | **Origin** |
|---|---|
| Malignant ascites | 5 different patients with high-grade serous ovarian cancer (FIGO IIIb/c/IV) |
| Cisplatin | Central pharmacy of the Klinikum rechts der Isar, Technische Universität München |
| *p*-nitrophenyl-*N*- acetyl-β-D-glucosaminide | Sigma-Aldrich |
| Sodium citrate | Roth |
| NaOH | Roth |

### 2.1.12 Instruments

**Table 2.22 Instruments used in laboratory**

| **Product** | **Origin** |
|---|---|
| Axiovert 25 microscope | Zeiss, Oberkochen, Germany |
| BIORAD Imager Gel Doc™ XR+ and ChemiDoc™ XRS+ Systems | Bio-Rad Laboratories, Munich, Germany |
| Cell counting chamber | Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen. Germany |
| Cell incubator Hera *Cell* | Heraeus, Hanau, Germany |
| Centrifuge 5417C & 5424R | Eppendorf, Hamburg, Germany |

| | |
|---|---|
| Centrifuge Labofuge 400R | Heraeus |
| Confocal laser scanning microscope (CLSM), Axio Observer. Z1 + LSM700 microscope | Zeiss |
| Electrophoresis chamber, combs and pour station | Bio-Rad Laboratories |
| Flow cytofluorometer FACS Calibur | BD Biosciences, San Jose, CA, USA |
| pH meter | Schott, Mainz, Germany |
| Protein elektrophoresis *Power-supply Power Pac* 300V | Bio-Rad Laboratories |
| Safety workbench Hera *Safe* | Heraeus |
| SLT Spectra Elisa Reader | SLT, Crailsheim, Germany |
| Vortex Genie 2 | Bender & Hobein AG, Zürich, Switzerland |
| Wet-blotting chamber | Bio-Rad Laboratories |

### 2.1.13 Software

**Table 2.23 Software for analysis of the results**

| **Product** | **Origin** |
|---|---|
| CellQuest | BD Biosciences, San Jose, CA, USA |
| FlowJo™ 10 | BD Biosciences |
| Image Lab™ | Bio-Rad Laboratories, Munich, Germany |
| ZEN Software | Zeiss, Oberkochen, Germany |

## 2.2 Methods

### 2.2.1 Cell culture technologies

#### 2.2.1.1 Cell cultivation and splitting

Human OV-MZ-6 cells were cultured until they reached a confluency of appr. 70-80% in complete or selection medium at 37°C/5% (v/v) $CO_2$. Then, the medium was aspirated and splitting solution added, followed by further cell incubation at 37°C/5% (v/v) $CO_2$ for 4 min.

Cells were centrifugated at 1300 x g for 3 min and cell supernatant aspirated. Cell pellets were resuspended in medium and transferred in a new cell culture flask.

#### 2.2.1.2 Cell cultivation of suspended cells

For anchorage-independent cell cultivation in suspension, cells resuspended either in cell culture medium or in ascites of patients with high grade malignant ovarian cancer were transferred into cell culture flasks put into an upright position to avoid cell adhesion to the cell growth area in the flask. Prior to the experiments, only floating cells were harvested and, if necessary, cell aggregates dissoluted by treating them with 200 µl trypsin for 1-2 min.

#### 2.2.1.3 Cell counting

For cell counting, adherent cells were detached by adding splitting solution and cell suspensions were transferred into a tube. After centrifugation and washing in PBS, cells were diluted in 10 ml cell culture medium. Then, 10 µl of each cell suspension were pipetted into an Eppendorf tube. In order to exclude counting of dead cells, trypane blue was added and only unstained cells registered by using a Neubauer-hemocytometer.

#### 2.2.1.4 Cryoconservation of cells

For preparing frozen cell stocks, cells were detached from the flask as described above, washed in PBS and centrifugated at 1300 x g for 3 min. Cell supernatants were aspirated and cell pellets resuspended in cryoconservation medium (Table 2.8). Aliquots of cell suspensions were transferred to cryo tubes on ice. Thereafter, cells were first put to -80°C for 1 to 2 days and then stored in liquid nitrogen at -196°C for longer-term storage.

Cells were thawed and instantaneously resuspended in cell culture medium. After washing the cells in PBS and resuspension in cell culture medium, they were passed to cell culture flasks. Before applying cells to experimental studies, they were at least twice passaged.

#### 2.2.1.5 Use of malignant ascites

Malignant ascites was provided from five different patients diagnosed with high-grade serous ovarian carcinoma. The tumor stages FIGO IIIb/c and IV were included. The ascites were collected under sterile conditions. After centrifugation at 300 x g for 10 min, the cell-free supernatants were stored at -80°C. The sampling and processing of the human ascites were approved by the ethics committee of the Medical Faculty of the Technical University in Munich. The patients consented in writing to the use for scientific purposes according to the Declaration of Helsinki.

### 2.2.2 Characterisation of the proliferation activity of EOC cells cultivated grown in suspension

The proliferative activity of ovarian cancer cells grown in suspension in ascites was determined within 3 and 48 hours. The samples were prepared in quadruplets. After each cultivation period, cell suspensions were transferred into tubes, cell clusters dissoluted by treatment with trypsin and counted in a Neubauer hemocytometer (2.2.1.3).

### 2.2.3 Capability of suspended cells to regain cell adhesive capacity

Cell transfectants were incubated under suspension either in ascites or in DMEM for 48 hours. At each time point 10000 cells in 100 µl ascites or rather selection/complete medium were transferred in 96-well culture plates. The samples were prepared in quadruplets. After further incubation for 2 hours at 37°C the supernatant was removed and the adherent cells were gently washed in PBS, followed by an addition of 50 µl of PBS and 50 µl of a substrate (Table 2.10), which determines the N-acetyl-ß- D-hexoaminidase activity. The reaction was stopped after 90 min by addition of 100 µl of buffer solution (Table 2.11). Optical density as a measure of number of adherent cells was evaluated at 405 nm. By using a standard dilution series with increasing cell numbers, the linearity of the absorbance range to the adherent cell numbers was demonstrated (Hapke et al., 2001).

### 2.2.4 Western blot analysis

#### 2.2.4.1 Preparation of gels for Western blot analysis

For preparation of the separating gel the reagents described in Table 2.5 were mixed. Afterwards, the mixture was poured in between two glass plates, which build a 1.5 mm-chamber. Ethanol 80% (v/v) was put on top of the separating gel mixture. The ethanol inhibits the contact of oxygen with the gel, which could block the correct gel polymerization process. Thereafter, the ethanol was removed from the polymerized separating gel and the stacking gel poured onto it by using a comb to form gel slots for different samples to be loaded onto the gel.

#### 2.2.4.2 Preparation of cell lysates

OV-MZ-6 cells, either suspended for 3, 24, or 48 hours in cell culture medium, or ascites, or grown adherently in complete or selection medium until cell monolayer reached a confluency of appr. 80%, followed by a detachment from the cell culture flasks, were passed into tubes and centrifuged at 1300 x g for 3 min.

After discarding the supernatant, cell pellets were washed in PBS, centrifuged again and the cell pellets resuspended on ice in cold cell lysis buffer. This buffer (Table 2.13) contained in addition a protease inhibitor cocktail (EDTA-free), which suppresses the cellular proteases. Cell proteins were extracted by stirring the lysates fixed on a rotating wheel at 4°C for 15 min. For separating the membrane fraction, the lysates were centrifuged at 14,000 x g at 4°C for 15 min. Afterwards, aliquots of the supernatants were pipetted into Eppendorf tubes and stored in the refrigerator at -20°C. Aliquots were taken to determine the protein concentration.

#### 2.2.4.3 Quantification of protein content in cell lysates

Cellular protein concentrations were determined by using the bicinchoninic acid (BCA)-method of the Pierce™ BCA Protein Assay Kit according to the manufacturers´ protocol. This process is based on the so called 'Biuret-reaction'. $Cu^{2+}$ is reduced to $Cu^{1+}$ by proteins in alkaline solution. This reaction leads to a violet colour complex. Its absorption can be measured with a spectrophotometer. At first, a TBS-stock solution (pH 8.5) (Table 2.14) was prepared. For determining the protein concentration, a calibration curve using BSA at distinct concentrations was established. 50 µl of the protein standard (BSA), or the samples of the protein lysates were pipetted into a 96-well-plate and afterwards added with 200 µl of the BCA reagent of the kit. The samples were prepared in triplets. The plate was then covered and incubated at 37°C/5% (v/v) $CO_2$ for 30 min, followed by the measurement of the absorbance at 570 nm in the spectrophotometer. The protein content of the analyzed solution was evaluated by means of the values obtained from the standard curve by using the law of Lambert-Beer.

#### 2.2.4.4 Electrophoresis

Proteins of the cell lysates were separated via SDS-polyacrylamide-gel-electrophoresis (SDS-PAGE). The protein samples were diluted in lysis buffer and a 4-fold concentrated, reducing gel loading buffer with mercaptoethanol (Roti®Load) was added. After denaturation of the samples at 95°C for 4 min, they were transferred into the pockets of the prepared gel (2.2.4.1), which has already been clamped in the electrophoresis chamber. Besides the protein-samples, a prestained protein ladder, and a biotinylated marker for detection of the molecular protein weight were applied. After addition of the electrophoresis buffer (1x; Table 2.15), protein separation occurred, starting at 85 V up to 120 V, until the proteins had almost reached the end of the gel.

#### 2.2.4.5 Western blotting

For the identification of proteins in cell lysates, the separated proteins within the gel were transferred onto a polyvinylidene fluoride membrane (PVDF) by the so called 'wet-blotting'

process in an electrical field. At first, the PVDF-membrane was shortly incubated in methanol for equilibration. The necessary Whatman-filter-papers were soaked in transfer buffer (Table 2.16). Afterwards the wet-blotting-transfer was set up in the blotting device as follows: cathode – three Whatman-papers – electrophoresis-gel – PVDF-membrane – three Whatman-papers – anode. The air bubbles between the gel and the membrane were removed with a glass pipette. The whole blotting system was placed on ice. Transfer buffer was added, and the protein-transfer from the gel to the membrane was completed at 350 mA for 90 min.

#### 2.2.4.6 Detection of the proteins

After blotting, the unspecific protein binding sites were blocked by incubating the membrane in blocking buffer (Table 2.17) for one hour at room temperature (RT). After washing the membrane 3 times for 10 min in 1x TBS-T 0.1% (TBS, 0.1% (v/v) Tween-20), the respective antibody was added at 4°C overnight. The membrane was then washed again 3 times for 10 min in 1x TBS-T 0.1%, followed by its incubation with a secondary antibody (either goat-anti-mouse or goat-anti-rabbit 1:10000 in diluted BSA (w/v) or skimmed milk (w/v) in 1x TBS-T 0.1%; horseradish peroxidase (HRP)-conjugated and the antibody against the biotinylated marker for one hour at RT. After washing the membrane again 3 times for 10 min in 1x TBS-T 0.1%, the ECL solution, a non-radioactive, enhanced luminol-based chemiluminescent substrate, was prepared according to the manufacturers' recommendations, which detects HRP on the PVDF-membrane, and incubated with the membrane for 5 minutes at RT. Normalization of different protein loadings and blotting efficiency were performed by re-sampling the membranes with a mAb directed to GAPDH. Band intensity was analyzed by densitometry, and signal intensities were evaluated by normalization with GAPDH by using the BIORAD Imager Gel DocTM XR+ and ChemiDocTM XRS+ Systems with the software Image LabTM.

The depiction of the histograms occurred as a mean value ± standard deviation (SD) by setting the cells, expressing TMD-αvβ3 at each time point to '1'.

For reuse, the membranes were incubated in stripping buffer (Table 2.18) at 80°C for 20 min. So, the previously detected antibodies were removed and after blocking the non-specific binding sites the detection of new antibodies was enabled. This process was performed maximum 2 times.

### 2.2.5 Immunocytochemical analysis of cellular proteins

For the detection of integrin-mediated signaling molecules, OV-MZ-6 cells were incubated with the respective antibodies targeting the protein of interest and analyzed via immunocytochemistry. For this, cells were cultivated for 24 hours at 37°C/5% (v/v) $CO_2$ in

microchambers slides, which were pretreated before at 4°C for 30 min with the ECM protein VN (5 µg/ml PBS) or FN (5 µg/ml PBS). After removing the cell culture medium and washing the cells in PBS, the cells were fixed in PBS, 4% (w/v) PFA for 15 min or with methanol for 5 min at 4°C, then in Triton-X-100 for 15 min or acetone for 30 seconds at 4°C. In the first experiment, both fixation procedures were tried, and the best staining results were obtained by cell fixation in PFA/Triton-X. Cells were then washed in PBS and blocked in PBS, 2% (w/v) BSA for 30 min at RT. After repeated washing steps in PBS, the monoclonal antibody (Bcl-2, Table 2.19) in 1:100 in PBS, 1% (w/v) BSA was added for 2-3 hours at RT. Thereafter, an Alexa 488-conjugated goat-anti-mouse IgG was added as secondary antibody (diluted 1:1000 in PBS, 0.1% (w/v) BSA) and incubated in the dark for 45 min at RT. After washing the cells 5-6 times in PBS, the chamber slides were mounted in PBS prior to visual inspection of cells and fluorescence intensity by CLSM. Staining procedures in the absence of the primary antibody served as controls for possibly occurring unspecific binding of the secondary antibody.

## 2.2.6 Fluorescence activated cell sorting (FACS)

### 2.2.6.1 Principle

Fluorescence activated cell sorting (FACS) allows to separate cells in suspension depending on various characteristics like granulation or size. The cells pass a laser beam in a row. Before analysis, the cells can be incubated with a fluorescence-conjugated primary antibody, or a primary antibody plus a fluorescence-conjugated secondary antibody, which binds to an antigen of interest. By passing this laser beam, the cells emit a scattered light and a fluorescence signal, either due to their autofluorescence, or the binding of the fluorescence-conjugated antibody. There are different detectors, which determine the scattered light of the cells:

- Forward scatter (FSC): in the same line as the light path of the laser; information about the size of a cell
- Side scatter (SSC): out of the light path in different directions; information about the granularity of a cell, the size and the structure of the nucleus, and number of vesicles in the cell
- One or more fluorescent detectors: detection of cellular structures of interest

These data are converted into electronical signals and blotted on a scatter chart.

### 2.2.6.2 Detection of early apoptosis-events by Annexin -V-Fluos/PI apoptosis assay

The viability of a cell can be detected by staining with Annexin, which bind to phosphatidylserine (PS) within cell membranes, and propidium iodide (PI) staining of the

nucleus. A vital cell has an intact cell membrane consisting of a lipid bilayer. During early apoptotic processes, the PS within the cell membrane switches from the inner to the outer cell surface. In this case, the Annexin V-fluorescein isothiocyanate (FITC) can bind via calcium to the PS and so mark an apoptotic cell. However, a necrotic cell loses its membrane stability and becomes also permeable for Annexin. Then, Annexin V-FITC binds to the PS on the inside and a distinction between an apoptotic and a necrotic cell is no longer possible. So, the added PI penetrates the permeable membrane of a necrotic cell, where it binds to the deoxyribonucleic acid (DNA) in the nucleus. In sum, the staining of the cells with Annexin and PI and the following FACS measurement enables the differentiation between a viable, an apoptotic, and a necrotic cell. (Vermes et al., 1995).

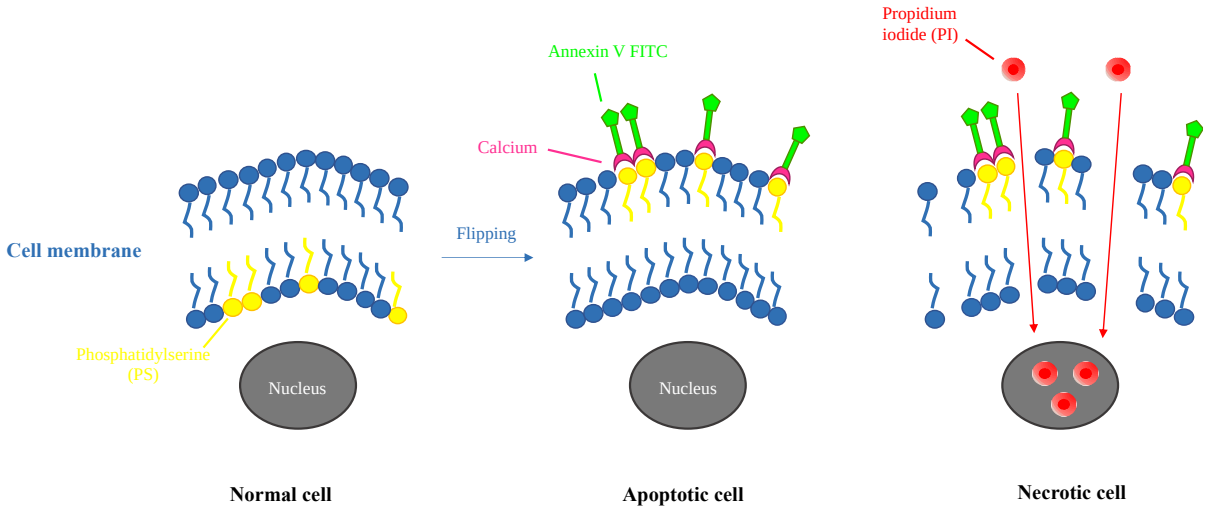


**Figure 9 Detection of apoptotic and necrotic cells**
A normal cell consists of a lipid bilayer as cell membrane. One phospholipid is the PS on the inside of the cell membrane. When a cell undergoes the apoptotic process, the PS switches from the inside to the outside of the cellular membrane. Now the Annexin V-FITC can bind via calcium to the PS and therefore mark an apoptotic cell. Necrotic cells also lose the integrity of the cell membrane and Annexin V-FITC is permitted to link to PS. To distinguish an apoptotic and a necrotic cell, PI, a DNA intercalator, is added. PI passes through the lipid bilayer directly in the nucleus, and thus only stains necrotic cells. (Vermes et al., 1995).

#### 2.2.6.3 Detection of early apoptotic events in suspended anchorage-dependent OV-MZ-6 cells

For the investigation of early apoptotic events of suspended but *per se* anchorage-dependent epithelial type ovarian cancer cells, the Annexin-V-Fluos Staining Kit was used. OV-MZ-6 cells were cultivated in suspension in T25 flasks for 48 hours. In each T25 suspension flask, 1.5 million cells were seeded in 4 ml human ascites, or selection cell culture medium. Thereafter, cells were transferred in a tube and washed in PBS. Because aggregated cell clusters

were observed, cells had to be treated prior to FACS analysis with trypsin for 1-2 min in order to obtain single cell solutions. Cells were then washed twice in PBS and smoothly centrifuged. The cell pellet was resuspended in 100 µl incubation buffer (IB) from the kit, portioned into three special FACS tubes and incubated for further 10 min at RT. Afterwards each tube was prepared for the FACS as follows:

**Table 2.24 Preparation of the cells for FACS analysis**

| Tube | Cell suspension | Add first | Add second |
|---|---|---|---|
| **1** | 30 µl | 200 µl IB | - |
| **2** | 30 µl | 200 µl IB | Annexin-FITC 1:20 in IB (10 min at RT in the dark) |
| **3** | 30 µl | 200 µl IB | 2.5 µl PI (short incubation at RT in the dark) |

All FACS experiments were accomplished with the flow cytometer *FACS Calibur*. For the data analysis the software CellQuest™ (Becton Dickinson) and FlowJo (Becton Dickinson) were used.

#### 2.2.6.4 Apoptosis assay after treatment of suspended cells with cisplatin

For evaluating the resistance of suspended epithelial-type OV-MZ-6 cells to chemotherapeutical treatment, one million cells were suspended for 30 hours either in ascites or in DMEM. After further incubation with 2.5 µg/ml cisplatin for 18 hours at 37°C/5% (v/v) $CO_2$, the cells were transferred into tubes, washed in PBS, and prepared for the Annexin-FLUOS/PI-staining as described in Table 2.24.

### 2.2.7 Statistical analysis

Data were statistically analyzed by Sigma Plot 14 (Systat Inc., Erkrath, Germany) by application of one-way or two-way ANOVA with a *post hoc* pairwise significance test. Datasets were tested for normality (Shapiro–Wilk; condition $P \geq 0.050$) and for equal variance (Brown–For- sythe; condition $P \geq 0.050$). After successful test completion, the ANOVA was conducted parametrical with *post hoc* pairwise Bonferroni *t*-test. Significances are given by * for $P < 0.050$, ** for $P < 0.010$, and *** for $P < 0.001$ in graphical artwork. (Dolinschek et al., 2021).

# 3 Results

## 3.1 Detection of early apoptotic events depending on the integrin αvβ3-TMD conformational activation state by Annexin-V-Fluos/PI staining

The apoptotic events of EOC cell transfectants, suspended for 48 hours either in ascites or in DMEM, depending on their integrin αvβ3-TMD activation state, were detected by usage of the Annexin/PI based apoptosis assay (2.2.6.2)

After 48 hours of cell suspension in ascites, highest number of vital cells were found for the TMD-GpA-I cell transfectants (appr. 71%), followed by TMD-αvβ3 (appr. 55%), and TMD-GpA expressers (appr. 34%). TMD-GpA expression led to appr. 42% of apoptotic cells, whereas the lowest rate of apoptotic events was noticed for TMD-GpA-I (appr. 12%). TMD-αvβ3 expression resulted in appr. 23% of apoptotic cells. In TMD-GpA expressers, the highest number of necrotic cells was detectable (appr. 37%). Necrotic cell fractions were smallest for TMD-GpA-I (appr. 16%), followed by TMD-αvβ3 transfectants (appr. 28%). (Figure 10)

After 48 hours of incubation of detached cells in culture medium, appr. 58% of viable TMD-GpA-I transfectants were determined, whereas appr. 18% TMD-GpA expressers were vital. TMD-αvβ3 transfectants took on an intermediate position of appr. 29% vital cells. Lowest apoptosis rate was detected in TMD-GpA-I expressers (appr. 29%), followed by the TMD-αvβ3 (appr. 53%), and the TMD-GpA transfectants (appr. 63%). Highest rate of necrotic events was detected in the TMD-GpA expressers (appr. 22%), when compared to TMD-αvβ3 (appr. 10%) and TMD-GpA-I transfectants (appr. 13 %). (Figure 11)

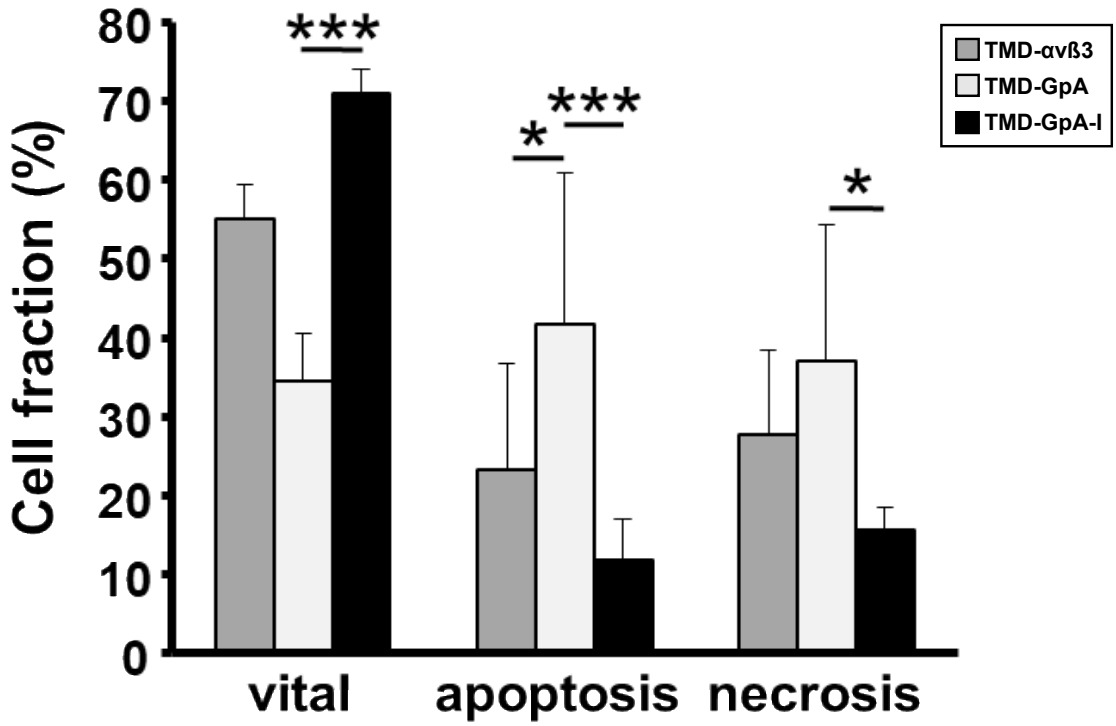


**Figure 10 Detection of early apoptotic events in detached EOC cells in ascites depending on the αvβ3 TMD conformational activation state**

After cultivation in suspension for 48 h in ascites, the cell transfectants were subjected to Annexin-V-Fluos/PI apoptosis assays in order to separate vital, apoptotic, and necrotic cell fractions, respectively. Data are indicated as cell fraction in % as mean values ± SD from 3 independent experiments. Significances for all experiments were determined by two-way ANOVA with *post hoc* Bonferroni *t*-test. Significance for all experiments is given by $*P < 0.050$, $**P < 0.010$, and $***P < 0.001$ (n. s., not significant).

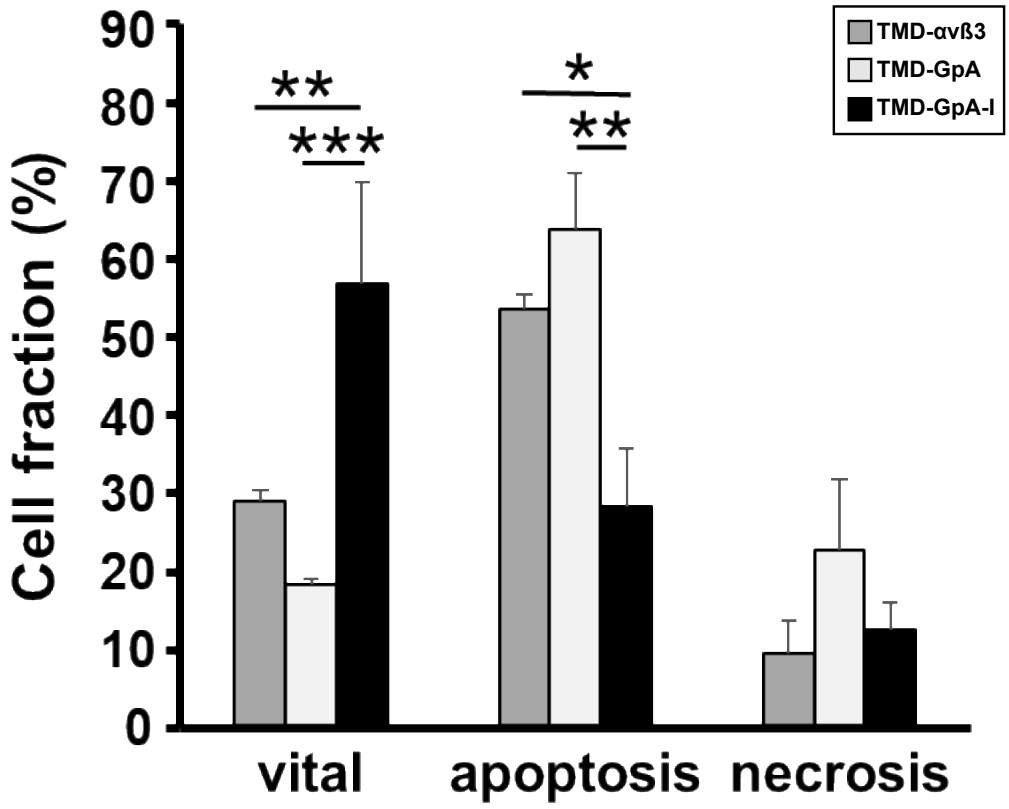


**Figure 11 Detection of early apoptotic events in detached EOC cells in DMEM depending on the αvβ3 TMD conformational activation state**

After cultivation in suspension for 48 h in DMEM the cell transfectants were subjected to Annexin-V-Fluos/PI apoptosis assays in order to separate vital, apoptotic, and necrotic cell fractions. Data are indicated as cell fraction in % as mean values ± SD from 3 independent experiments. Significance is given as outlined in legend to Figure 10.

Figure 12 shows the three cell transfectants suspended for 48 h in ascites or DMEM after application of the Annexin-V-Fluos/PI apoptosis assay. The evaluation of their behavior in terms of survival, apoptosis. and necrosis was performed by FACS analysis. The typical density plot quadrants and the cell division into vital, apoptotic, and necrotic cells are depicted. The lower left quadrant shows the vital cells (low Annexin and low PI staining), the lower right quadrant the apoptotic cells (high Annexin and low PI staining), and the upper right and upper left quadrants present the necrotic cells (high Annexin and high PI staining). (Figure 12)

The cell suspensions applied to Annexin/PI-FACS analysis were in parallel inspected by CLSM. As already found by performing Annexin-V-Fluos/PI apoptosis assays, in the cellular presence of the TMD-GpA cell transfectant, a high fluorescence signal intensity was noticed for Annexin-FITC and PI. In contrast, the TMD-GpA-I cell transfectants show a significantly lower signal intensity, both for Annexin and PI staining, which also accompanies with the results of the FACS analysis. (Figure 13)

All in all, these results indicate that cells expressing the constitutively active αvβ3 have a survival advantage associated with a delayed onset of apoptosis, even more after detached growing in ascites.

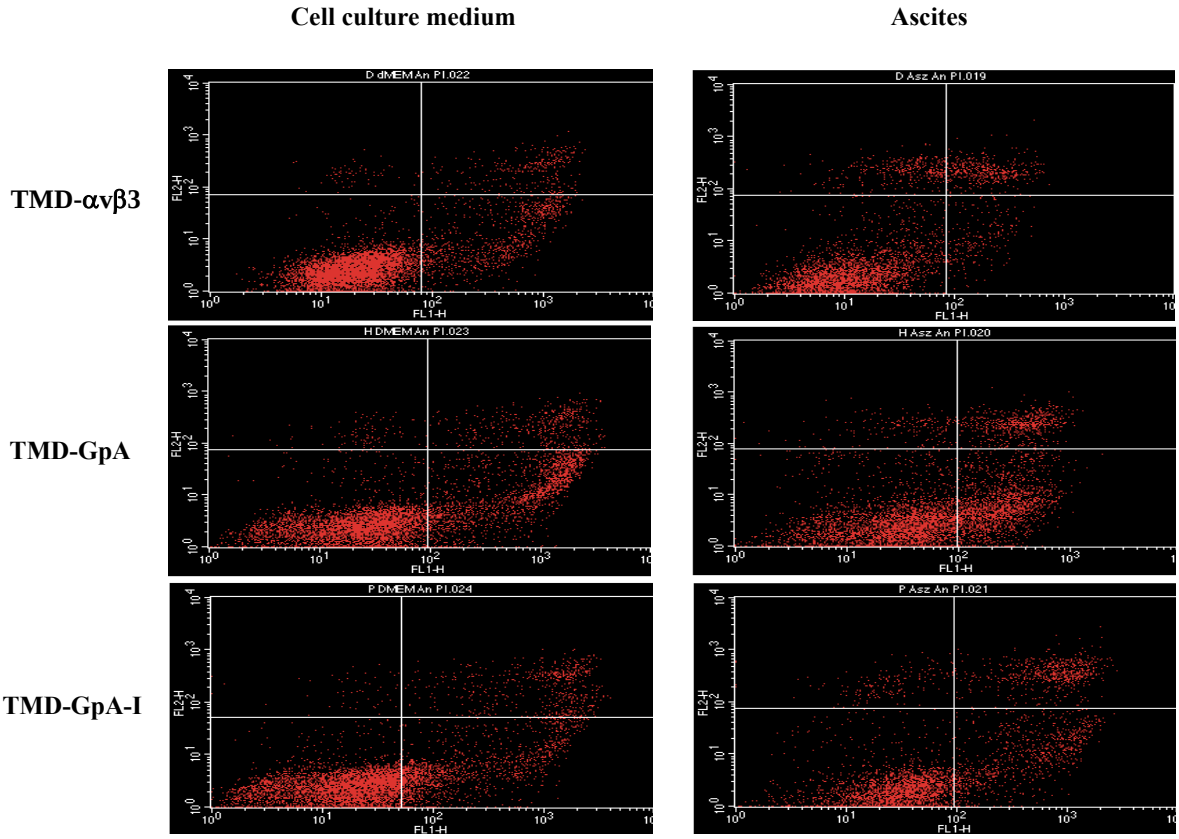


**Figure 12 Detection of vital, apoptotic, and necrotic cell fractions in EOC cells as a function of the αvβ3-TMD conformational activation state obtained by FACS analysis**

A typical density plot quadrant analysis of an Annexin-V-Fluos/PI staining is depicted for all three cell transfectants suspended for 48 h in ascites or DMEM; lower left quadrant: vital cells (low Annexin and low PI staining), lower right quadrant: apoptotic cells (high Annexin and low PI staining), upper right and upper left quadrants: necrotic cells (high Annexin and high PI staining).

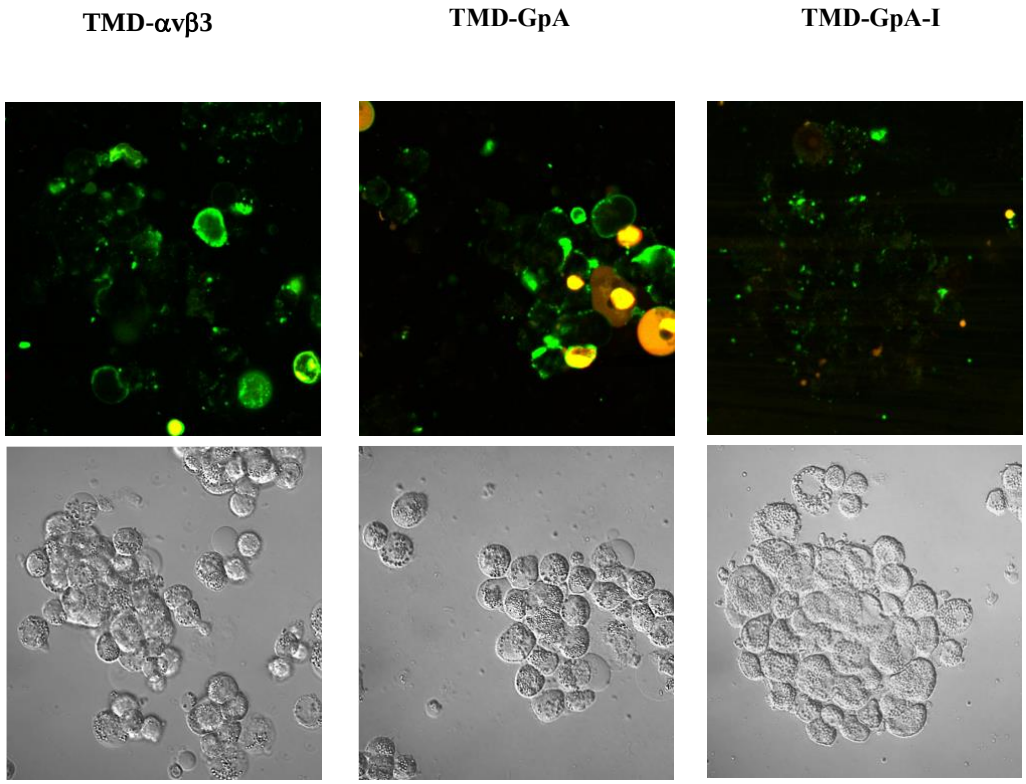


**Figure 13 Detection of the αvβ3-TMD cell transfectants after Annexin-V-Fluos/PI staining and visualization by CLSM**

After suspension for 48 h in ascites, early apoptotic (Annexin staining: green) and necrotic (PI staining: red; Overlay of Annexin and PI staining: yellow) events of the different EOC cell transfectants were visualized by CLSM. Representative fluorescence images and the associated differential interference contrast images are depicted.

## 3.2 Chemoresistance of anchorage-independently grown ovarian cancer cells as a function of the integrin αvβ3 TMD conformational activation state

Chemoresistance of EOC cells plays a crucial role in cancer progression especially for the survival of floating carcinoma cells in ascites which are shed from the primary tumor in the course of peritoneal metastasis. For the determination of the cellular effects of chemotherapeutics on cells expressing integrin αvβ3 in the different TMD conformational activation states, cells were suspended in ascites or cell culture medium for 30 hours, followed by the addition of 2.5 µg/ml cisplatin for another 18 hours.

As expected, the viability of all different transfectants dropped after treatment with cisplatin.

The cells (after growth in ascites) expressing the TMD-GpA-I still presented the highest number of vital cells with an only 2.9-fold drop. However, in TMD-GpA and TMD-αvβ3 expressers, a drop of appr. 5.6-fold was noticeable. Due to the effect of cisplatin, an 1.8-fold increase of cells within the apoptotic fraction of TMD-GpA expressers compared to the absence of cisplatin was observed The TMD-αvβ3 transfectants showed an appr. 3.5-fold enhancement of apoptotic cells under the influence of cisplatin. Concerning the TMD-GpA-I expressers, which had without the effect of cisplatin 2 and 1.3-fold more cells alive that can be affected by cisplatin than TMD-GpA and TMD-αvβ3 expressers, here, the apoptotic cell fraction still remained lowest (appr. 48%). However, the TMD-GpA-I transfectants presented a high necrotic cell fraction (appr. 29%) compared to the TMD-GpA (appr. 20%) and the TMD-αvβ3 expressers (appr. 15%), respectively. (Figure 14)

These data indicated that in ascites cellular expression of TMD-GpA-I was associated with a survival benefit after treatment with cisplatin compared to the effect of cisplatin on the other cell transfectants.

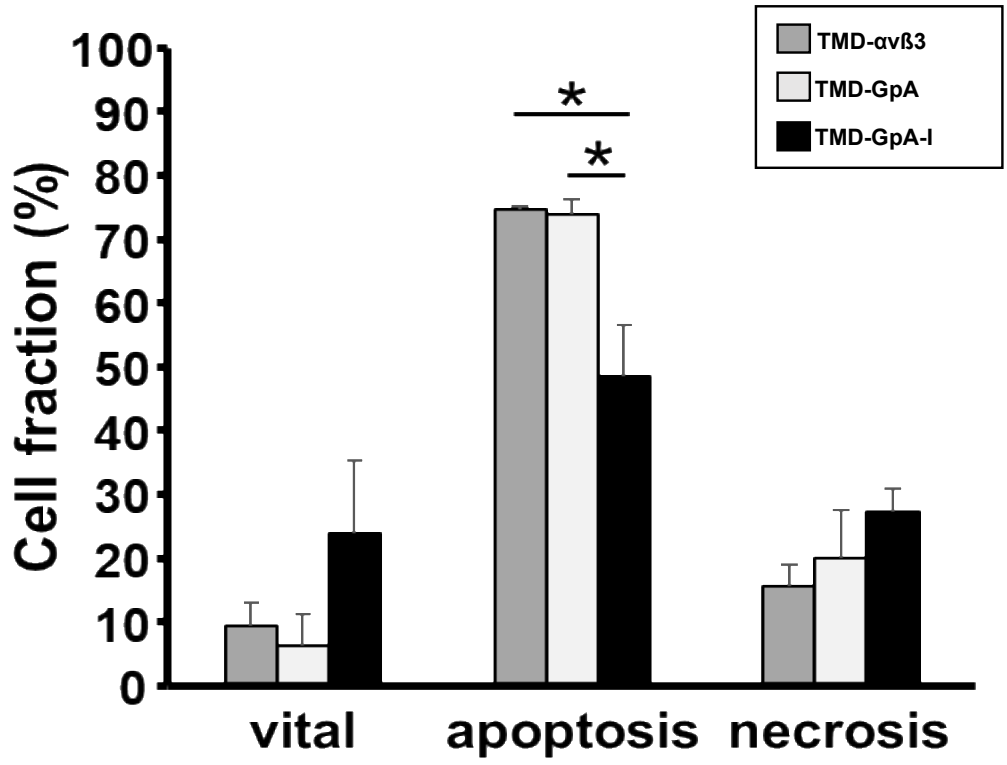


**Figure 14 Detection of early apoptotic events in detached EOC cells in ascites as a function of the αvβ3 TMD conformational activation state after treatment with cisplatin**

After 30 h of suspension in ascites, 2.5 µg/ml cisplatin were added for another 18 h. Data are indicated as fractions of vital, apoptotic, and necrotic cells in % as mean values ± SD from 3 independent experiments. Significance is given as outlined in legend to Figure 10.

In cell culture medium, apparently the difference between the different αvβ3 cell transfectants in terms of response to chemotherapy was abrogated. The cell vitality showed a comparable decrease in all cell transfectants (appr 29% for TMD-GpA-I, appr. 32% for TMD-GpA, and appr. 35% for TMD-αvβ3 expressers). The apoptotic fractions of the cell transfectants upon cisplatin treatment also presented an almost comparable range from appr. 51% (TMD-αvβ3) to appr 55% (TMD-GpA), and appr. 60% (TMD-GpA-I). The number of necrotic TMD-GpA-I and the TMD-αvβ3 transfectants, respectively, did not significantly differ from that in the absence of cisplatin. Only in the TMD-GpA expressers, a decrease of necrotic cells by appr. 10% was measured. (Figure 15)

In contrast to the results for cells suspended in ascites, those suspended in DMEM, did not reveal significant differences in the cell fractions as a function of cisplatin treatment.

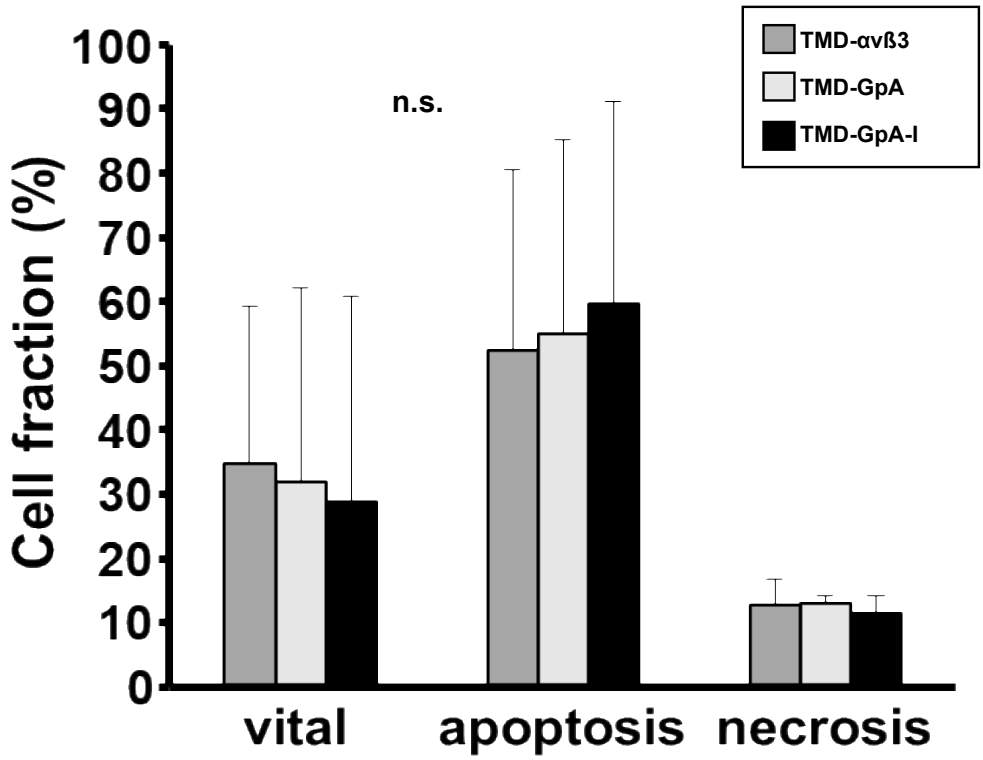


**Figure 15 Detection of early apoptotic events in detached EOC cells in DMEM as a function of the αvβ3 TMD conformational activation state after treatment with cisplatin**

After 30 h of suspension in DMEM 2.5 µg/ml cisplatin were added for another 18 h. Data are indicated as cell fraction in % as mean values ± SD from 3 independent experiments. Significance is given as outlined in legend to Figure 10.

## 3.3 Proliferative activity of detached OV-MZ-6 cells expressing different αvβ3-TMD conformation states

Proliferative activity of detached ovarian cancer cells as a function of their expression of integrin αvβ3 in its different TMD conformational activation states was evaluated by cell counting using a Neubauer hemocytometer after a suspension time of 3 and 48 hours in ascites. After 3 hours, an approximately comparable decline in the absolute cell number of all three cell transfectants was observed, which further decreased after a cultivation time of 48 hours. The most significant drop of vital cells was noticed in TMD-GpA-I expressers (appr. 67%), followed by TMD-αvβ3 cell transfectants (appr. 50%), and TMD-GpA expressers, the latter disclosing the smallest decrease in vital cells (appr. 33%). (Figure 16)

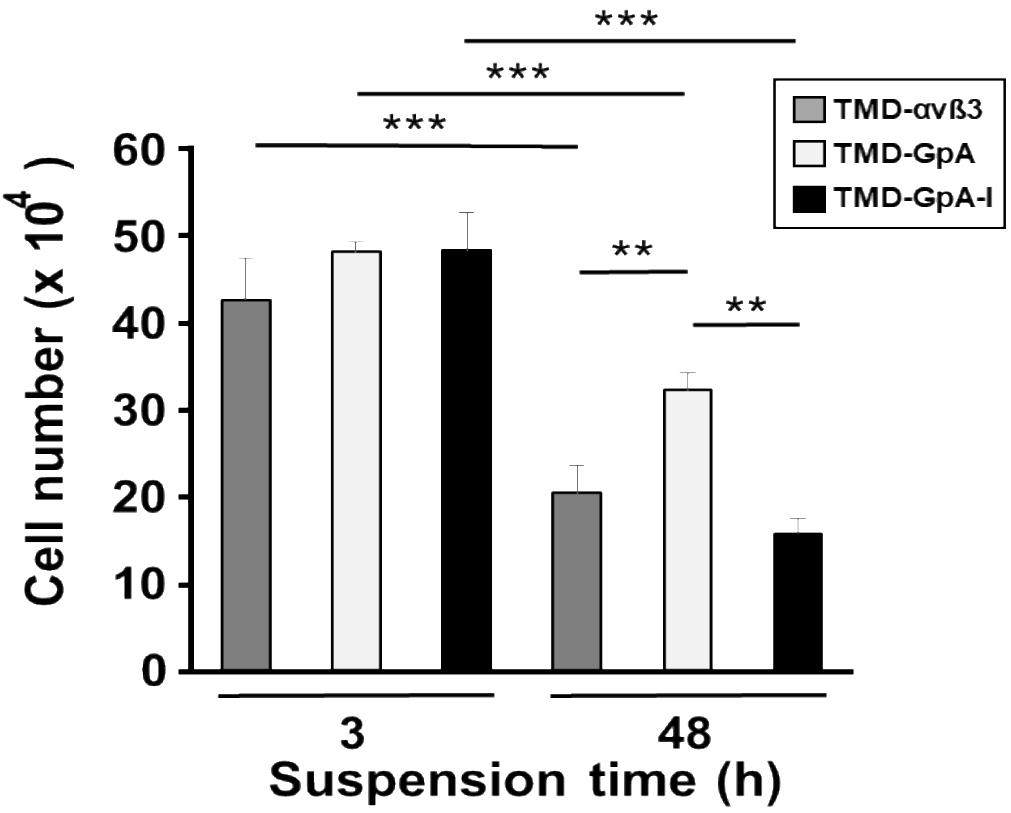


**Figure 16 Cell proliferation of floating human EOC cells as a function of the αvβ3-TMD conformational activation state**

Proliferative activity was detected after growth in suspension for 3 and 48 h in ascites by cell counting using a Neubauer hemocytometer. Data are given as mean values of cell numbers ± SD from 3 independent experiments. Significance is given as outlined in legend to Figure 10.

### 3.3.1 $p27^{Kip-1}$ expression

Alterations in the proliferative activity of the different cell transfectants floating in ascites was in addition analyzed by measurement of the expression of the cyclin/cyclin-dependent kinase inhibitor $p27^{Kip-1}$ by Western blot, known to take over a huge role in various cancer types, including ovarian cancer (Chu et al., 2008).

After 3 and 24 hours of cell suspension in ascites, TMD-GpA-I expressing cells showed the lowest expression levels of $p27^{Kip-1}$. However, in TMD-GpA-I transfectants, $p27^{Kip-1}$ expression increased within 48 hours in line with the observed lowest proliferative activity of this cell transfectant by appr. 2.6-fold and appr. 1.7-fold when compared to TMD-αvβ3 and TMD-GpA transfectants, respectively. (Figure 17)

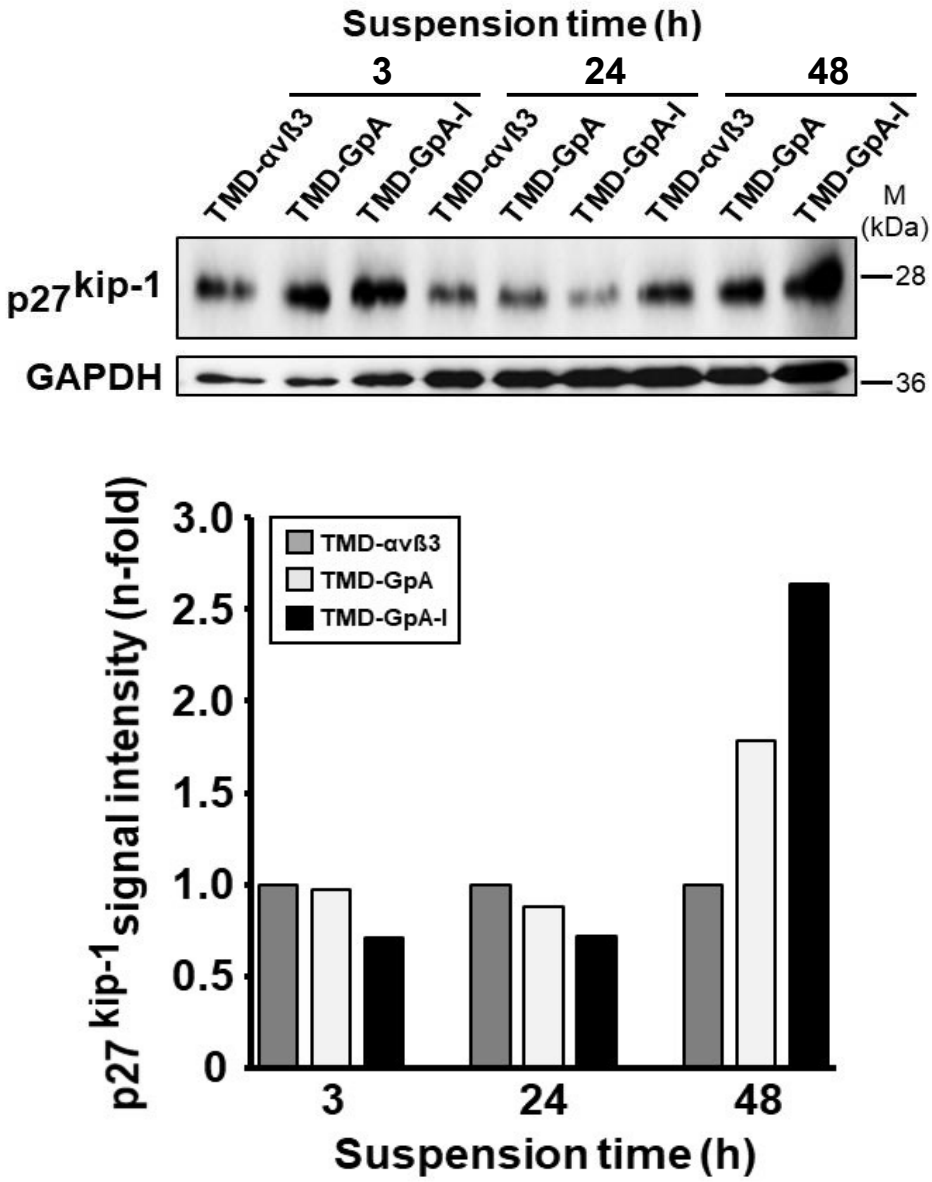


**Figure 17 $p27^{Kip-1}$ expression in human EOC cells suspended in ascites as a function of the αvβ3-TMD conformational activation state**

The expression of $p27^{Kip1}$ was determined by Western blot after cells were grown suspended in ascites for 3, 24, and 48 h, respectively. A typical Western blot is shown together with the corresponding histogram depicting GAPDH-normalized fluorescence signal intensities for $p27^{Kip-1}$ by setting data for the TMD-αvβ3 transfectant at each time point to '1'.

## 3.4 Spheroid formation by detached OV-MZ-6 cells expressing different αvβ3-TMD conformation states

EOC cells shed from the primary tumor are known to form multicellular spheroids within the ascites of ovarian cancer patients. In order to investigate this cellular behavior in the

experimental cell model used in this thesis, the three cell transfectants were cultured in ascites for 48 hours and cellular aggregation in dependence on the αvβ3-TMD conformational activation state was subsequently analyzed by CLSM.

Indeed, after 48 hours of cell suspension in ascites, the formation of cell clusters was noted for the different cell transfectants. Differences in the sizes of this multicellular aggregates were observed as a function of the αvβ3 activation state. The TMD-GpA-I expressers displayed small spheroids with many suspended single cells, followed by an increase of the size of the cellular aggregates in cells expressing the TMD-αvβ3. TMD-GpA transfectants were shown to form the biggest cell clusters under floating conditions in ascites. (Figure 18)

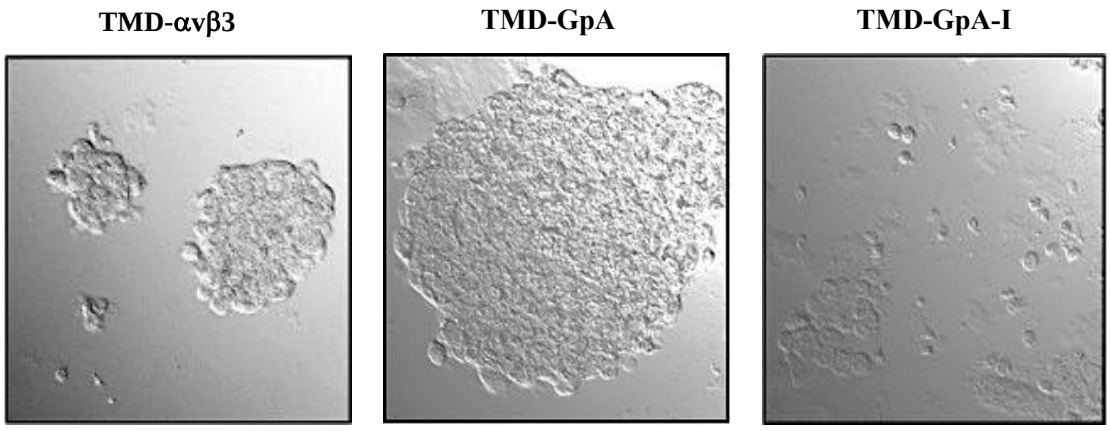


**Figure 18 Spheroid formation by TMD-αvβ3, TMD-GpA, and TMD-GpA-I transfectants, respectively, grown suspended in ascites**

After 48 h of cell cultivation under anchorage independent conditions in ascites, the cellular aggregation of the different cell transfectants as a function of the αvβ3 TMD conformational activation state was investigated by CLSM. The formation of the cell clusters is depicted by representative differential interference contrast images.

## 3.5 Capability of suspended cells to regain cell adhesive capacity as a function of the integrin αvβ3 TMD conformational activation state

In order to build secondary tumor knots and to form intraperitoneal metastasis, shed EOC cells from the primary tumor floating in patient ascites have eventually to re-gain the ability to adhere in different locations of the peritoneal cavity. Here, the influence of the time of EOC cell growth in suspension and the αvβ3 activation on the re-adhesive capacity of those cell transfectants

were studied. For this, cells were cultured for 48 hours under non-adherent conditions in ascites or DMEM, followed by an adhesion period of 2 hours onto cell culture plates.

Under both conditions, the TMD-GpA transfectants presented the lowest capacity for re-adhesion, whereas the other two cell transfectants re-gained their adhesive ability. In DMEM, the highest adhesive capacity was recovered by TMD-αvβ3 cell transfectants, followed by TMD-GpA-I expressers. After suspension in ascites, the TMD-GpA-I transfectants displayed the strongest cell adhesive capacity, which even exceeded TMD-GpA by appr. 6-fold and TMD-αvβ3 cells by appr. 1.2-fold. (Figure 19)

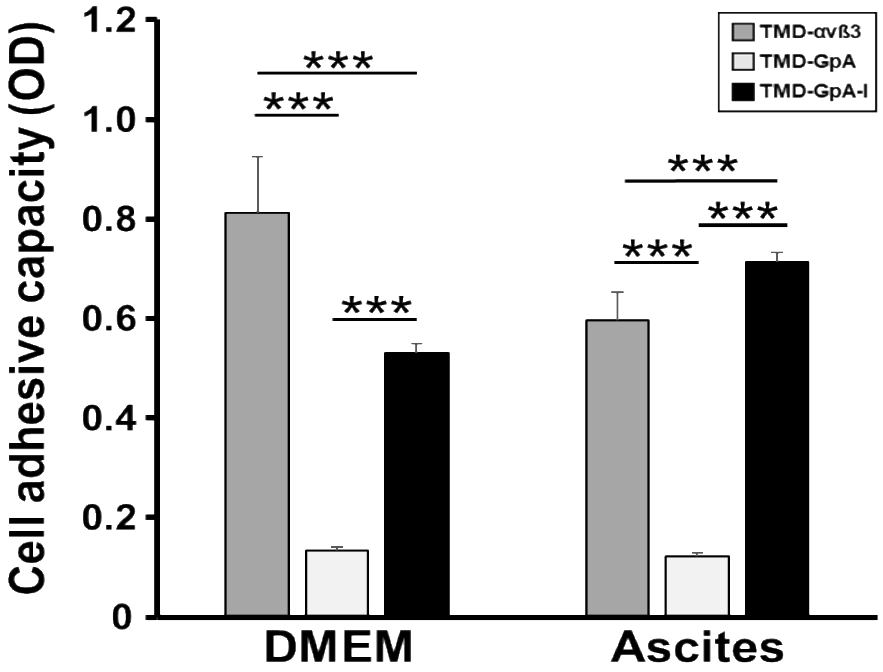


**Figure 19 Cell adhesive capacity after growing under nonadherent conditions in ascites or DMEM**
The different αvβ3 cell transfectants were cultured under suspension for 48 h in ascites or DMEM, followed by further growth for 2 h onto cell culture plates. Their ability to readhere was determined as described. Data are indicated as mean values of optical density (OD) ± SD from 4 independent experiments. Significance is given as outlined in legend to Figure 10.

## 3.6 EGF-R expression and activation in suspended ovarian carcinoma cells depending on the αvβ3 activation state

EGF-R is a member of the erythroblastosis oncogene B (ErbB) family (Herbst, 2004).

In a previous study with adherent OV-MZ-6 cells, an upregulation of the EGF-R expression and activation in correlation to the expression level of αvβ3 had been determined (Lossner et al., 2008). Based on these findings, the EGF-R expression and activation of EOC cells, grown anchorage-independently, depending on the αvβ3 activation states were here investigated. For this, the different cell transfectants were cultured under non-adherent conditions in ascites for 3, 24, and 48 hours, followed by the evaluation of the expression and activation levels of the EGF-R as a function of the αvβ3 activation state by Western blot analysis.

Cellular expression of TMD-GpA-I led to increased EGF-R expression levels, reaching an appr. 12-fold at 24 hours and an appr. 18-fold higher expression level after 48 hours compared to the EGF-R content in TMD-αvβ3 expressers. The TMD-GpA transfectants presented after 3 hours of flotation in ascites a similar expression profile for the EGF-R like TMD-GpA-I expressers, in contrast to the lower expression levels in TMD-αvβ3 cells. The EGF-R content in TMD-GpA transfectants decreased over time, with still appr. 2-fold higher expression levels after 24 hours compared to the TMD-αvβ3, however without further changes until 48 hours suspension time. (Figure 20)

Concerning the activation of the EGF-R, similar results were found. After 24 hours of cultivation in ascites, for TMD-GpA-I cell transfectants a decreased EGF-R activation was observed, followed by a significant higher activation after 48 hours (up to 4.7-fold over that in TMD-GpA and TMD-αvβ3 expressers, respectively). (Figure 21)

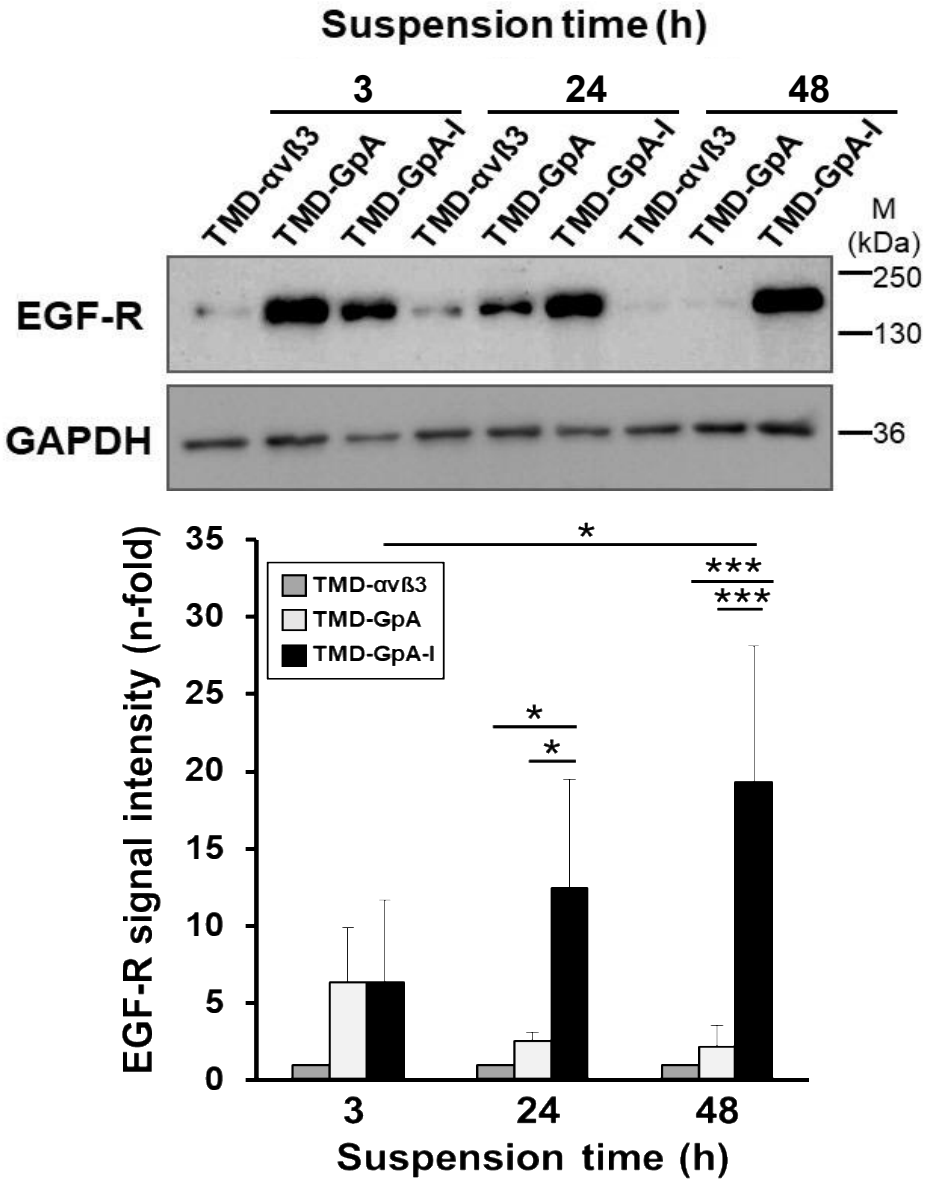


**Figure 20 EGF-R-expression of human EOC αvβ3-TMD transfectants after anchorage-independent growth in ascites**

The expression levels of the EGF-R were determined by Western blot analysis after growth of cells for 3, 24, and 48 h, respectively, suspended in ascites. A typical Western blot is shown together with the corresponding histogram. The histogram depicts GAPDH-normalized fluorescence signal intensities ± SD (*n*=3) for the EGF-R by setting the data for the TMD-αvβ3 transfectant at each time point to '1'. Significance is given as outlined in legend to Figure 10.

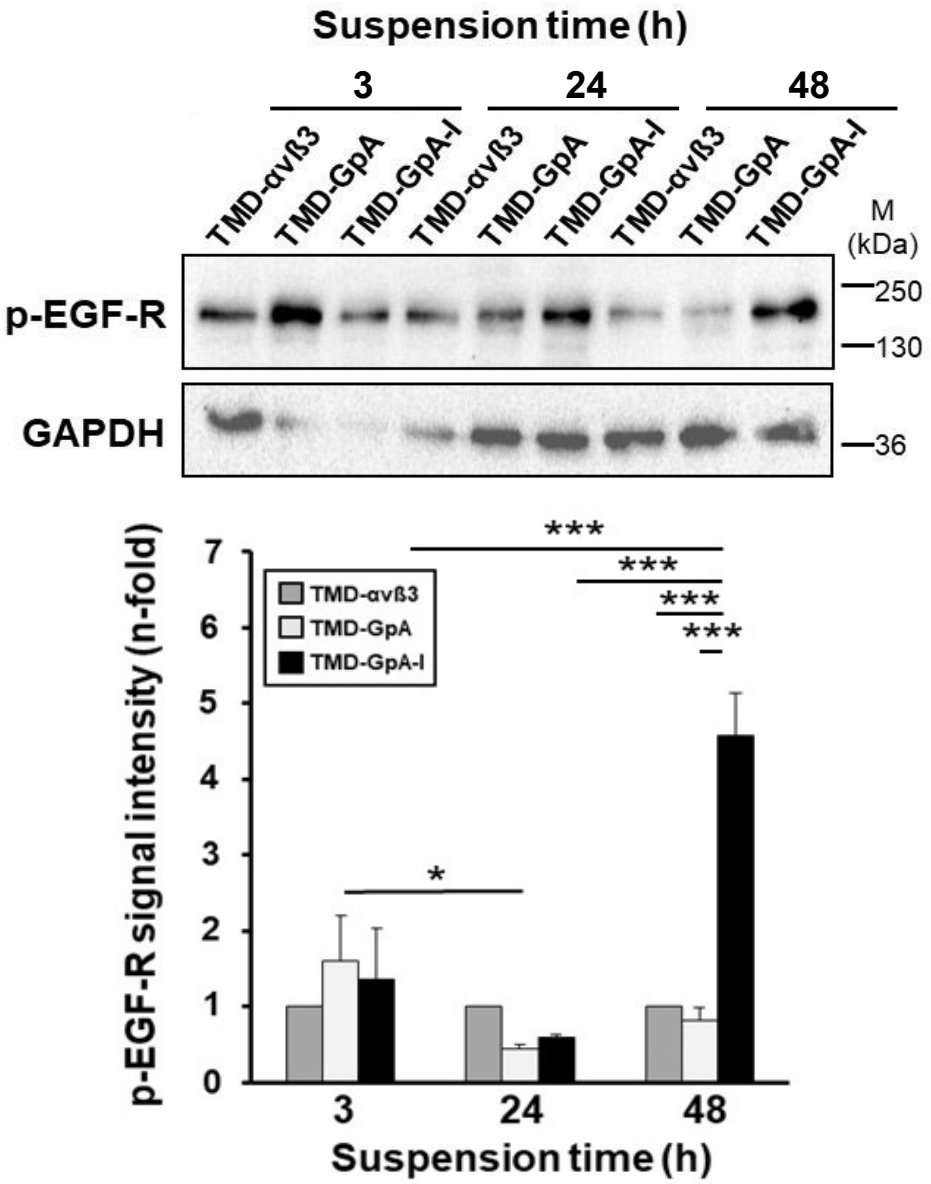


**Figure 21 EGF-R-activation of human EOC αvβ3-TMD transfectants after anchorage-independent growth in ascites**

The activation levels of the EGF-R were determined by Western blot analysis after growth of cells for 3, 24, and 48 h, respectively, suspended in ascites. A typical Western blot is shown together with the corresponding histogram. The histogram depicts GAPDH-normalized fluorescence signal intensities ± SD (*n*=3) for the p-EGF-R by setting the data for the TMD-αvβ3 transfectants at each time point to '1'. Significance is given as outlined in legend to Figure 10.

## 3.7 Activation of integrin-related signaling molecules in suspended EOC cell transfectants as a function of the αvβ3 activation state

Integrins act as transmembrane adhesion receptors capable of transducing signals bi-directionally across cell membranes. After binding to the ECM, integrins activate intracellular signaling cascades, finally leading to alterations in cellular proliferation, migration, or cell survival (reviewed in Cooper & Giancotti, 2019).
In this thesis, the activation of these integrin-dependent signaling pathways in detached EOC cells as a function of the αvβ3 TMD conformational activation state was studied.

### 3.7.1 FAK expression and activation

Since FAK is a very important downstream signaling molecule within the integrin signal transduction cascade, its expression levels and activation status were determined by Western blot analysis as a function of the expression of integrin avß3 in its different activation states. Highest FAK expression was noticed in suspended TMD-GpA-I expressers with comparable levels in their adherently grown cell counterparts. The TMD-αvβ3 expressers showed similar levels of FAK expression. For suspended TMD-GPA transfectants, decreased FAK expression was observed which, however, did not reach significance. Also, the adherently grown cell transfectants presented similar expression patterns. (Figure 22)
Moreover, in TMD-GpA-I expressers, also highest activation of FAK (appr. 2.5-fold elevated over TMD-GpA and TMD-αvβ3 transfectants, respectively) was detected, very similar to the content of p-FAK in adherent TMD-GpA-I expressers. (Figure 23)

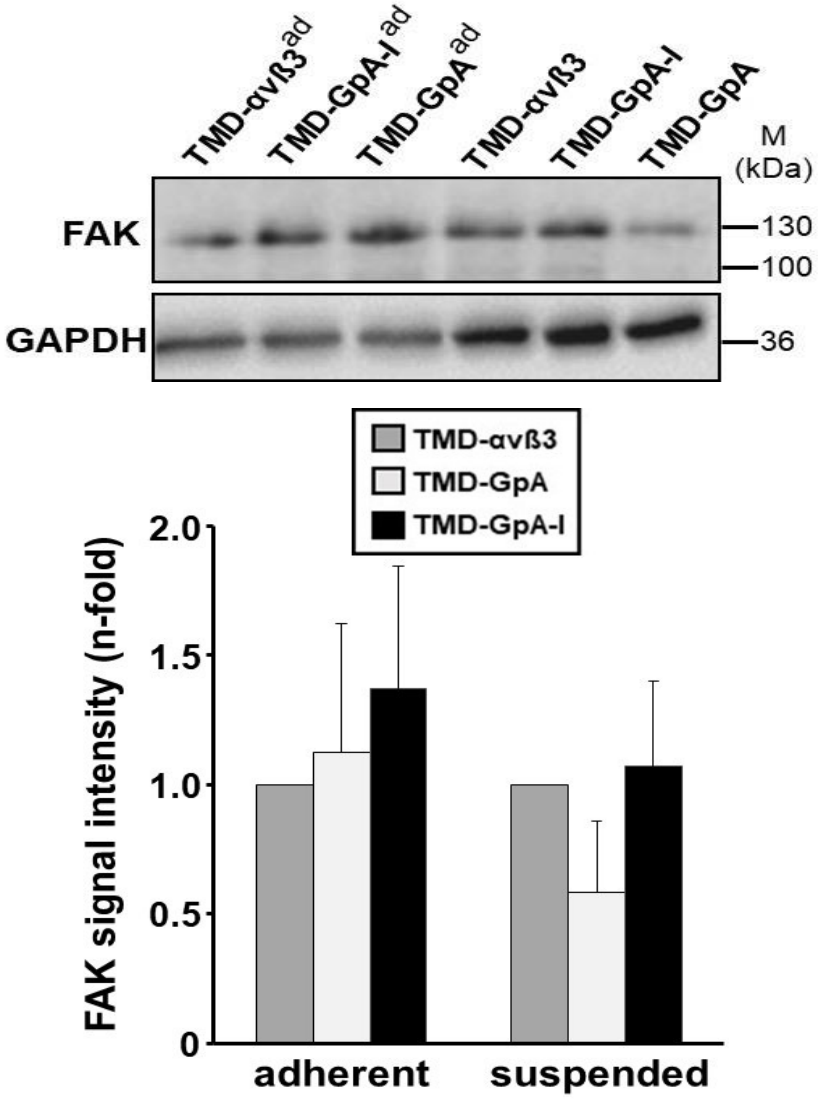


**Figure 22 FAK expression in EOC αvβ3-TMD transfectants**

FAK expression levels were investigated by Western blot analysis in cells grown suspended for 48 h in DMEM or grown adherent to cell culture plates. A typical Western blot is shown together with the corresponding histogram. The histogram depicts GAPDH-normalized fluorescence signal intensities ± SD (*n*=3) for the FAK by setting the data for the TMD-αvβ3 transfectants under each culture condition to '1'.

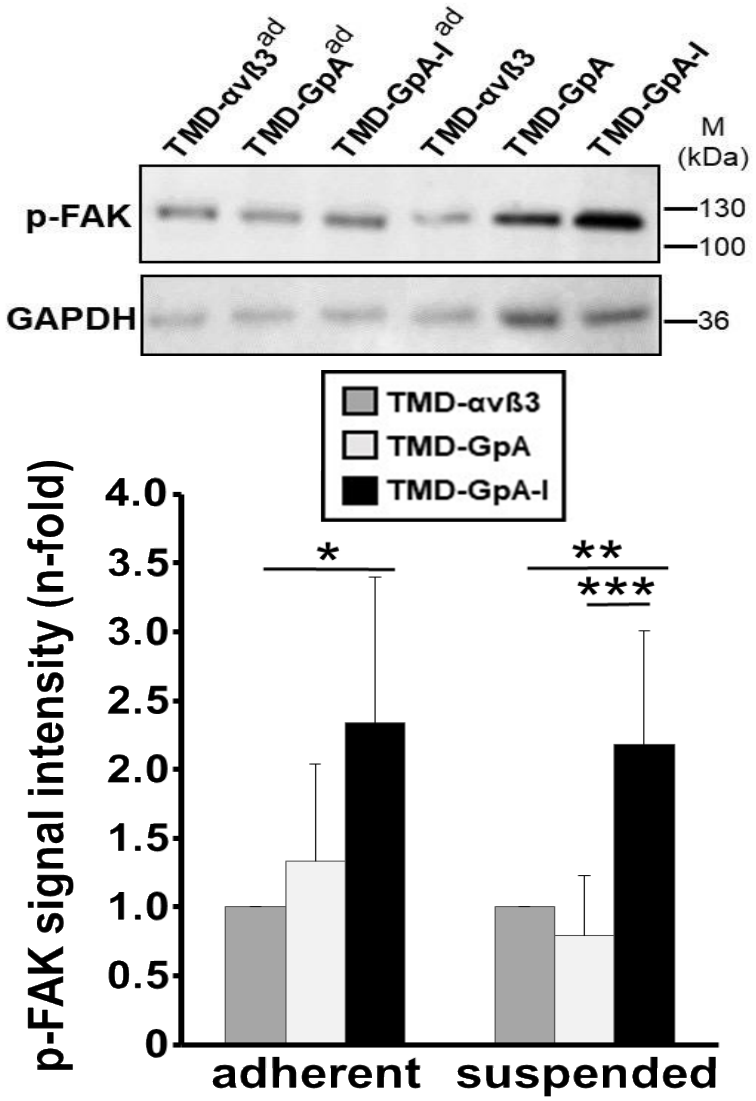


**Figure 23 FAK activation in EOC αvβ3-TMD transfectants**
FAK activation levels were investigated by Western blot analysis in cells grown suspended for 48 h in DMEM or grown adherent to cell culture plates. A typical Western blot is shown together with the corresponding histogram. The histogram depicts GAPDH-normalized fluorescence signal intensities ± SD (*n*=3) for the p-FAK by setting the data for the TMD-αvβ3 transfectants under each culture condition to '1'. Significance is given as outlined in legend to Figure 10.

## 3.7.2 Src expression and activation

Src is a non-receptor tyrosine kinase which plays a huge role in cell proliferation, migration, and survival (Roskoski, 2004). Moreover src is correlated to tumorigenicity, tumor progression, and metastasis (Irby & Yeatman, 2000).

Thus, the expression and activation of src in detached EOC cells as a function of the αvβ3 TMD activation state were investigated.

Src expression and activation depending on the αvβ3 TMD conformational activation state was determined after cultivation of cells suspended in ascites. After 48 hours, the TMD-GpA-I cell transfectants exhibited up to appr. 2-fold higher src expression and activation levels than cells displaying the TMD-GpA variant or the TMD-αvβ3 (Figure 24).

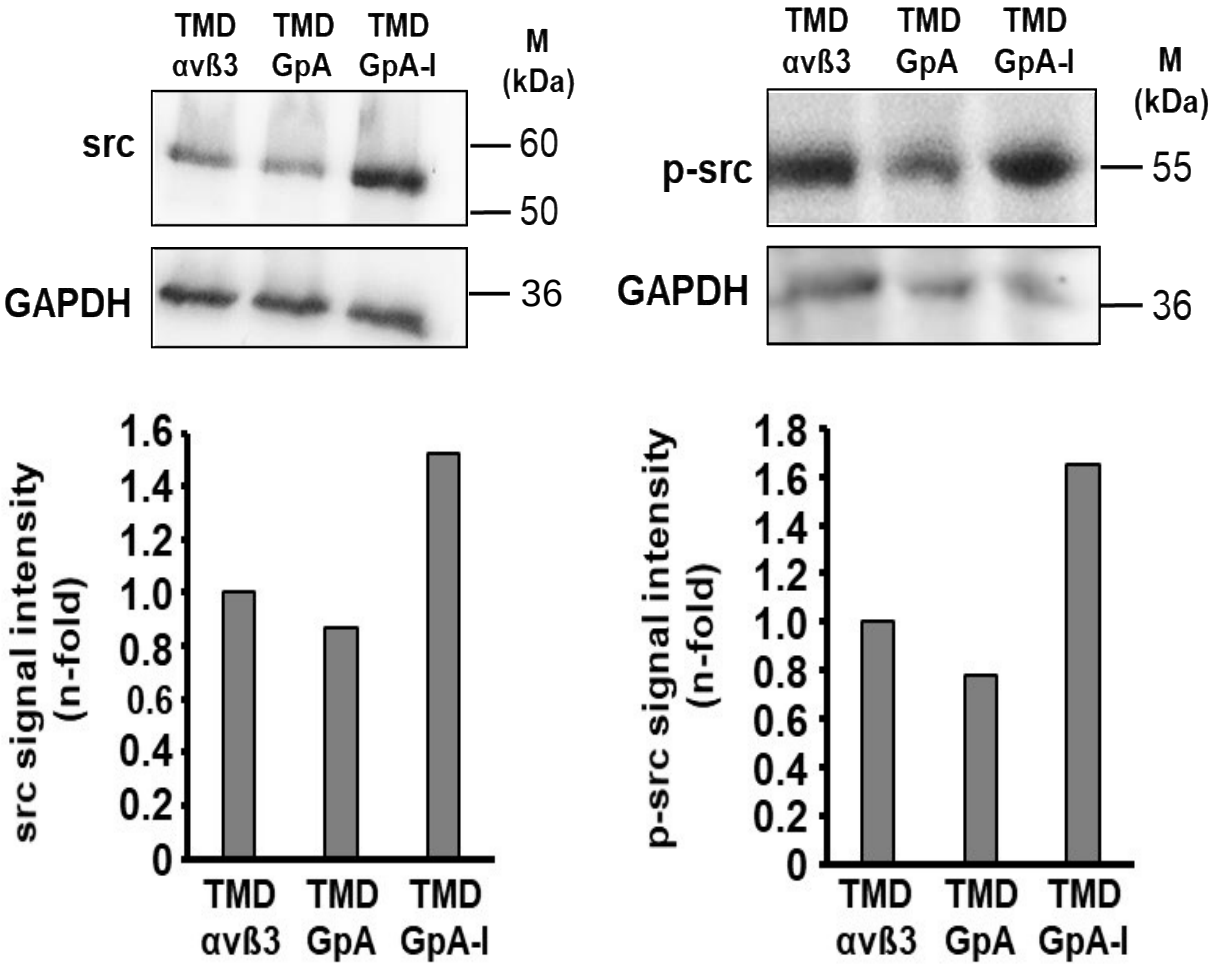


**Figure 24 Src expression and activation in EOC αvβ3-TMD transfectants**

Src expression and activation levels were investigated by Western blot analysis in cells grown suspended for 48 h in ascites. A typical Western blot is shown together with the corresponding histogram. The histogram depicts GAPDH-normalized fluorescence signal intensities for the src and the p-src by setting the data in the TMD-αvβ3 to '1'.

### 3.7.3 PKB/Akt expression and activation

PKB/Akt is a serine-threonine kinase, which activation plays a crucial role in various cell survival mechanisms, thus also in oncogenesis (Fresno Vara et al., 2004). Here, the expression and activation of Akt in suspended EOC cells in dependence on their αvβ3 activation state was studied and compared to their adherent counterpart.

The expression levels of PKB/Akt were not noticeably altered among all transfectants (Figure 25). However, the activation of PKB/Akt in TMD-GpA-I transfectants was increased up to appr. 3-fold and appr. 3.7-fold in adherently grown cells compared to TMD-GpA and TMD-αvβ3 transfectants, respectively. In suspended cells, PKB/Akt activation in TMD-GpA-I expressers was appr. 2.7- and 2.3-fold higher than in TMD-GpA and TMD-αvβ3 transfectants, respectively. (Figure 26)

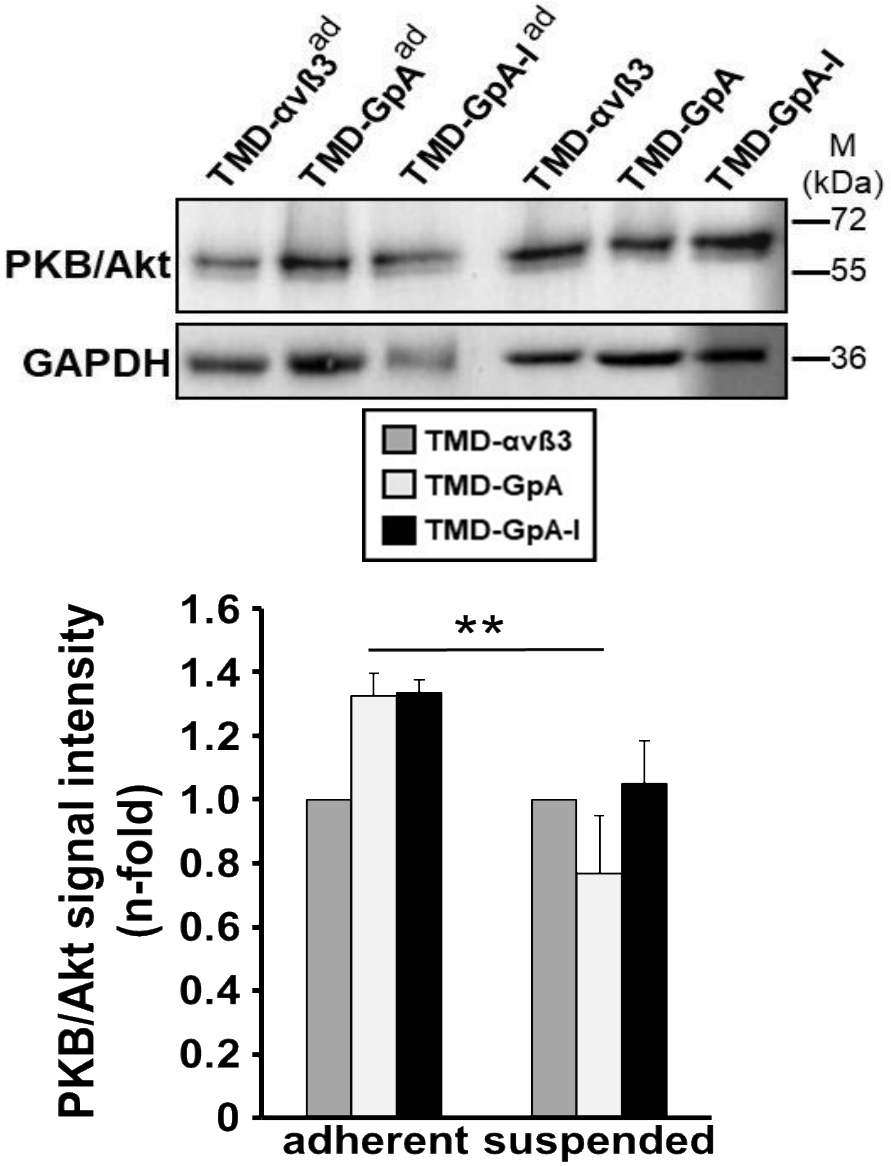


**Figure 25 PKB/Akt expression in EOC αvβ3-TMD transfectants**

PKB/Akt expression levels were investigated by Western blot analysis in cells grown suspended for 48 h in DMEM or grown adherent to cell culture plates. A typical Western blot is shown together with the corresponding histogram. The histogram depicts GAPDH-normalized fluorescence signal intensities ± SD (*n*=3) for the PKB/Akt by setting the data for the TMD-αvβ3 transfectants under each culture condition to '1'. Significance is given as outlined in legend to Figure 10.

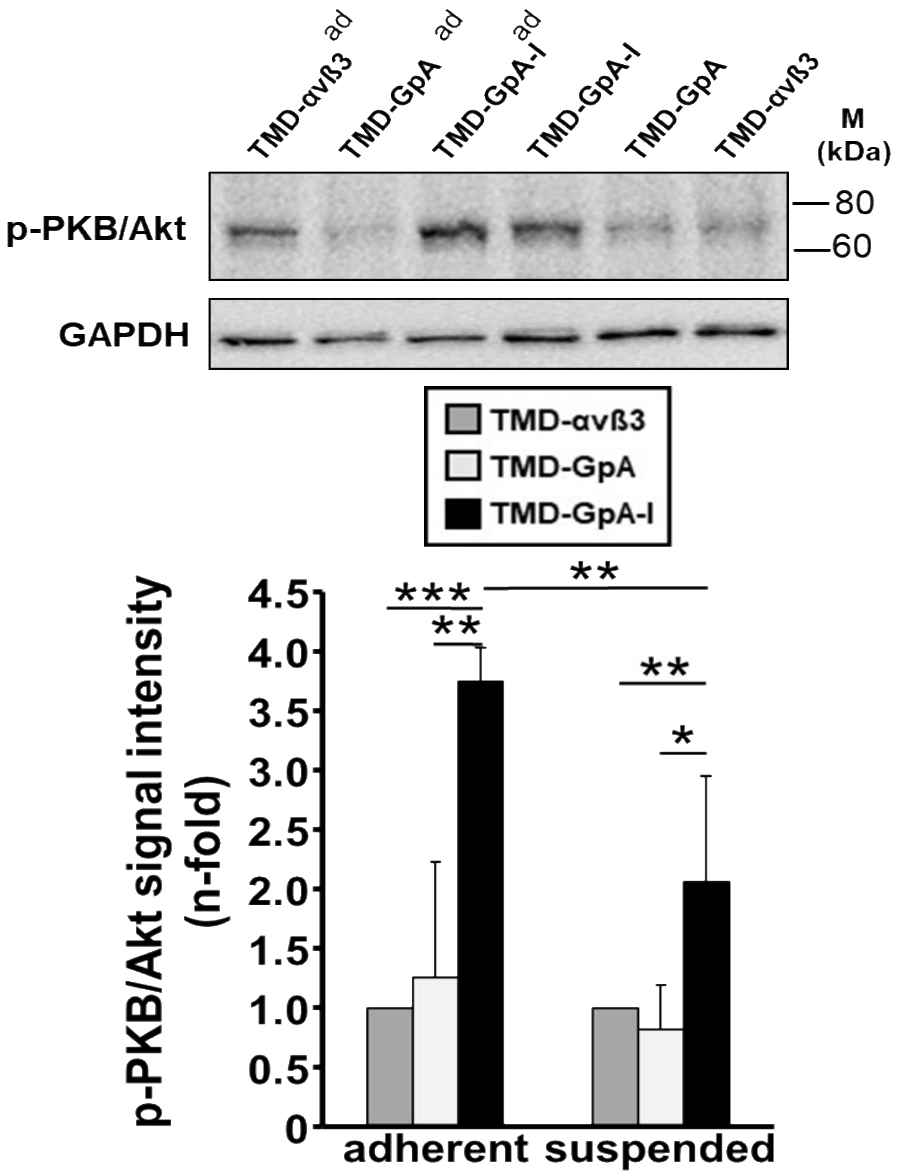


**Figure 26 PKB/Akt activation in EOC αvβ3-TMD transfectants**

PKB/Akt activation levels were investigated by Western blot analysis in cells grown suspended for 48 h in DMEM or grown adherent to cell culture plates. A typical Western blot is shown together with the corresponding histogram. The histogram depicts GAPDH-normalized fluorescence signal intensities ± SD (*n*=min. 3) for the p-PKB/Akt by setting the data for the TMD-αvβ3 transfectants under each culture condition to '1'. Significance is given as outlined in legend to Figure 10.

## 3.8 Expression of anti-apoptotic factors depending on the integrin αvβ3-TMD conformational activation state

### 3.8.1 Anti-apoptotic Bcl-2

The pro-survival protein Bcl-2 is a member of the Bcl-2 protein family, which plays a crucial role in the regulation of cellular apoptotic processes and thus in human cancer growth (Delbridge & Strasser, 2015).

Based on the results of the present study referring to the prolonged survival and delayed onset of apoptosis in non-adherent TMD-GpA-I expressers, it was next investigated whether changes in the expression of the anti-apoptotic protein Bcl-2 occurred correlating possibly with the anti-apoptotic behavior of the different cell transfectants. For this, EOC cells were grown suspended in ascites for 48 hours and the Bcl-2 content - as measured by Western blot analysis compared to that in adherently grown cells.

The suspended TMD-GpA-I cell transfectants presented a significant appr. 2- and appr. 4-fold higher expression levels compared to the TMD-αvβ3 and the TMD-GPA expressers, respectively. In adherent cell transfectants, no significant alterations in Bcl-2 expression levels were detected among all different cell transfectants. (Figure 27)

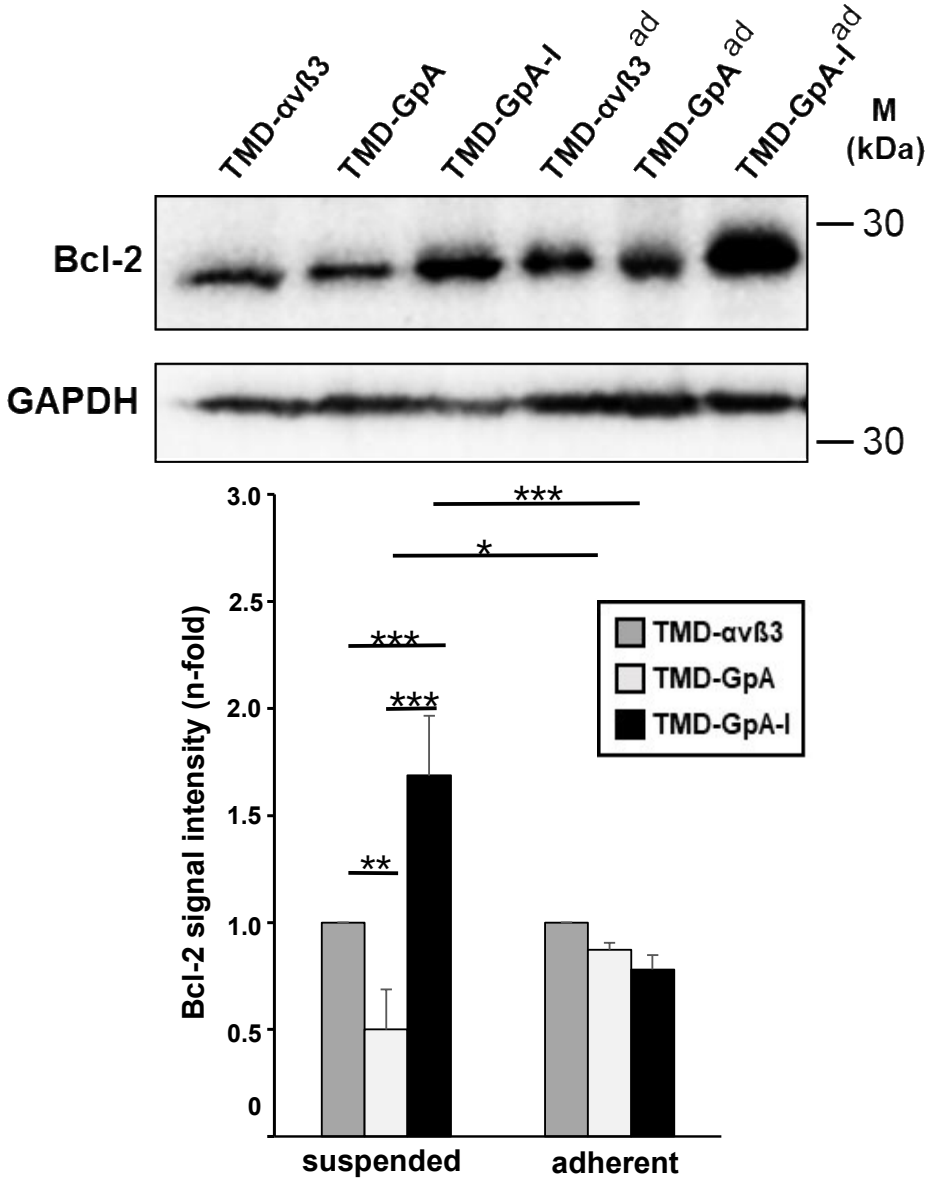


**Figure 27 Bcl-2 expression in EOC cells as a function of the αvβ3-TMD conformational activation state**

Bcl-2 expression levels were investigated by Western blot analysis in cells grown suspended for 48 h in ascites or grown adherent to cell culture plates. A typical Western blot is shown together with the corresponding histogram. The histogram depicts GAPDH-normalized fluorescence signal intensities ± SD (*n*=min. 3) for the Bcl-2 by setting the data for the TMD-αvβ3 transfectant under each culture condition to '1'. Significance is given as outlined in legend to Figure 10.

Additionally, Bcl-2 expression was assessed by immunocytochemical staining. Cells expressing TMD-GpA-I presented the strongest staining and hence the highest Bcl-2 level compared to the TMD-GpA and the TMD-αvß3 transfectants. (Figure 28)

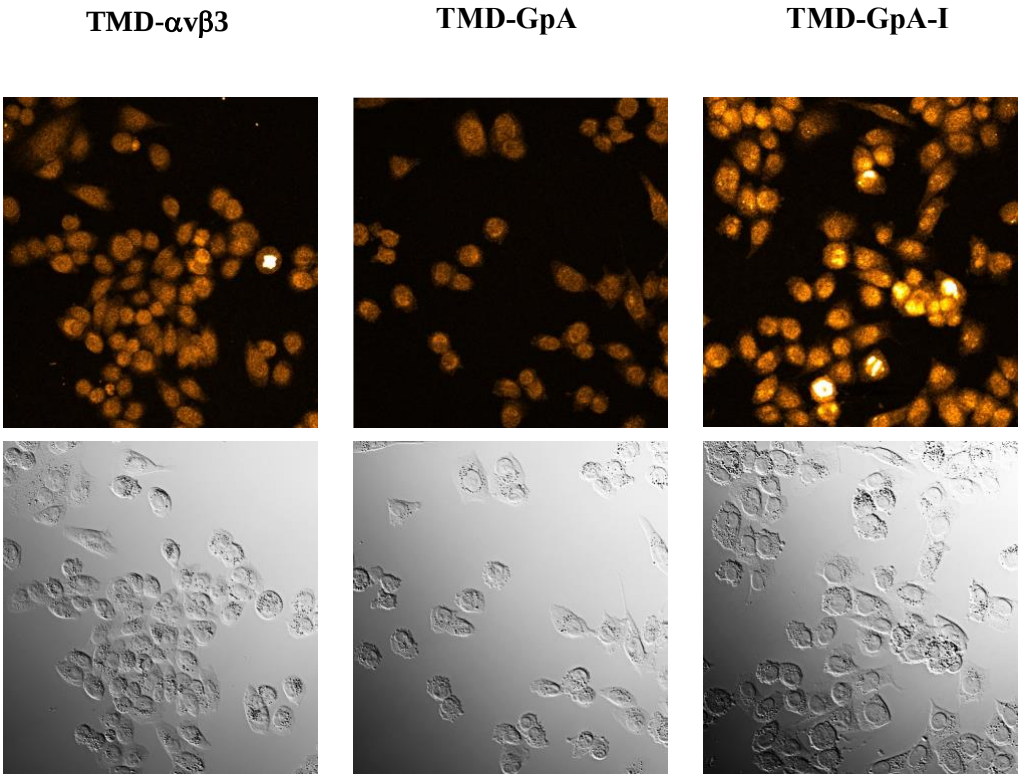


**Figure 28 Immunocytochemical detection of Bcl-2 in adherent EOC αvβ3-TMD transfectants.** After growth under adherence the Bcl-2 expression of the different EOC cell transfectants as a function of the αvβ3 TMD conformational activation state was done by immunocytochemical staining. Fluorescence signal intensities were visualized by CLSM using an Alexa 488-conjugated secondary antibody. Presented are representative fluorescence images and the corresponding differential interference contrast images.

### 3.8.2 Anti-apoptotic survivin

Survivin belongs to the inhibitor of apoptosis (IAP) protein family (Altieri, 2003; Li et al., 2018). Via binding and therefore inhibition of active caspase-3 and -7, it leads to an interruption of the apoptosis process (Tamm et al., 1998). Its expression levels in cancer cells are also associated with the patient's outcome and correlates with higher aggressiveness (Altieri, 2003; Li et al., 2018). Thus, the expression of survivin in floating and adherent EOC cell transfectants was also assessed, particularly in relation to the expression differences depending on their αvß3 activation state.

For this, EOC cells were grown anchorage-independently in ascites for 48 hours. Their expression levels of survivin were determined by Western blot analysis and compared to that in cells grown adherently

In floating TMD-GpA-I expressers, survivin expression level presented a significant appr. 2.7- and appr. 4-fold increase over levels in TMD-GpA and TMD-αvβ3 transfectants, respectively. In contrast to adherent TMD-GpA-I cells, the expression level of survivin was appr. 2-fold higher in detached transfectants. Among all adherent EOC cell transfectants no significant differences in survivin expression were noticed. (Figure 29)

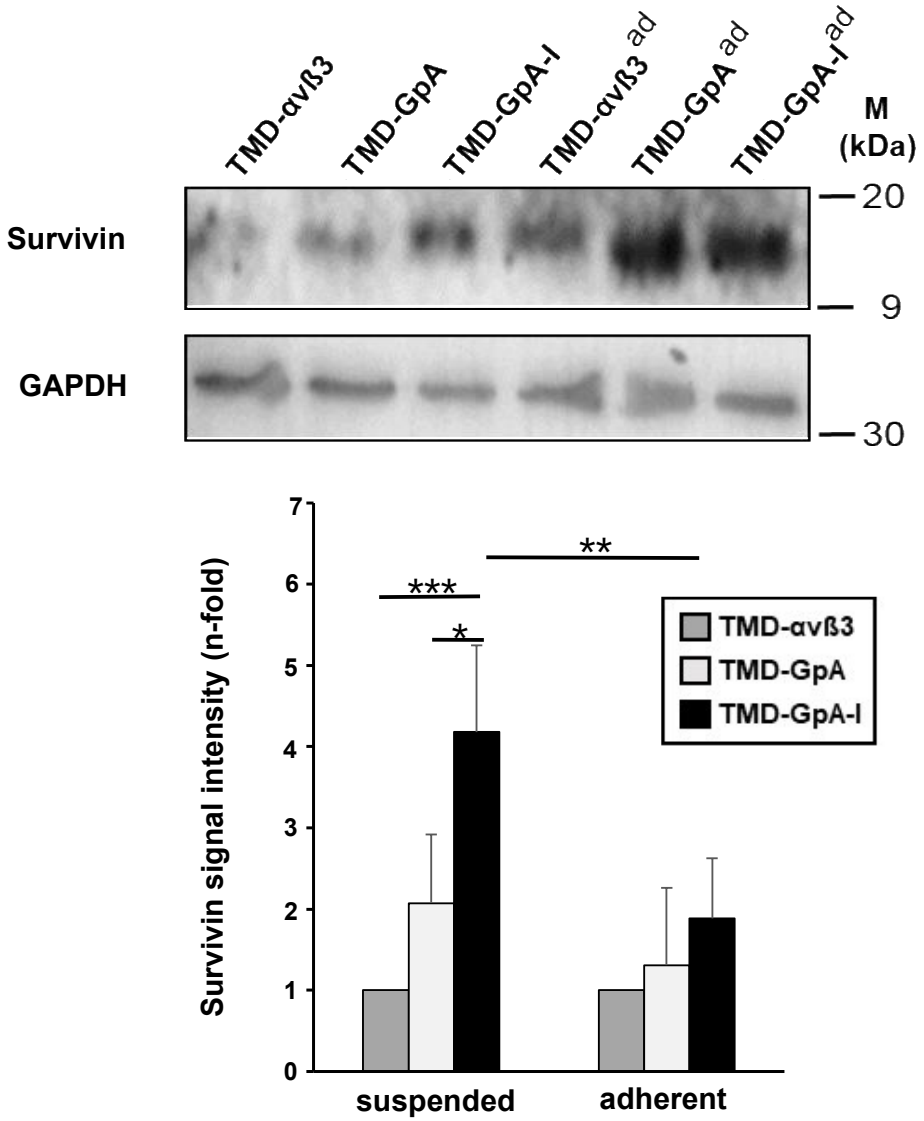


**Figure 29 Survivin expression in EOC cells as a function of the αvβ3-TMD conformational activation state**

Survivin expression levels were investigated by Western blot analysis in cells grown suspended for 48 h in ascites or grown adherent to cell culture plates. A typical Western blot is shown together with the corresponding histogram. The histogram depicts GAPDH-normalized fluorescence signal intensities ± SD (*n*=4) for the survivin by setting the data for the TMD-αvβ3 transfectants under each culture condition to '1'. Significance is given as outlined in legend to Figure 10.

# 4 Discussion

Under physiological conditions, epithelial and endothelial-type cells, which detach from the ECM or lose contact to adjacent cells undergo a special form of apoptosis, the anoikis (Frisch & Francis, 1994; Meredith et al., 1993; Simpson et al., 2008). However, malignant anchorage-dependent cells may survive under anchorage-independent conditions allowing them to invade adjacent tissue, to be distributed within the blood or lymph stream or - in the case of ovarian cancer - within the peritoneal fluid or ascites to sites of metastasis. Here, they regain their adhesive properties and reattach in locations within different compartments of the body to form secondary tumors. Hence, this resistance to anoikis plays a crucial role in the development of metastasis and, consequently, in cancer therapy. (Chiarugi & Giannoni, 2008; Frisch & Screaton, 2001; Zhong & Rescorla, 2012). In most of these processes, members of the integrin superfamily of adhesion and signaling receptors, among those αvβ3, are majorly involved. The detailed mechanisms of the contribution of integrin activation and signaling to anoikis resistance of cancer cells are not fully resolved yet. Over the last decades, accumulating evidence has been collected about conformational alteration during the integrin activation process, which leads to increased integrin affinity to ECM ligands and thus enhanced adhesive capacity as well as signal transduction capability (*outside-in* and *inside-out*) (Gottschalk, Adams, et al., 2002; Gottschalk & Kessler, 2002; Hughes et al., 1996; Hynes, 2002; Mas-Moruno et al., 2016; Nieberler et al., 2017; Shattil et al., 2010; Yamada & Geiger, 1997).

## 4.1 Anoikis resistance of *per se* anchorage-dependent human ovarian carcinoma cells as a function of the αvβ3 conformational activation state

In the present study, anoikis and chemotherapy resistance of human ovarian cancer cells had been analyzed as a function of different αvβ3 TMD conformational activation states by using the described ovarian cancer cell transfection model (Muller et al., 2013). For human ovarian cancer cells, which expressed αvβ3 in its highly affine conformational state with constitutively active signaling competence, lowest apoptosis rates and prolonged survival rates upon their flotation in ascites or DMEM compared to the low affinity state of αvβ3 lacking also signaling capacity as well as unactivated wild type αvβ3 were demonstrated. This was accompanied by a decline of the activation of the executioner caspase-3 (Dolinschek et al., 2021). These findings emphasize the importance of a fully active αvβ3 displaying dissociated integrin TMD to promote delayed onset of anoikis, thereby allowing detached cancer cells to survive and

instigate tumor growth at distant site of metastasis. Consequently, this leads to enhanced tumor progression and finally a poor patient outcome in accordance with findings of higher malignancy and aggressiveness of tumors expressing αvβ3 (reviewed in Nieberler et al., 2017). In fact, earlier studies in breast cancer cells have also suggested a role of an activated αvβ3 state in cancer progression and tumor metastasis (Sloan et al., 2006; Takayama et al., 2005). In these mentioned publications, the activated αvβ3 was shown to play a crucial role in hematogenous metastasis to the lungs and other organs. Also, activation of integrin αvβ3 was shown to be associated with spreading of breast cancer cells in bones where it interacts inter alia with osteoclasts (Sloan et al., 2006; Takayama et al., 2005). Moreover, via interaction of a high-affinity αvβ3 with platelets, the extravasation of tumor cells was observed, which represents a crucial step in the hematogenous metastatic process (Weber et al., 2016). Also, enhanced progression of leukemia was demonstrated to depend in part on constitutive integrin activation, most likely based on a dysregulated inside-out signaling (Brandsma et al., 2006). All of these results are also in accordance with previous findings for the platelet integrin αIIbβ3, where a point mutation in the integrin cytoplasmic domain led to the disruption of a putative salt bridge thereby abrogating the restrains for integrin subunit separation and conformational integrin activation, followed by enhanced cell growth and migration (Hughes et al., 1996). In prostate cancer, the activation of β1 integrin signaling was also shown to correlate with a higher metastatic potential due to anoikis resistance (Lee et al., 2013).

Similar to the observed spheroid formation of shed tumor cells in patient ascites, the deprivation of anchorage in those different αvβ3 cell transfectants upon cultivation in suspension also led to an obvious formation of cell clusters. Most interestingly, they differed in size, revealing that transfectants representing a low-affinity and signaling-incompetence in αvβ3 (TMD-GpA) formed large cell clusters, compared to the constitutively active TMD-GpA-I transfectants. This observation came somehow as a surprise, because there was expected to be a survival advantage of spheroid-like cell aggregates over single cells by enhanced cell-cell adhesion in order to compensate the loss of ECM anchorage. Thus, one might possibly suggest that expression of TMD-GpA-I might overrule the need for enhanced cell-cell contacts, whereas the TMD-GpA transfectant tend to form larger cell clusters fighting anoikis upon loss of ECM adherence.

During the metastatic process, shed cancer cells may regain the ability to attach to and invade tissue at locations within different compartments of the body to form secondary tumor knots (Chiarugi & Giannoni, 2008; Frisch & Screaton, 2001; Zhong & Rescorla, 2012). For those reasons, it was investigated whether the different αvβ3 cell transfectants of the used cell model were also capable of re-adhesion following a culture period upon loss of anchorage. Indeed,

fully active TMD-GpA-I expressers still exhibited best cell adhesive capacity among all different transfectants after a flotation period of 48 hours in ascites. These findings corresponded well with former data obtained by the research group of Prof. Dr. Reuning, who showed highest adhesive capacity of the TMD-GpA-I transfectant (Muller et al., 2013).

## 4.2 Chemotherapy resistance of *per se* anchorage-dependent human ovarian carcinoma cells as a function of the αvβ3 conformational activation state

For the prognosis of the outcome of ovarian cancer patients, radicality of the surgery with the aim of an R0 resection of the tumor represents the greatest significance and best prognosis predictor. Besides the surgical pillar, of course, chemotherapy plays a crucial role in the therapy of ovarian cancer. However, one major problem is the poor response of ovarian cancer cells to chemotherapy due to various resistance mechanisms. It had been verified that detached ovarian cancer cells after having formed spheroids exhibit resistance to some chemotherapy regimens, such as Taxol, an antimitotic drug, and also to radiation treatment (Filippovich et al., 1997; Makhija et al., 1999). The fact of development of chemoresistance, accompanied by changes in the cytoskeleton and integrin-dependent cell adhesion, is a major problem in cancer treatment (Aoudjit & Vuori, 2012; Luo et al., 2018; Maubant et al., 2002). Thus, the resistance of EOC cells to cisplatin as a function of the cellular expression of integrin αvβ3 in its different conformational activation states was here investigated. In both, ascites and DMEM, a drastic drop of the viability of detached cells in the presence of cisplatin was noticed, no matter what αvβ3 TMD was expressed. Still, highest cell viability and lowest number of apoptotic cells was found in cells floating in ascites and expressing TMD-GpA-I. On the opposite, lowest cell survival and a high apoptotic rate were determined in TMD-GpA transfectants. These results suggest the impact of the expression of constitutively active αvβ3 on chemotherapy resistance and thus of prolonged survival, even under non-adherent conditions in ascites. Also, malignant ascites is supposed to contribute to cell survival of shed tumor cells, since it contains various pro-survival factors, including growth factors, chemokines, cytokines, but also stromal, and immune cells (Piche, 2018). Thus, malignant ascites represents an additional factor for the survival of detached cancer cells providing a suitable environmental milieu for tumor progression (Latifi et al., 2012). These data are in accordance with studies by others, who characterized the role of integrins in cancer regarding their impact on chemoresistance. As such, resistance to Taxol had been noticed in human breast cancer cells due to the interaction of αvβ3

and CYR61, an angiogenic inducer overexpressed in breast cancer cells (Menendez et al., 2005). Another study in human breast cancer cells (MDA-MB-231) suggested that the activation of β1 integrins upon engagement of ECM ligands led to a delayed onset of apoptosis when treated with paclitaxel and vincristine via prevention of the cytochrome c release mediated by the PI3-kinase/Akt-pathway (Aoudjit & Vuori, 2001). Apart from that, Sethi et al. (1999) described another mechanism regarding chemoresistance of small lung cancer cells to etoposide, cyclophosphamide, or daunorubicin and protection from apoptosis, which was shown to involve β1 integrin. Here, via integrin β1-mediated adhesion to fibronectin, laminin, or collagen type IV, small lung cancer cells displayed reduced apoptotic events and gained the ability to survive longer (Sethi et al., 1999). Another study with human glioma cell lines described that the activation of αvβ3 or αvβ5 upon VN engagement resulted in chemoresistance (Uhm et al., 1999). In human laryngeal carcinoma cells, expression of αvβ3 enhanced their resistance to cisplatin or doxorubicin (Brozovic et al., 2008).

Thus, the major impact of overexpression and activation of αvβ3 on cancer cell survival, apoptosis, and drug resistance makes it a promising target in cancer therapy.

## 4.3 Proliferative activity of floating human ovarian cancer cells as a function of their expression of different integrin αvß3 activation states

Factors of the cell cycle are also involved in the regulation of cell proliferation and apoptosis due to their influence on proper cell division (Harbour & Dean, 2000; Nahle et al., 2002). Key players of the cell cycle are G1 phase cyclin-depended kinases (CDKs), which are regulated by integrins. Moreover, cell cycle progression depends on integrin-mediated cell adhesion to the ECM. Thus, following loss of ECM attachment, *per se* anchorage-dependent epithelial-type cells normally undergo cell cycle and thus growth arrest. (Schwartz & Assoian, 2001).

In this study, the proliferative activity of detached human ovarian cancer cells as a function of their expression of integrin αvβ3 was investigated. For the TMD-GpA and the TMD-GpA-I expressers floating in ascites for 3 hours, comparable proliferation rates were observed. However, after 48 hours of cell detachment, cell loss in all three cell transfectants was found, with lowest cell numbers for the TMD-GpA-I expressers, followed by cells harboring TMD-αvβ3. Highest cell numbers were determined for TMD-GpA transfectants. These data were confirmed by proliferation data obtained by culturing the cell transfectants in a 3D-hydrogel model. This model of cell cultivation is considered as a measure of the cellular metabolic

activity and its DNA content. Also, by that experimental approach, TMD-GpA expressers exhibited lowest metabolic activity in the presence of a coupled RGD motif, which changed after day 10, where lowest metabolic activity was found in cells expressing TMD-GpA-I. In addition, lowest DNA-content was found in TMD-GpA-I cell transfectants cultivated for up to 14 days in RGD-functionalized 3D-hydrogels, whereas it increased after 14 days in cells expressing integrin αvβ3 wild type or TMD-GpA. Also, CLSM images of ovarian cancer cell transfectants cultured for 14 days in 3D-hydrogels revealed larger spheroid sizes of the TMD-αvβ3 and TMD-GpA expressers compared to the TMD-GpA-I cell transfectants. (Dolinschek et al., 2021).

These results showed that – even highest percentage of viable cells was found in TMD-GpA-I expressers under anchorage-independent cultivation for appr. 48 hours – they still disclosed a low proliferative activity leaving them at least in part in a more quiescent/dormant state.

Also in murine lung cancer solitary cells detached from the primary tumor, a dormant state had been proven by others, which may contribute to the metastatic process (Cameron et al., 2000). The phenomenon of dormant cells is also described in breast cancer cells, where solitary cells rest in tissues, leading to a lower response to chemotherapeutic drugs and, consequently, recurrence of cancer (Naumov et al., 2002). Various chemotherapeutic drugs target highly proliferating cells, such as tumor cells (reviewed in Haschka et al., 2018). Thus, not only in quiescent cells a reduced response on chemotherapeutics is observed (reviewed in Mitchison, 2012), but also a low proliferation rate of tumor cells already favors chemoresistance (Wilson et al., 1997). This is also in accordance with the findings of the present study, showing that detached TMD-GpA-I transfectants with reduced proliferative activity exhibited highest resistance to cisplatin (4.2).

Moreover, expression of $p27^{Kip-1}$, a CDK inhibitor, was found in various cancer types, including ovarian cancer and takes over a substantial role in cell proliferation and cell death (Chu et al., 2008). In the present study, in constitutively active TMD-GpA-I cell transfectants $p27^{Kip-1}$ levels were elevated in line with their lower proliferative activity. Similar findings in ovarian tumor have been published by Korkolopoulou et al., who described lower levels of $p27^{Kip-1}$ in carcinomas with low malignancy in comparison to tumors with higher aggressiveness (Korkolopoulou et al., 2002). However, there are conflicting data on the role and the functions of $p27^{Kip-1}$ in cancer. On one hand, low $p27^{Kip-1}$ expression levels were shown to correlate with higher malignancy in ovarian carcinoma, on the other, it had been reported that its expression is associated with a better patient prognosis (Sui et al., 1999). Indeed, benign and borderline

tumors show higher $p27^{Kip-1}$ expression levels than malignant ovarian carcinomas (Sui et al., 2001).

Also, there are conflicting results from different studies regarding the impact of $p27^{Kip-1}$ expression on cell cycle progression. $p27^{Kip-1}$ had already been characterized regarding two functions: On one hand, in the nucleus it acts as a tumor suppressor, on the other, as an oncoprotein in the cytoplasm. There are two possible explanations for this assumption - the nuclear protein is inactivated after transport to the cytoplasm as so-called cytoplasmic re-localization. However, it is also possible that a protein has different functions in the cytoplasm and in the nucleus. (Blagosklonny, 2002). In agreement with the results of the present study, $p27^{Kip-1}$ expression correlated with a higher grade and consequently a poorer patient prognosis in endometrioid adenocarcinomas (Watanabe et al., 2002). Moreover, detached MCF-10A mammary epithelial cells, which overexpress $p27^{Kip-1}$ showed an arrest in the G1/S phase in order to avoid anoikis (Collins et al., 2005). Furthermore, also β1 integrins were shown to promote anoikis resistance due to a decreased expression of $p27^{Kip-1}$ (Hodkinson et al., 2006). Taken together, the results in this work are in accordance with previously published data underlining the important role of $p27^{Kip-1}$ in cell cycle regulation and anoikis.

## 4.4 EGF-R expression and activation in human ovarian cancer cells grown in suspension depending on the integrin αvβ3 activation state

Integrins are known to functionally cooperate with the EGF-R for pro-tumorigenic signaling in a synergistic manner, consequently, increasing cell adhesion, migration/invasion, and proliferation (Giancotti & Ruoslahti, 1999). Integrin-mediated cellular processes may accompany with a ligand-independent EGF-R activation and, *vice versa*, EGF-R activation results in ligand-independent integrin signaling (Cabodi et al., 2004; Moro et al., 2002; Moro et al., 1998; Sieg et al., 2000). EGF-R initiates cell signaling upon binding of its ligand EGF, leading to enhanced cell proliferation and survival (Rajaram et al., 2017). EGF-R is also overexpressed in various cancers, including ovarian cancer, and correlates with a low response to chemotherapeutic agents and radiation and thus poor patient prognosis (Herbst, 2004). Additionally, integrin-mediated cell adhesion leads to enhanced EGF-R cell surface density (Moro et al., 2002). Since previous data by the research group of Prof. Dr. Reuning had shown that EGF-R expression and activation increased in adherent ovarian cancer cells when they overexpressed αvβ3 (Lossner et al., 2008), it was next studied whether EGF-R expression and

activation was modulated as well in cells grown in ascites upon loss of ECM anchorage as a function of the αvβ3 conformational activation state. Indeed, the high affine and fully signaling TMD-GpA-I cell transfectants displayed also in this experimental cell model a significant time-dependent increase of EGF-R expression and activation compared to the TMD-GpA expressers, which presented a particularly low EGF-R expression and activation.

Thus, constitutive signaling competent αvβ3 promotes high EGF-R expression and activation levels in ovarian carcinoma cells grown under anchorage-independent cultivation conditions, which may be involved in anoikis resistance and formation of metastasis.

Reginato et al. (2003) indicated that in normal cells EGF-R expression depends on β1 integrin binding to an ECM ligand. If cell attachment to the ECM is lost (or blocked by an integrin antagonist), EGF-R levels were shown to be decreased, promoting the activation of pro-apoptotic proteins, such as Bim, and finally anoikis. Though, overexpression of EGF-R prevents its integrin-mediated downregulation after loss of ECM ligand binding and thus the onset of anoikis. Apparently, overexpression of EGF-R in cancer cells disrupts their regular interaction with integrins and thus averts integrin-mediated cell death. (Reginato et al., 2003).

Another study published the important role of integrin/EGF-R correlation concerning anoikis resistance in colon carcinoma cells (Guha et al., 2019). Guha et al. (2019) showed that anchorage-independent colon carcinoma cells, which are resistant to anoikis, displayed higher integrin α2, β1, and active EGF-R expression levels, respectively, than adherent colon cancer cells. Moreover, they observed an enhanced co-localization between integrin α2/-β1 or rather α5/-β and EGF-R on the surface of anoikis-resistant colon cancer cells. Besides that, they showed that the interaction of an unligated integrin with EGF-R led to an activation of further downstream signaling pathways like Erk and Akt, fostered the increase of the anti-apoptotic factor Bcl-2, and reduced the activation of caspase-3, thereby enhancing anoikis resistance (Guha et al., 2019). In hepatocellular cancer cells, the β4-EGF-R correlation exhibited enhanced development of metastasis due to anchorage-independent growth by activation of the integrin-mediated FAK-Akt signaling pathways (Leng et al., 2016).

To sum up, a constitutive active αvβ3 promotes high EGF-R expression and activation levels in ovarian carcinoma cells grown under anchorage-independent conditions, which seems to be involved in anoikis resistance and formation of metastasis.

## 4.5 Impact of the αvβ3 conformational activation state on integrin-related signaling molecules in suspended human EOC cells

Since it is well known that αvβ3-mediated cell signaling majorly contributes to various cellular processes, such as proliferation and cell survival, in the present study the influence of the αvβ3 TMD activation state in anchorage-independent EOC cells on the activation of various integrin-mediated signaling cascades was investigated. It was shown that the escape of anoikis by a tumor cell seems to be related to the constitutive activation of integrin-associated signaling pathways, involving FAK (Sulzmaier et al., 2014), src kinases (Parsons & Parsons, 2004; Roskoski, 2015), and PI3K/PKB/Akt kinases (Fresno Vara et al., 2004).

### 4.5.1 FAK

In epithelial-type anchorage-dependent cells, the integrin signaling pathway gets activated following integrin engagement by ECM ligands. One of the first downstream signaling molecules within the integrin signaling cascade is the non-receptor protein tyrosine kinase FAK, which is overexpressed in various cancer types (Weiner et al., 1993) correlating with higher cancer cell aggressiveness (reviewed in Sulzmaier et al., 2014). FAK is activated via autophosphorylation at the tyrosine residue Y397, leading to the activation of further downstream signaling molecules (reviewed in Sulzmaier et al., 2014). To this end, here, the role of FAK in ovarian cancer cells grown upon loss of ECM anchorage by measuring its expression as well as its phosphorylation and thus activation status was studied. These results disclosed an enhanced FAK expression and activation in both, adherent and suspended TMD-GpA-I cell transfectants. The lowest FAK expression and activation were noticed in cells expressing the TMD-GpA or the TMD-αvβ3. These findings confirmed previous data by the research group of Prof. Dr. Reuning, who showed before elevated FAK activation as a function of the αvβ3 activation state in adherent cells (Muller et al., 2013). In agreement to that, highest FAK expression and activation in EOC cells are apparently associated with constitutive αvβ3 activation, not only due to adherence to the ECM, but also after deprivation of cell attachment. This suggests a crucial participation of FAK in gaining anoikis resistance via integrin-mediated cell survival upon loss of ECM anchorage.

These findings are in accordance with a study in MDCK cells, in which FAK has also been demonstrated to inhibit anoikis, designating it as an important kinase of the integrin signaling pathway contributing to anoikis resistance and anchorage-independent cell growth (Frisch et al., 1996). A further study also described the induction of apoptosis following FAK inhibition

or its blockade in human breast cancer cells (Golubovskaya et al., 2002). Apart from that, in human breast cancer cells, the onset of apoptosis was observed to be correlated with dephosphorylation of FAK (Beviglia et al., 2003).
Taken together, the results of this work and other studies underline the role of FAK as an important player in the avoidance of anoikis/apoptosis.

### 4.5.2 Src

Also, the non-receptor tyrosine kinase src, which is overexpressed with enhanced activity in various human cancers, is linked to enhanced tumor cell growth, metastasis, and survival, since its expression and activation also affect integrin-mediated bidirectional signaling (reviewed in Irby & Yeatman, 2000). Based on a study by Degrosellier et al., who showed in anchorage-independently grown αvβ3-expressing tumor cells enhanced c-src activation after binding to the β3 cytoplasmic domain (Desgrosellier et al., 2009), in the present work the expression and activation of src in suspended EOC cells as a function of the αvβ3 activation state was investigated.
In this thesis, highest src expression and activation levels were determined in TMD-GpA-I cell transfectants compared to lowest levels in cells expressing TMD-GpA. These findings were in line with the results published by Degrosellier et al. (2009). However, in contrast to the present results, this was proven to happen in a FAK-independent fashion (Desgrosellier et al., 2009).
Also, Loza-Coll et al. (2005) published a transient upregulation of src activation after the detachment of normal intestinal epithelial cells, followed by a decrease of src expression to levels, which are comparable to those of adherent cells. This study described that further intracellular survival signals in these anchorage-independent cells were induced through src-mediated activation of the PI3-K/Akt pathway. So, this temporary increase of src expression levels was also shown to be involved in gaining resistance to anoikis (Loza-Coll et al., 2005). The relation of increased src expression/activation and the following enhanced impact on pro-survival Akt-signaling pathways leading to a lower sensitivity to anoikis was also shown in human colon carcinoma cells (Windham et al., 2002). In addition to that, also an interaction between the EGF-R and c-src had been determined. Thus, in a previous publication, enhanced src expression had been described as mediator of a higher malignant potential of the EGF-R (Maa et al., 1995). Maa et al. (1995) showed that c-src interacted with EGF-R through phosphorylation of the receptor, which led to a hyperactivation of the EGF-R, and, consequently, to a higher aggressiveness of various human tumors. Hence, EGF-R activation might also occur without ligand binding (Xu et al., 2014). These findings correspond well to

the obtained results in this present research project emphasizing the role of the EGF-R as a key contributor to cancer cell malignancy and survival and thus to anoikis resistance due to its interaction with integrins and its participation in the activation of intracellular integrin pro-survival signaling pathways.

### 4.5.3 PKB/Akt

Another integrin-related signaling molecule studied here is PKB/Akt. Its activation plays a huge role in various cellular processes, such as cell survival or cell growth (Hemmings & Restuccia, 2012). However, it has also been shown that its activation in cancer cells is associated with a possible development of resistance to apoptosis (Fresno Vara et al., 2004).

Based on this, in the present study, PKB/Akt expression and activation in adherent as well as suspended human EOC cells as a function of the expression of differently active αvβ3 conformational TMD states was analyzed. Neither in adherent nor in suspended cells in DMEM altered protein levels for PKB/Akt depending on the αvβ3 activation state were found. However, PKB/Akt activation was enhanced in adherent and suspended (in DMEM) TMD-GpA-I expressers. Same results relating to PKB/Akt activation had previously been reported in adherent OV-MZ-6 cancer cells (Muller et al., 2013).

The results of the present study are consistent with a previous publication demonstrating that overexpression or constitutively activated PKB/Akt was proven to inhibit anoikis in suspended MDCK cells (Khwaja et al., 1997).

Moreover, Cruet-Hennequart et al. (2003) published that activation of PKB/Akt is mediated by integrin αv through activation of the integrin-linked kinase (ILK). After blocking αv, a decrease of PKB/Akt phosphorylation at serine-473 was determined, pointing to a decreased proliferation of human ovarian carcinoma cells (Cruet-Hennequart et al., 2003). Moreover, PKB/Akt activation had been observed to be induced by ascites-mediated FAK activation, which leads to a decreased Akt phosphorylation after FAK deprivation (Lane et al., 2010).

Furthermore, in human epithelial cells PI3K/Akt signaling was linked to regulation of proteins of the Bcl-2 family, which also has an impact on cellular survival (Gauthier et al., 2001). Datta et al. published that the PI3K/Akt pathway averts cellular apoptosis due to its phosphorylation of the pro-apoptotic Bcl-2 family member Bad (Datta et al., 1997).

## 4.6 Impact of the αvβ3 conformational activation state on the expression of anti-apoptotic factors in detached human ovarian cancer cells

### 4.6.1 Bcl-2

Based on the results showing prolonged survival and delayed onset of apoptosis in suspended EOC cells expressing the constitutively activated αvβ3, the influence of the αvβ3 TMD activation state on the expression of anti-apoptotic factors was next investigated.

Bcl-2 is a member of the Bcl-2 family and acts as an anti-apoptotic protein. Its expression and especially its overexpression take over essential roles in cancer cell survival and tumor progression in many different cancer entities (reviewed in Schenk et al., 2017). By measuring its expression in ovarian cancer cells grown under flotation in ascites, highest Bcl-2 expression levels were documented in TMD-GpA-I transfectants thus favoring avoidance of anoikis. These results corroborate with previously published data from Matter and Ruoslahti (2001) that in adherent chinese hamster ovary (CHO) cells regulation of Bcl-2 expression depended on the initiation of intracellular signaling pathways via Shc and FAK. Subsequently, this resulted in the activation of Ras followed by a Ras-dependent PI3K/Akt pathway activation finally leading to increased Bcl-2 transcription (Matter & Ruoslahti, 2001). Furthermore, in αvβ1-transfected CHO cells adherent to fibronectin, enhanced Bcl2 expression had been demonstrated (Zhang et al., 1995). Moreover, blocking of the αv-subunit in breast cancer cells was shown to decrease Bcl-2 levels (Cheuk et al., 2020). However, Bcl-2 does not only interfere with the apoptotic cell pathway, but has also an impact on cell cycle progression, since it delays the G1-phase and the transition to the S-phase due to retarded activation of the cyclin-dependent protein kinases cdk2 and cdk4 by increasing the expression of the cyclin/cyclin-dependent kinase inhibitor $p27^{Kip-1}$ (Greider et al., 2002; Vairo et al., 2000).

### 4.6.2 Survivin

Since in floating αvβ3 cell transfectants a correlation between the cellular expression of a fully activated αvβ3 and Bcl-2 expression was found, in addition, another anti-apoptotic protein possibly involved, survivin, was determined. Survivin inhibits apoptosis induced by Fas or Bax and abrogates the transformation of procaspase 3 and 7 into active caspases thereby protecting cells from apoptosis (Tamm et al., 1998).

In adherent αvβ3 cell transfectants, no differences in survivin expression levels were noticed in TMD-GpA or TMD-GpA-I expressers. However, after loss of anchorage, TMD-GpA-I expressing cells showed increased survivin expression exceeding its levels in TMD-αvβ3 and TMD-GpA cell transfectants by several fold. In accordance with these findings, it was shown that survivin expression in cancer was mediated by the enhanced activation of the PI3K pathway (reviewed in Rafatmanesh et al., 2020). Moreover, in pancreatic cancer cells, also FAK activation followed by activation of PKB/Akt led to an enhanced survivin expression (Aoudjit & Vuori, 2012). This provoked a decrease of cellular apoptosis and a reduced response to the chemotherapeutic agent gemcitabine (Huanwen et al., 2009). Also, after etoposide treatment in 60 different human tumor cells, survivin overexpression was associated with their prolonged survival by the inhibition of caspase activity (Tamm et al., 1998). Therefore, survivin expression is associated with tumor progression, drug resistance, and poor patient prognosis in various carcinomas (reviewed in Rafatmanesh et al., 2020). Furthermore, Wang et al. (2019) described that expression of αvβ3 in prostate cancer was accompanied by resistance to ionizing radiation caused by the presence of survivin. *Vice versa*, blocking of β3 decreased survivin expression and, subsequently, enhanced the sensitivity of tumor cells to radiation (Wang et al., 2019). In addition, in suspended keratinocyte stem cells, inhibition of β1 led to downregulation of survivin and, consequently, promoted the anoikis cascade (Marconi et al., 2007).

# 5 Conclusions

EOC cells are known to detach from their primary tumor, survive under anchorage-independent conditions in patient ascites, and eventually colonize at secondary sites as metastases. During the period of suspension, epithelial-type cancer cells may acquire the ability to avoid/escape anoikis, implicating integrins, including αvβ3. The previous establishment of an EOC cell transfectant model harboring αvβ3 in different TMD conformational activation states and grown under loss of ECM detachment, allowed the investigation of the impact of αvβ3 on cell survival and anoikis avoidance.

The results of the present work demonstrated that a high-affinity αvβ3 receptor displaying full constitutive signaling competence majorly contributes to cell survival under threat of anoikis due to missing ECM attachment. This is achieved by αvβ3 pro-survival signaling, which favors the activation of important players involved in anoikis resistance, such as the EGF-R and important molecules of the integrin signaling cascade, including FAK, src, and PKB/Akt, respectively. Adding to this, the anti-apoptotic factors Bcl-2 and survivin were documented to contribute to survival of detached *per se* anchorage-dependent cells. The molecular mechanisms of anoikis avoidance or at least its delay in order to allow possible re-adhesion and subsequently outgrowth to distant tumor nodules, represent important issues in cancer research and will allow the identification of important players as valuable target structures. This will inspire the development of e.g., allosteric inhibitors for clinical translation capable of blocking integrin activation and thus its pro-survival signaling in order to reverse anoikis resistance also by downregulating anti-apoptotic factors and enabling the action of caspases.

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