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Review

Intracellular Proteins Displayed on the Surface of Tumor Cells as Targets for Therapeutic Intervention with Antibody-related Agents

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Abstract. *The identification of targets which are located intracellularly in normal cells and are exposed on the surface of malignant cells is an issue in the target selection process for the development of anticancer agents. Targets with these characteristics should increase the specificity of intervention and the corresponding therapeutic window. We discuss targets such as heat-shock protein 70 (HSP70) and heat-shock protein 90 (HSP90), glucose-regulated protein 78 (GRP78), actin, cytokeratins, vimentin, nucleolin, nucleosomes, estrogen receptor-alpha variant 36 (ER-α36) and feto-acinar pancreatic protein (FAPP). Involvement of these targets in cellular processes, tumor specificity and tractability with antibody-related agents, are discussed.*

The identification and validation of targets for therapy of cancer is the first step of the drug discovery and development process. Dependent on the target, intervention with small molecules or biologics such as antibody-related biologics, conjugates or both is possible, as in the case of transmembrane receptors with enzymatic activity. Criteria for target selection are: overexpression in tumors in comparison to normal tissues, the role of the target in the pathogenesis of cancer, and its tractability with small molecules and/or biologics. Most of the previously identified targets have a tumor-suppressive role and are down-regulated. Reconstitution of the function of these types of targets is difficult from a technical point of view. Drug development therefore focuses on targets which are up-regulated or activated by mutations. The function of the targets match the seven hallmarks of cancer such as tissue invasion and metastasis, evasion of apoptosis, sustained angiogenesis, limitless replicative potential, self-sufficiency of growth signals, insensitivity to growth inhibitors and inflammatory microenvironment (1, 2).

Target validation investigates the consequences of inhibition by small molecules, antibodies or interfering RNAs, with respect to proliferation, migration and survival of tumor and stromal cells, such as endothelial cells, macrophages and fibroblasts, and the impact on other tumor-related parameters *in vitro* and *in vivo*. In this paper, we focus on targets which are located intracellularly in non-transformed cells and are exposed to the cell surface in malignant cells. The targets discussed in this review and their cellular locations are displayed in Figure 1.

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Plasma Membrane-associated HSP70

Heat-shock protein 70 (HSP70) is a chaperone with ATPase activity and is found in cellular compartments involved in the folding of nascent proteins, prevention of protein aggregation and assisting transport across membranes (3). It exhibits high affinity for unfolded proteins when bound to ADP and low affinity for folded proteins when bound to ATP. Plasma-membrane association of expression of HSP70 on tumor cells has been demonstrated (4-7) by selective membrane protein iodination and flow cytometry. It was concluded that 15-20% of the cellular HSP70 protein is plasma-membrane associated (7).The stress-induced variant is referred to as HSP72 and does not seem to be expressed on normal cells. Two testis-specific HSP70 isoforms have been detected on the surface of human sperm (8). Autologous tumor cell lines, generated

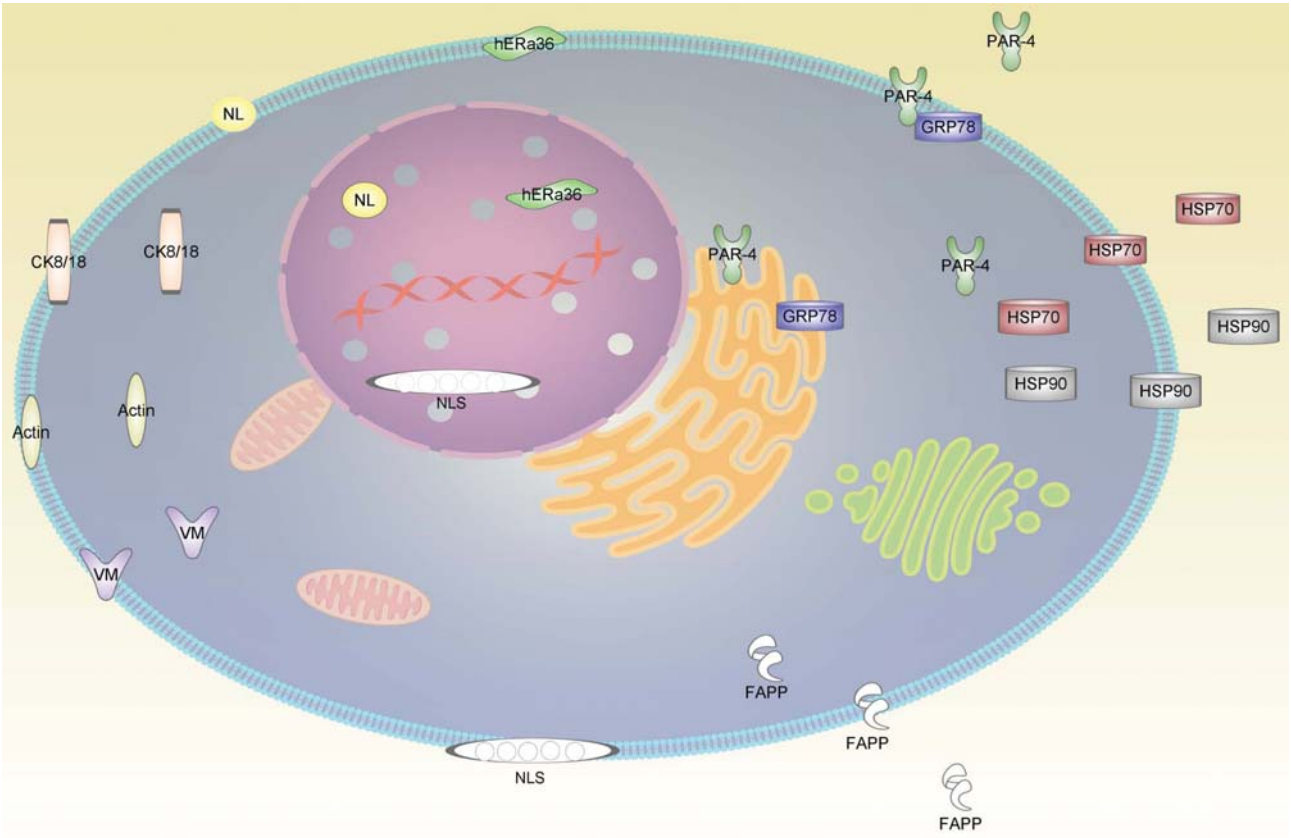

Figure 1. *Cellular localization of targets discussed in this review. Ingenuity Pathway Analysis (IPA) Path Designer tool was used to create an overview of molecules and their locations within the cell. HSP70: Heat-shock protein 70; HSP90: heat-shock protein 90; PAR-4: prostate apoptosis response 4 protein; GRP78: glucose-regulated protein 78; hERa-36: estrogen receptor alpha, 36 kDa isoform; NL: nucleolin; CK8/18: cytokeratin 8 and 18; Actin: actin; VM: vimentin; NLS: nucleosomes; FAPP: fetoacinar pancreatic protein, glycoform of bile salt-dependent lipase (BSDL).*

by antibody-based cell sorting, express membrane-associated HSP70 differentially and it has been shown that high-level expressors are more sensitive to natural killer (NK) cell-mediated killing than low expressors (9). The mechanism by which HSP70-positive tumor cells are lysed by NK cells is not clear, however, HSP70 NK cell interaction was demonstrated in binding studies and a 14-mer peptide termed TKD which is derived from the *C*-terminal substrate binding domain, exhibits immunomodulatory activity on NK cells (10). Cluster of differentiation 94 (CD94) is a candidate NK receptor for interaction with plasma membrane-associated HSP70, which has also been detected on exosomes of tumors with high HSP70 on their plasma membrane (11). It can be hypothesized that NK cells might be attracted to and activated by tumors *in vivo via* the secretion of exosomes presenting HSP70 on their lipid membrane. It was shown that HSP70 membrane-positive tumors differ from their membrane negative counterparts by containing higher amounts of the glycosphingolipid Gb3 in the plasma membrane and additionally that HSP70 predominantly binds to artificial liposomes containing Gb3 (12). A mouse anti-HSP70 monoclonal antibody was generated by immunizing mice with a 14-mer peptide derived from HSP70 (cmHSP70.1) (13). This antibody detects membrane HSP70 on viable mouse and human tumors *in vitro* and *in vivo* and it was evaluated in the murine CT26 colon carcinoma model in more detail (13). The antibody translocates into early endosomes and lysosomes. Intraoperative and near-infrared fluorescence (NIRF) imaging revealed an enrichment of Cy5.5 conjugated antibody in the tumor after intravenous injection into the tail vein. Therefore, tumor cells expressing plasma membrane HSP70 might be targeted with this antibody, as it has been shown to mediate antibody-dependent cellular cytotoxicity (ADCC), or with an antibody conjugated with toxins or radionuclides. One thousand molecules of plasma-membrane associated HSP70 were detected on CT26 cells. Dose-dependent efficacy data are not yet available. It remains to be investigated whether the receptor density is sufficient to mediate *in vivo* efficacy of this antibody in different tumor models. Humanization of this antibody has been described, yet *in vivo* data has not been reported (14). The fragment of antigen binding (Fab) of HSP70.1 (IgG1/λ) has been

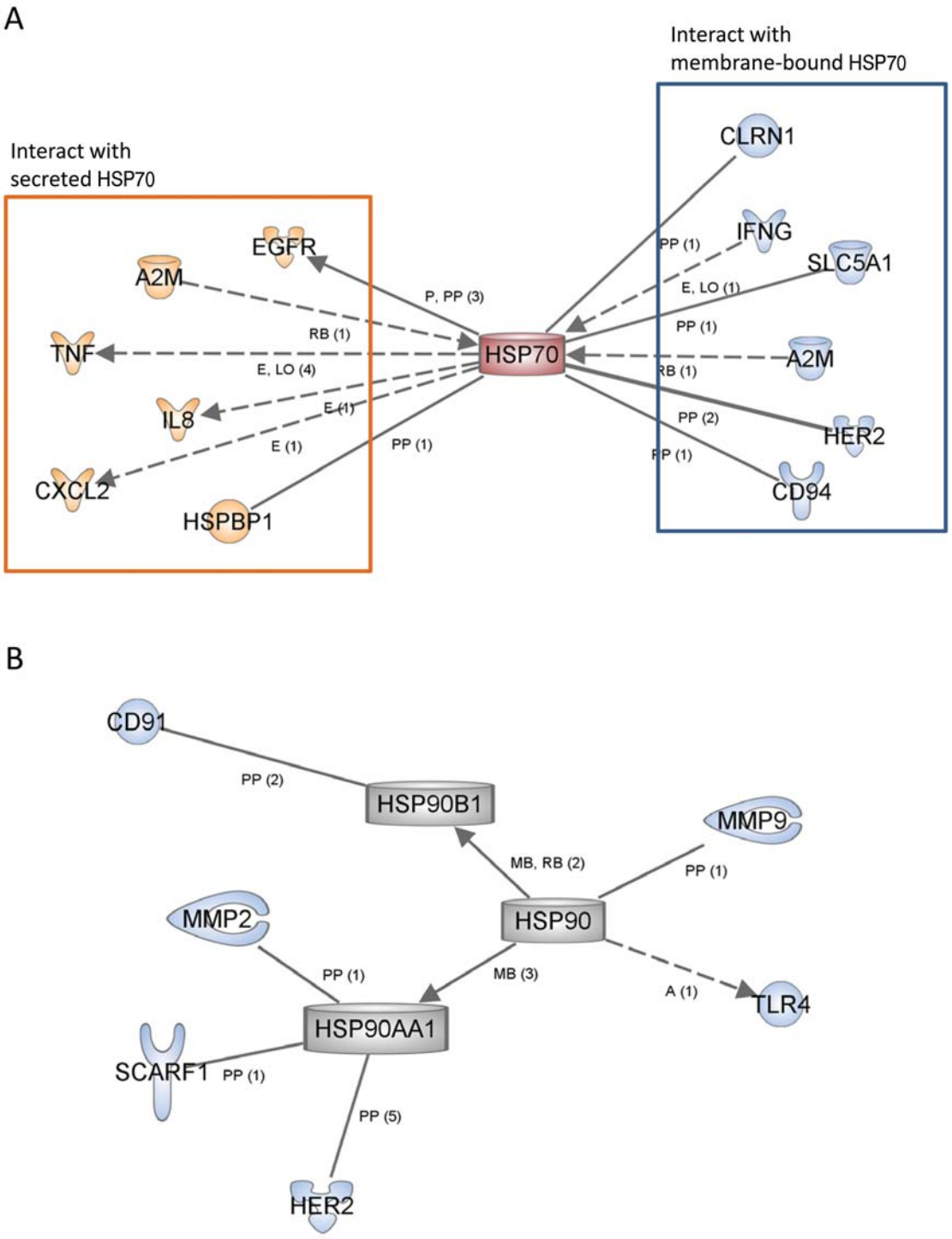

Figure 2. *(A): Display of proteins interacting with HSP70. Ingenuity Pathway Analysis (IPA) was used to find molecules which interact with secreted and membrane-associated HSP70 and (B) membrane-associated HSP90. Edges are annotated by the relationship type of two molecules: A – activation; E – expression; LO – location; MB – membership; P – phosphorylation; PP – protein-protein interaction; RB – regulation of binding. Number of journal references supporting the relationship is shown in brackets. Abbreviations are as follows: A2M: alpha-2-macroglobulin; CD94: Cluster of Differentiation 94, member 1; CLRN1: clarin 1; CXCL12: chemokine (C-X-C motif) ligand; EGFR: human epidermal growth factor receptor; HER2: human epidermal growth factor receptor 2; IFNG: interferon, gamma; IL8: Interleukin 8; SLC5A1: solute carrier family 5 (sodium/glucose cotransporter), member 1. b: Display of proteins interacting with HSP90. CD91: low density lipoprotein receptor-related protein 1; HER2: human epidermal growth factor receptor 2; HSP90: heat-shock protein 90; HSP90AA1: heat-shock protein 90α isoform; HSP90B1: heat-shock 90 kDβ member 1; TLR4: toll-like receptor 4; MMP2; matrix metalloproteinase 2; MMP9: matrix metalloproteinase 9; SCARF1: scavenger receptor class F member 1.*

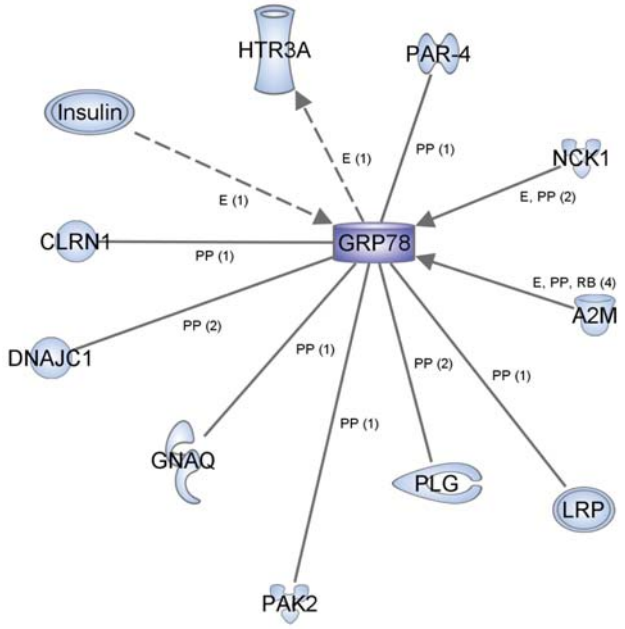

Figure 3. *Display of molecules interacting with GRP78. Ingenuity Pathway Analysis (IPA) was used to find molecules which interact with membrane-associated GRP78. Edges are annotated by the relationship type of two molecules: E – expression; PP – protein–protein interaction; RB – regulation of binding. Number of journal references supporting the relationship is shown in brackets. A2M: Alpha-2-macroglobulin; CLRN1: clarin 1; DNAJC1: DnaJ (HSP40) homolog, subfamily C, member1; GNAQ: guanine nucleotide binding protein, q polypeptide; HTR3A: 5-hydroxytryptamine (serotonine) receptor 3A; Insulin: insulin; LRP: low density lipoprotein receptor-related protein; NCK1: NCK adaptor protein 1; PAK2: p21 protein (Cdc42/Rac)-activated kinase 2; PAR-4: prostate apoptosis response 4 protein; PLG: plasminogen.*

expressed as a potential reagent for tumor diagnosis in the periplasm of *Escherichia coli* after co-expression of two periplasmic oxidoreductases and two chaperones with proline-cis/trans isomerase activity (15). The Fab fragment sensitively recognizes membrane-associated HSP70 on tumor cells with immuno-fluorescence microscopy and flow cytometry, thus highlighting the potential for tumor detection *in vitro* and *in vivo* (15). Figure 2A displays interactions of membrane-associated and secreted HSP70 with other proteins, as derived by Ingenuity Pathway Analysis (IPA). We noted interactions with receptors such as epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2), solute carrier family 5, member 1 (SLC5A1) sodium/glucose cotransporter and transmembrane protein Clarin 1 (CLRN1) which contains an endoplasmic reticulum (ER) membrane retention signal. Interaction between secreted and membrane-associated HSP70 and α2-macroglobulin (α2-M) with several cytokines and chemokines such as tumor necrosis factor (TNF), interleukin 8 (IL8), stromal-derived factor 1 (SDF1, CXCL2), interferon γ (IFNγ) and HSP70 binding protein 1 (HSPBP1) have also been noted, indicating the involvement of membrane-associated and secreted HSP70 in several signal transduction pathways.

Plasma Membrane-associated HSP90

HSP90 is a molecular chaperone that assists client proteins in proper folding. More than 200 proteins are referred to as clients of HSP90, many of which have an oncogenic function (16). Client proteins belong to different functional categories such as protein kinases, ribonucleoproteins, steroid hormone receptors, transcription factors, chromatin remodeling factors and members of the kinetochore complex. Prime examples are B-type rat fibrosarcoma kinase (BRAF), casein kinase (CK2), nuclear serine-threonine kinase (WEE-1), HER2, cyclin-dependent kinase 4 (CDK4), androgen receptor (AR), hypoxia inducible factor-1α (HIF-1α), oncogene fusion protein consisting of bcr and abl (bcr-abl), mutant p53 and protein kinase B (AKT). The first HSP90 inhibitor 17-AAG (17 allylamino-17-dimethoxygeldanomycin, tanespimycin) entered clinical trials in 1999, followed in 2004 by 17-dimethylaminoethylamino-17-dimethoxygeldanomycin (17-DMAG). Based on structural modeling, new scaffold discovery and other drug discovery efforts, 13 HSP90 inhibitors are currently under clinical investigation (16).

However, HSP90 is not located exclusively intracellularly, it can also be expressed on the cell surface and can be secreted into the extracellular space. It was shown that secretion of HSP90 is determined by the phosphorylation status of Thr-90, that the plasma level of HSP90α is positively correlated with tumor malignancy in cancer patients and that secretion of HSP90α is a prerequisite of its proinvasive function (17). Furthermore, it was shown that HSP90α localizes to the leading edge of endothelial cells and promotes their angiogenic activities, whereas a monoclonal antibody directed against HSP90α can reverse the effect (18). In 1986, HSP90 was identified as a cell-surface tumor-specific transplantation antigen (19). The immunization of mice with HSP90 inhibited tumor growth, suggesting involvement of HSP90 in the host antitumor response. Surface HSP90 was dramatically up-regulated in malignant melanoma when compared to benign melanocytic lesions and it was expressed on the surface of 7 out of 10 melanoma metastases (20). Cell-surface expression of HSP90 also has been described in small-cell lung cancer cells (21), fibrosarcoma (22), neuroblastoma (23) and lymphoma cells (24). Bortezomib was shown to induce surface expression of HSP90 on dying melanoma cells, following stimulation of the activity of dendritic cells and the enhancement of antitumor immunity (25). Cell surface expression of HSP90 has also been found on different cells of the nervous system, such as oligodendrocyte precursor cells, microglial, cerebellar and Schwann cells (26).

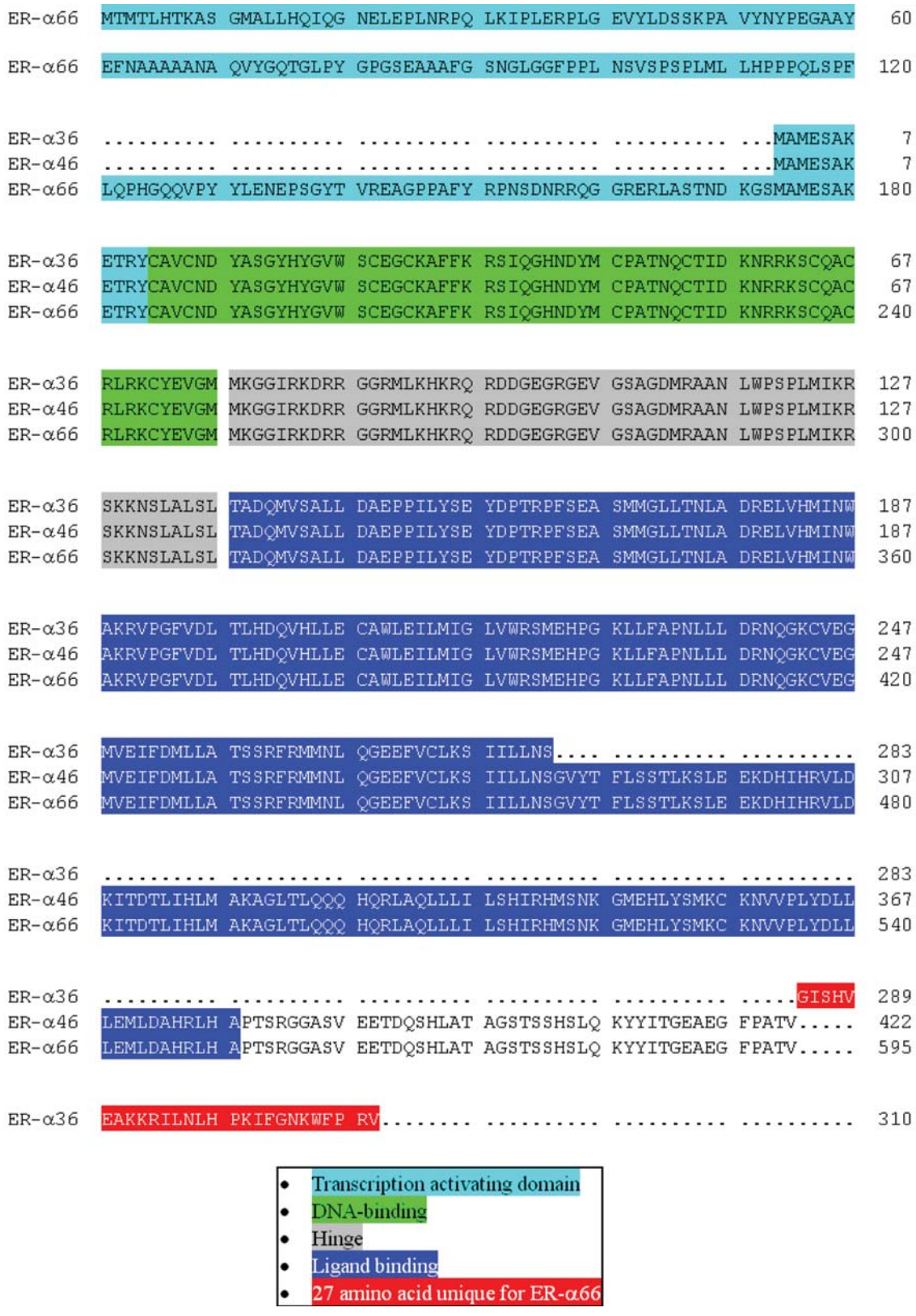

Figure 4. *Amino acid sequences of BSDL and FAPP. BSDL sequence was derived from Swissprot CEL_HUMAN (phasing of tandem repeats was modified), FAPP sequence was derived from TrEMBL. Discrepancy at residue #599 (Gly to Asp) was noted, no signal sequence is displayed.*

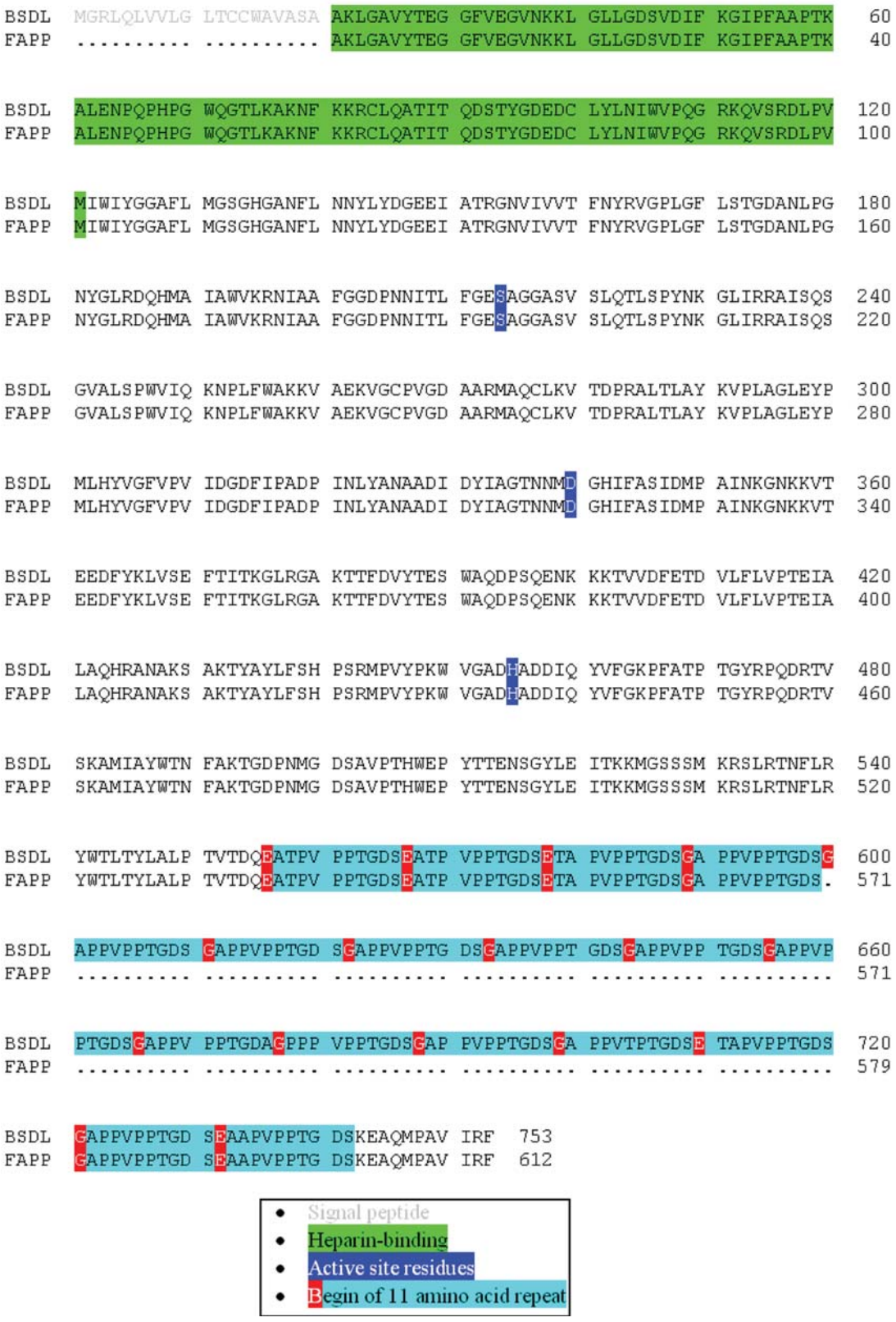

Figure 5. *Amino acid sequences of ER isoforms ER-α66, ER-α46 and ER-α36. Sequences were derived from Swissprot ESR1_HUMAN (ER-α66 and ER-α46) and TrEMBL Q6MZQ9_HUMAN for ER-α36. A discrepancy at residue #279 (Leu to Ile) was noted.*

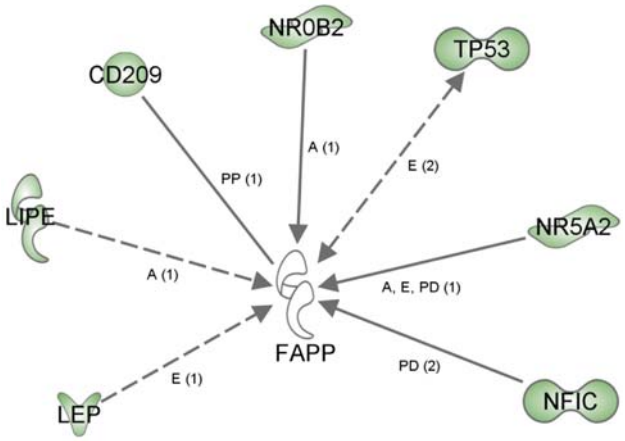

Figure 6. *Display of proteins interacting with FAPP. Ingenuity Pathway Analysis (IPA) was used to find molecules which interact with FAPP. Edges are annotated by the relationship type of two molecules: A – activation; E – expression; PD – protein–DNA interaction; PP – protein–protein interaction. Number of journal references supporting the relationship is shown in brackets. CD209: Cluster of differentiation 209; LEP: leptin; LIPE: lipase, hormone-sensitive; NFIC: nuclear factor I/C; NR0B2: nuclear receptor subfamily 0, group B, member 2; NR5A2: nuclear receptor subfamily 5, group A, member 2; TP53: tumor protein p53.*

The molecular mechanisms underlying secretion and the association of HSP90 with the cell surface are not fully understood. It is not clear how HSP90 gets to the cell surface and the extracellular space. A signal sequence for targeting HSP90 to the secretory pathway or the cell membrane has not been identified. However, it can not be excluded that an alternative splice variant of the cytoplasmic molecule is responsible for these phenomena (27). Involvement of an unconventional pathway is supported by the identification of HSP90 in exosomes (28). As shown with neutralizing antibodies, extracellular HSP90 is crucial for cell mobility in neuronal cells (29) and mobility of human dermal fibroblasts (30). Several groups have described involvement of cell surface HSP90 in migration, invasion and metastasis of tumor cells. Interaction of surface HSP90 with surface receptor CD91 was shown to mediate cell migration (31). This activity is independent of the ATPase function of HSP90. The pool of extracellular HSP90 was shown to be involved in cancer cell invasion. A monoclonal antibody recognizing both the α and the β isoforms of HSP90 (mab 4C5) mediates inhibition of melanoma invasion and metastasis due to interaction with the extracellular HSP90 pool (29). One of the underlying principles is the inhibition of actin reorganization which is known to be an important component of cell migration. In addition to CD91, toll-like receptor 4 (TLR4) has been identified as a receptor for cell surface HSP90 (32). Both seem to be crucial for the activity of NK and antigen presenting cells (APCs) such as macrophages and dendritic cells. Experimental evidence indicates that the extracellular domain of HER2 specifically interacts with cell surface HSP90 and that this interaction is necessary for HER2 activation and heterodimerisation with human epidermal growth factor receptor 3 (HER3), resulting in mitogen-activated protein kinase (MAPK) and phosphoinosite 3 kinase-AKT (PI3K-AKT) signaling leading to actin re-arrangement and cell mobility. It was shown that these functions can be inhibited with cell impermeable mab 4C5 (33, 34) directed against HSP90. This antibody inhibits heregulin (HRG)-mediated signaling, does not reduce HER2 expression in the cell membrane and leads to a significant reduction of phosphorylated forms of HER2, indicating that the antibody does not affect the stability of HER2 but that it interferes with its activation. It was shown that mab 4C5 had no effect on endosomal trafficking of the receptor. In addition, it was demonstrated that mab 4C5 prevents activation of MMP2 and MMP9 by disrupting their interaction with extracellular HSP90, resulting in inhibition of formation of metastatic breast cancer cell deposits (35, 36). Present gaps in the current knowledge base are: the quantitation of cell surface-associated HSP90, the mechanism of plasma membrane association, *in vivo* efficacy experiments (tumor growth inhibition) in models of established xenografts, the impact of surface HSP90 on cell proliferation and systematic immunohistochemistry studies for surface associated HSP90 in normal and tumor tissues. As shown in Figure 2b, in addition to the interaction of plasma-membrane associated HSP90 with HER2, TLR-4, MMP2 and MMP9, IPA revealed an interaction of HSP90 isoforms with scavenger receptor class F member 1 (SCARF1), an endocytic receptor for calreticulin expressed on endothelial cells.

Plasma Membrane-associated GRP78

Glucose-regulated protein 78 (GRP78), also referred to as BiP is an ER protein is involved in protein folding and assembly, targeting of misfolded proteins for degradation and in the regulation of transmembrane ER stress sensors (37). GRP78 is composed of two functional domains: a 44 kDa *N*-terminal ATPase domain and a 20 kDa *C*-terminal polypeptide-binding domain. GRP78 is a master regulator of ER function and the unfolded protein response (UPR), a cytoprotective signal transduction pathway (38).

In a variety of tumor types GRP78 is up-regulated and correlates with malignancy and in addition GRP78 has been identified as a mediator of drug resistance (37, 38). A number of studies have demonstrated that GRP78 is localized in the ER, but also on the cell surface, especially in tumor cells (37, 38). GRP78 can function as a transmembrane protein based on hydrophobic domains which can form transmembrane helices (37, 38). Fingerprinting of

the circulating repertoire of antibodies from cancer patients revealed GRP78 as a surface-expressed target (39). Synthetic, chimeric peptides composed of a GRP78-binding motif fused to an apoptosis-inducing sequence suppress tumor growth in prostate and breast cancer xenografts (39). It was shown that the peptides undergo internalization. High levels of plasma membrane-associated GRP78 were found on a variety of carcinomas such as gastric, breast, lung, hepatocellular and prostate cancer (40) and GRP78 expression correlates with prognosis in breast, prostate and gastric cancer (40). Antibodies directed against the COOH-terminal domain of GRP78 were shown to promote apoptosis in DU-145 prostate cancer and A375 melanoma cells (41). In addition, antibodies inhibit thymidine uptake and cellular proliferation and act as receptor antagonists by blocking autophosphorylation and activation of GRP78 (41). The proteinase inhibitor α2-macroglobulin (α2M) can bind to cell-surface GRP78 of 1-LN human prostate cancer cells and induce mitogenic signaling and cellular proliferation and enhances the metastatic potential of these cells (42). Making use of IPA, we identified further proteins which interact with membrane-associated GRP78: α2 macroglobulin receptor (a low density lipoprotein receptor) which is involved in receptor-mediated endocytosis and plasminogen. Coupling of membrane-associated GRP78 signaling is supported through its interaction with CLRN, a multiple helical transmembrane protein of the ER, GNAQ, a guanine-nucleotide binding protein which couples cell-surface 7-transmembrane receptors to intracellular signaling events, NCK1, a tyrosine kinase adapter protein, serine/threonine protein kinase 2 (PAK2), an effector that links Rho GTPases to cytoskeleton reorganization and nuclear signaling as well as 5-hydroxytryptamine receptor (HTR3A), the receptor of serotonin. These interactions are highlighted in Figure 3. However it should be kept in mind that GRP78 also has been found on the surface of normal cells such as fibroblasts, macrophages and proliferating endothelial cells (43,44) pointing to possible side-effects of GRP78-related approaches of cancer therapy. The interaction between cell-surface GRP78 and prostate apoptosis response-4 (PAR-4) has been identified as a mediator of apoptosis selectively in tumor cells (45-48). PAR-4 is a tumor suppressor and a pro-apoptotic protein which is down-regulated in several types of cancer (47). The human protein contains 342 amino acids, two putative nuclear localization sequences (NLS) in the *N*-terminal region, a leucine zipper domain (LZ) and a nuclear export sequence (NES) in the LZ domain (47). In addition, PAR-4 possesses conserved sites for phosphorylation by kinases, such as protein kinase A (PKA) and AKT. Overexpression of PAR-4 is sufficient to induce apoptosis in many types of tumor cells in the absence of a second apoptotic signal, but does not cause apoptosis in normal and immortalized cells (47). Recent findings indicate that PAR-4 can be secreted by tumor cells and that extracellular PAR-4 induces cancer cell specific apoptosis by interaction with cell-surface receptor GRP78 (48). Analysis of several deletion mutants from the *N*- and *C*-terminus of human PAR-4 resulted in the identification of a domain spanning amino acids 137-195 which induces apoptosis selectively in tumor cells, referred to as selective for apoptosis in cancer cells (SAC domain). This segment contains the NSL2 domain which facilitates its nuclear translocation and the T155 PKA phosphorylation site which is responsible for its activation. In normal and immortalized cells, the SAC domain fails to be adequately phosphorylated at the T155 residue because of low level of PKA activity in these cells (47). Green fluorescent protein (GFP)-tagged SAC transgenic mice show pronounced resistance to formation of spontaneous tumors, consistent with its apoptotic function (49). PAR-4 induces apoptosis by translocation of Fas and Fas ligand (Fas/FasL) to the plasma membrane, which recruits the Fas-dependent death domain (FADD) adapter protein and induces the formation of the (DISC), thereby initiating the caspase cascade (47, 48). Antibodies mimicking PAR-4 function, recombinant PAR-4, SAC domain or related molecules with improved pharmacokinetic properties are candidates for evaluation as possible anticancer agents.

Since GRP78 can also be surface-associated in normal cells as outlined above, the identification of a tumor specific glycotope version of GRP78 is an important achievement (40, 50, 51) regarding the identification of tumor-specific targets. A fully human monoclonal IgM antibody (SAM-6) was isolated from a gastric cancer patient and shown to bind to a new variant of cell-surface GRP78 *via* an *O*-linked carbohydrate moiety which seems to be specific for malignant cells (50, 51). The epitope recognized by SAM-6 is specifically expressed in malignant tissues and absent in non-transformed tissues (40). Binding of SAM-6 to cancer cells induces cell death by lipoptosis caused by accumulation of intracellular lipids, cholesteryl ester and triglycerides due to lipotoxicity (50, 51). It is not yet reported whether these properties can be recapitulated with IgG-based monoclonal antibodies.

Plasma Membrane-associated Vimentin

Vimentin belongs to the intermediate family of proteins which form the cytoskeleton together with microtubules and microfilaments, is a marker for mesenchyme-derived tissues and is useful as an immunohistochemical marker for sarcomas (52). Vimentin is involved in maintainance of the cell shape, integrity of the cytoplasm and stabilization of cytoskeletal interactions and was shown to be associated with the nucleus, the mitochondria and the ER (52). Vimentin expression correlates with epithelial mesenchymal transition

(EMT) during tumor progression (53). In soft tissue-sarcoma an interaction between AKT-1 and vimentin resulting in its phosphorylation at Ser 39 has been shown to mediate motility and invasiveness (54).

Cell surface-associated vimentin was found in endothelial cells (55) and its recruitment to the cell surface is mediated by β3 integrin and plectin, resulting in adhesion strength through focal adhesions. Recruitment of vimentin to the cell surface is regulated by tyrosine residues in the β3 cytoplasmic domain. In Chinese hamster ovary (CHO) cells which lack β3 integrin, vimentin seems to be collapsed around the nucleus (55).

A monoclonal antibody directed against cell-surface vimentin has been described (56). Pritumumab (also known in the literature as CLNH11, CLN-IgG and ACA-11) is a natural human IgG1, kappa antibody that was derived from B lymphocytes obtained from a regional draining lymph node from a patient with cervical carcinoma (57). It interacts with a 226 kDa plasma protein in A549 cells consisting of α (60 kDa) and β (53 kDa) subunits which are disulfide-linked and correspond to vimentin. In a panel of 20 normal tissues, no cross-reactivity of Pritumumab was observed, however, endothelial cells were not included in this panel (56). The following tumors scored positive for cell-surface vimentin expression: brain (21/32), thyroid (2/2), esophagus (1/1), lung (3/5), stomach (3/5), renal (1/1), pancreas (2/2), gall bladder (2/2), breast (5/15), ovary (6/8), cervical (7/10) and uterus (2/3). Pritumumab scored positive for ADCC and mediated significant tumor growth inhibition in a glioblastoma xenograft model after multiple injections (56). A daunomycin-pritumumab conjugate was shown to inhibit cell proliferation of A549 and HeLa 229 cells; no inhibition of control cells (Flow 200 fibroblasts) was observed.

In various clinical trials in Japan, Pritumumab was evaluated in 249 brain cancer patients treated with a low dose regimen of 1 mg twice a week for 24 weeks (56, 57). Overall response rate was 25-30%, with several patients surviving more than 5 years post treatment. The half-life of the antibody was 73 h. The treatment group preferentially included patients with astrocytoma, anaplastic astrocytoma and glioblastoma. Correlation between cell-surface expression of vimentin and response to treatment was not investigated. In preclinical experiments, the question whether pritumumab has an impact on proliferation of cell-surface positive tumor cells in the absence of immune effector cells was not addressed. Data covering quantitation of surface vimentin are also not available. Seven patients were treated with 131-I labeled pritumumab and partial remissions were observed in two patients (56). Despite promising clinical data the antibody has to be investigated in standardized clinical studies in order to evaluate its potential as an anti-tumoral agent. Also the range of vimentin-positive tumor cells within defined tumor sub-types should be investigated in more detail.

Plasma Membrane-associated Cytokeratins

Cytokeratins are proteins of intermediate filaments of the intracytoplasmic cytoskeleton of epithelial tissues. The localization and distribution of cytokeratin-8 (CK8) on carcinoma and normal cells was investigated by immunofluorescence and confocal laser scanning and revealed its homogeneous expression on the surface of head and neck, cervix, breast and colon carcinoma cells (58). Healthy tissues do not express surface-associated CK8, with the exception of liver and heart which displayed sporadic and weak CK8 membrane association. During mitosis, membrane-associated CK8 redistributes to speckles and is phosphorylated at serine 73 (58). Membrane-associated CK8 can interact with plasminogen and tissue-type plasminogen activator (tPA) thus establishing a protease system on the cell surface (59, 60). COU-1, a monoclonal IgM antibody was obtained by fusion of a human B lymphoblastoid cell line with lymphocytes from a colon cancer patient (61, 62) and intact COU-1 as well as its recombinant Fab fragment bind to modified forms of CK8 and cytokeratin 18 (CK18) (62). Immunohistochemical analysis shows that the antibody reacts with malignant tissues of epithelial origin such as carcinomas of the colon, ovary, pancreas and breast (62). COU-1, in contrast to murine antibodies directed against CK8 and CK18 can differentiate between malignant and normal colon epithelia and between colon cancer metastasis in the liver and surrounding normal hepatocytes (62). The modified cytokeratins on the cell surface undergo endocytosis upon binding of COU-1 or its recombinant Fab fragment, highlighting modified cytokeratin at the cell surface of carcinoma cells as a possible target for therapy with antibodies or immunoconjugates (62). However, other investigators reported expression of CK18 on the surface of hepatocytes and its interaction with thrombin-antithrombin complexes (63).

Expression of cytokeratin 1 (CK1) on the cell surface was reported (64) and identified as an endothelial cell receptor for high molecular weight kininogen (65). Also expression of actin has been noticed on the cell surface. Actin is a component of microfilaments and thin filaments which are part of the contractile apparatus in muscle cells. Cell surface actin binds to factor Va, angiogenin, plasminogen, tissue-type plasminogen activator and lipoprotein a (66-68). Binding of cell-surface α-actinin to thrombospondin (69) and cell-surface annexin II to plasminogen (70) was noted. It remains to be investigated whether CK1, actin, α-actinin and annexin II can be found on the surface of tumor cells.

Plasma Membrane-associated Nucleolin

Nucleolin is a nucleolar protein involved in regulation of proliferation, cytokinesis, replication, embryogenesis and nucleogenesis (71, 72). Nucleolin is expressed on the surface

of tumor-related blood vessels in contrast to mature vessels or capillaries (73). Interaction of nucleolin with endostatin was demonstrated (74). Endostatin is a naturally occurring 20 kDa *C*-terminal fragment which is derived from type XVIII collagen with anti-angiogenic properties similar to angiostatin and thrombospondin (75). Blockage of nucleolin by RNA interference results in loss of the anti-angiogenic properties of endostatin (74). Nucleolin and endostatin are co-localized on the surface of angiogenic blood vessels (74). The following pathway has emerged: nucleolin mediates internalization of endostatin and transports it into the cytosol and the nucleus and endostatin inhibits vascular endothelial growth factor (VEGF) and basic-fibroblast growth factor (bFGF) stimulated phosphorylation of nucleolin thus inhibiting proliferation and survival of endothelial cells (74). Endostar, a recombinant endostatin expressed in *E. coli* with an additional nine amino acid sequence and forming another his-tag structure suppresses VEGF-mediated proliferation, migration and tube formation of human umbilical vein endothelial cells (HUVECs) (75). The anti-angiogenic effect of endostar was exerted by inhibition of VEGF-induced phosphorylation of KDR/Flk1 of endothelial cells (76). Unfortunately, comparative experiments with endostatin are not available. Non-muscle myosin heavy chain 9 (MyH9), an actin-based motor protein was identified as a physical linker between nucleolin and the cytoskeleton thus modulating translocation of endostatin (77). Knocking-down of MyH9, inhibition of myosin activity or overexpression of functionally deficient MyH9 disrupts the organization of cell-surface nucleolin and inhibits its anti-angiogenic function (77). Voltage-dependent anion channel 1 (VDAC1) is involved in endothelin-induced endothelial cell apoptosis (78). Knocking down VDAC1 by RNA interference attenuates endostatin-induced apoptosis while overexpression of VDAC1 enhances sensitivity of endothelial cells to endostatin. A 27 amino acid synthetic peptide corresponding to the NH_2-terminal zinc-binding domain of endostatin is responsible for its antitumor activity (79). This peptide contains three histidines that are mediators of Zn-binding. Mutation of the Zn-binding histidines abolished its antitumor and antimigration function, but not its antipermeability properties (79). Endostatin, however does not inhibit proliferation of surface-nucleolin positive tumor cells, probably they do not internalize nucleolin in the presence of endostatin (74). The anti-angiogenic activity of endostatin is heparin-dependent which is now explained by the finding of the interaction of the heparin-binding motif of endstatin with cell-surface associated nucleolin (74). The arginine clusters in the heparin-binding motif of endostatin significantly contribute to the interaction with nucleolin and mediate its anti-angiogenic and antitumor efficacy (79).

The molecular details of the presentation of nucleolin on the cell surface are not yet resolved. It also is not yet clear whether nucleolin acts as a transmembrane protein. The presented findings explain why endostatin specifically targets angiogenic blood vessels with virtually no toxicity in animal studies and clinical trials. Endostar has received approval for anticancer therapy in China.

It might be of interest whether an agonistic monoclonal antibody to cell-surface associated nucleolin with improved pharmacokinetic and pharmacodynamic properties compared to endostatin can be identified. Since expression of cell-surface associated nucleolin seems to be restricted to proliferating endothelial cells and tumor cells also antibody-cytotoxic conjugate approaches might be considered. The number of cell-surface associated nucleolin molecules per cell might be a critical issue for these approaches.

hERα-36 as a Target

Estrogen signaling in humans is mediated by receptors designated as hERα-66, hERα-46 and hER-β (80, 81). The hERα-66 and hER-β share a common structural architecture. The hERα-66 *N*-terminal domain has a ligand-independent activation function (AF1) involved in interactions with co-activators and transcriptional activation of target genes, the DNA binding domain contains two zinc-finger like structures involved in receptor dimerisation and binding to ERE's (estrogen responsive elements) on DNA and the *C*-terminal domain is the ligand-binding domain that mediates receptor dimerisation, nuclear localization and excerts a ligand-dependent transactivation function (80, 81). ERα66 largely mediates 17β-estradiol mediated cell proliferation. However, complimentary studies have suggested that 17β-estradiol binds to a cell surface receptor and stimulates generation of cAMP (82). Recently a new isoform of the estrogen receptor, designated as hERα-36 (83) has been described. hERα-36 is generated from a promoter in the first intron of hERα66, lacks both transcriptional transactivation domains, retains the DNA binding and partial dimerisation and ligand-binding domains and possesses an extra 27 amino acid domain at the *C*-terminus. The protein sequences of ERα-66, ERα-46 and ERα-36 are displayed in Figure 5. It was shown that hERα-36 is a membrane based estrogen receptor (84). The mechanism by which hERα-36 is localized to the plasma membrane is unclear. It has no *N*-terminal signal sequence and contains two nuclear localization signals and three additional *N*-myristoylation sites. The surface localization of hERα-36 might be associated with the generation of tumor-specific surface epitopes for therapeutic intervention with small molecules and monoclonal antibodies or antibody-based conjugates. hERα-36 possesses a broader ligand binding spectrum than hERα-66. It mediates binding of estrogens such as E2α, E2β, E3 and E4 as well as tamoxifen classifying it as a potentially more potent mediator of estrogen signaling (84). Stimulation of growth by estrogens and anti-estrogens mediated by hERα-36 occurs through activation of

the MAPK/ERK signaling pathway (84). This finding is not anticipated. hERα-36 inhibits transactivation of both estrogen-stimulated and non-stimulated hERα-66 and hER-β (84) as a dominant-negative effector. The estrogen and anti-estrogen signaling pathways mediated by hERα-36 provide an alternative explanation why some breast cancers are worsened by anti-estrogen therapy. hERα-66 seems to repress hERα-36 (85). hERα-36 expression was investigated in specimen of 896 patients with breast cancer (86). The results indicate that patients with hERα-66 positive breast cancers are more likely to benefit from tamoxifen treatment than patients who do not. In patients with hERα-66-positive breast tumors who received tamoxifen treatment, hERα36 expression was associated with poorer survival. These finding raise the possibility that hERα-36 is a therapeutic target because tamoxifen cannot block hERα-36-mediated membrane initiated estrogen signaling pathways. Immunohistochemistry-based analysis has revealed that hERα-36 is expressed in ER-positive and ER-negative human breast carcinomas (87). Breast cancer tissues only expressing hERα-36 may represent the subset of patients that are diagnosed as ER-negative, but still could respond to estrogen signaling and anti-estrogen signaling (87). hERα-36 also mediates testosterone-stimulated ERK and AKT activation in endometrial cells (88). It is possible that the estrogen produced locally from testosterone in endometrial cells binds to hERα-36 and subsequently activates MAPK/ERK and PI3K/AKT pathways. Letrozole, an aromatase inhibitor abrogated testosterone-induced ERK and AKT phosphorylation, suggesting that aromatase might be involved in testosterone-mediated carcinogenesis.

Cell surface-associated FetoAcinar Pancreatic Protein (FAPP)

FAPP was identified as another surface-associated protein in pancreatic adenocarcinoma as a possible target for antibody-related treatment (89). It is an oncofetal glycoform of the pancreatic enzyme bile-salt dependent lipase (BSDL) which is a 100 kDa glycoprotein secreted by the pancreas into the duodenum and is involved in cholesterol and lipid-soluble vitamin ester hydrolysis and adsorption (90, 91). Its enzymatic activity is non-specific with regard to the substrate, as it can hydrolyse cholesterylesters, tri-, di- and mono-acylglycerols and esters of fat-soluble vitamins (92). FAPP is expressed in human embryonic and fetal pancreas (93). Earliest expression of this protein was noticed in undifferentiated mesenchymal cells and in nascent acini at the beginning of the morphological differentiation of the pancreas (93). Molecular cloning of FAPP has indicated that it is a variant of BSDL (94). The *N*-terminal domain encoded by exons 1-10 was identical to that of BSDL. In BSDL, exon 11 encodes for 17 tandem repeated identical mucin-like sequences. In FAPP, 330 base pairs are deleted in exon 11 resulting in expression of only six of these repeated sequences. FAPP is different from BSDL in many aspects: FAPP is much less active than BSDL, the *N*-linked glycosylation is of a high-mannose-type, whereas that of BSDL is complex, the amount of *O*-linked glycosylation is largely decreased in FAPP and carbohydrate accounts for 47% of the FAPP mass instead of 20% for BSDL. FAPP (characterized by its reactivity with mAbJ28) can not be detected in conditional medium of pancreatic tumor cells (95). The antibody is directed against the J28 glycotope, a carbohydrate-dependent antigenic structure located within the *O*-glycosylated *C*-terminal domain of FAPP. Immunohistochemistry experiments have indicated that the J28 glycotope is located at the cell surface. The formation of the J28 glycotope requires the expression of two glycosyltransferases, the expression of which is activated during the pancreatic neoplastic process: 2-β-1,6-*N*-acetylglucosaminyl-transferase and the α-1,3/4 fucosyltransferase (96). Consequently the J28 glycotope represents a potential target for the diagnosis and therapy of pancreatic adenocarcinoma. Further investigations indicated that FAPP also distributes within the ER and the Golgi apparatus (97), is subsequently degraded, and the COOH-terminal domain of FAPP that carries the oncofetal epitopes is presented to the surface of tumor cells such as SOJ-6 cells (97). The amino acid sequences of BSDL and FAPP are displayed in Figure 4. A 32 kDa protein resulting from FAPP degradation is specifically recognized by mAb J28 and mAb16D10, two mAbs directed against the *O*-glycosylated COOH-terminal domain of FAPP. The epitope recognized by mAb16D10 is referred to as 16D10 and is identical to the glycotope recognized by mAb J28. Twenty-two pancreatic tumor tissues scored positive with mAb16D10 independent of the tissue pretreatment such as frozen sections or formalin-fixed, paraffin embedded tissues (97). mAb16D10 did not cross-react with normal tissues or with tissues derived from other types of cancer. Cell surface expression of FAPP was investigated in more detail in SOJ-6 pancreatic tumor cells (97). SOJ-6 cells express a 32 kDa protein at their cell surface corresponding to the *C*-terminal domain of FAPP. Making use of the endoproteinase Lys-C and geldanomycin it was found that the 32 kDa surface-associated protein is derived from degradation of FAPP. In a preventative SOJ-6 xenograft model, mAb16D10 inhibits the growth of the SOJ-6 xenograft (97). *In vitro* mAb16D10 has no impact on proliferation of SOJ-6 tumor cells (9). It is not yet resolved how the 32 kDa FAPP derived protein is presented on the tumor cell surface. The unique specificity of mAb16D10 suggests further preclinical validation of mAb16D10 or other monoclonal antibodies directed against the *C*-terminal FAPP protein for treatment of pancreatic tumors. Interactions between BSDL and/or FAPP with other proteins as derived by IPA are displayed in Figure 6. Unfortunately IPA does not differentiate between BSDL and FAPP. We note interactions with three nuclear factors (NROB2, NR5A2 and NFIC), C-type lectin receptor present

on macrophages and dendritic cells (CD209), adipose derived hormone leptin (LEP) and hormone-sensitive lipase (LIPE), indicating possible involvement of BSDL (FAPP) in signaling processes.

Conclusion

As outlined, cell-surface associated tumor targets can be created by different mechanisms. In the case of ERα36 and FAPP, differential splicing is involved. FAPP in addition reveals new glycotopes. Surface-associated tumor-related glycotopes also have been identified in GRP78 and CK8/CK18 exposed in tumor cells (62). It remains to be investigated whether post-translational modifications can be found in other tumor cell-associated surface antigens such as actin, HSP70, HSP90, nucleolin and nucleosomes. With the exception of the splice variants it is accepted that the membrane composition which differs in tumor cells compared to their corresponding non-transformed versions is an important contributor to differential display of antigens. Peptides binding to these targets can be identified with phage-displayed combinatorial libraries as well as single-chain antibodies or Fab fragments of antibodies with phage-based antibodies. In order to identify agents which target surface-associated post-transcriptional modifications such as tumor-specific glycosylation, hybridoma technology for identification of appropriate antibodies seems to be the method of choice. Another mechanism might be based on differential secretion in tumor *versus* non-transformed cells as noticed for PAR-4. The issue of membrane association versus transmembrane localization has to be investigated in more detail. Only for surface-associated GRP78, the transmembrane properties were experimentally substantiated (41). Expression of the identified surface antigens should be analysed in a broader panel of tumors and normal tissues and the copy number per cell of the surface-associated targets should be correlated with their expression in tumor samples. Internalization of the antigen by antibodies should also be investigated in more detail in order to define the optimal agent for therapeutic intervention such as monoclonal antibody *versus* antibody-fusion proteins or antibody–small molecule conjugates. Presently, clinical proof-of-concept studies for agents as discussed in the previous sections are not yet available.

References

1 Hanahan D and Weinberg RA: The hallmarks of cancer. Cell *100*: 57-70, 2000.

2 Colotta F, Allavena P, Sica A, Garlanda C and Mantovani A: Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. Carcinogenesis *30*: 1073-1081, 2009

3 Horváth I, Multhoff G, Sonnleitner A and Vígh L: Membrane-associated stress proteins: more than simply chaperones. Biochim Biophys Acta *1778*: 1653-1664, 2008.

4 Ferrarini M, Heltai S, Zocchi MR and Rugarli C: Unusual expression and localization of heat-shock proteins in human tumor cells. Int J Cancer *51*: 613-619, 1992.

5 Piselli P, Vendetti S, Poccia F, Cicconi R, Mattei M, Bolognesi A, Stirpe F and Colizzi V: *In vitro* and *in vivo* efficacy of heat shock protein specific immunotoxins on human tumor cells. J Biol Regul Homeost Agents *9*: 55-62, 1995.

6 Tamura Y, Tsuboi N, Sato N and Kikuchi K: 70 kDa Heat Shock cognate protein is a transformation-associated antigen and a possible target for the host's anti-tumor immunity. J Immunol *151*: 5516-5524, 1993.

7 Multhoff G, Botzler C, Wiesnet M, Müller E, Meier T, Wilmanns W and Issels RD: A stress-inducible 72-kDa heat-shock protein (HSP72) is expressed on the surface of human tumor cells, but not on normal cells. Int J Cancer *61*: 272-279, 1995.

8 Naaby-Hansen S and Herr JC: Heat-shock proteins on the human sperm surface. J Reprod Immunol *84*: 32-40, 2010.

9 Multhoff G: Activation of natural killer cells by heat-shock protein 70. 2002. Int J Hyperthermia 25: 169-175, 2009.

10 Multhoff G, Pfister K, Gehrmann M, Hantschel M, Gross C, Hafner M and Hiddemann W: A 14-mer HSP70 peptide stimulates natural killer (NK) cell activity. Cell Stress Chaperones *6*: 337-344, 2001.

11 Gastpar R, Gehrmann M, Bausero MA, Asea A, Gross C, Schroeder JA and Multhoff G: Heat-shock protein 70 surface-positive tumor exosomes stimulate migratory and cytolytic activity of natural killer cells. Cancer Res *65*: 5238-5247, 2005.

12 Gehrmann M, Liebisch G, Schmitz G, Anderson R, Steinem C, De Maio A, Pockley G and Multhoff G: Tumor-specific HSP70 plasma membrane localization is enabled by the glycosphingolipid Gb3. PLoS One *3*: e1925, 2008.

13 Stangl S, Gehrmann M, Dressel R, Alves F, Dullin C, Themelis G, Ntziachristos V, Staeblein E, Walch A, Winkelmann I and Multhoff G: *In vivo* imaging of CT26 mouse tumors by using cmHSP70.1 monoclonal antibody. J Cell Mol Med *14*: 992-1002, 2010.

14 Zettlitz KA, Seitter J, Müller D and Kontermann RE: Humanization of a mouse monoclonal antibody directed against a cell surface-exposed epitope of membrane-associated heat shock protein 70 (HSP70). Mol Biotechnol *46*: 265-278. 2010

15 Friedrich L, Stangl S, Hahne H, Küster B, Köhler P, Multhoff G and Skerra A: Bacterial production and functional characterization of the Fab fragment of the murine IgG1/lambda monoclonal antibody cmHSP70.1, a reagent for tumour diagnostics. Protein Eng Des Sel *23*: 161-168, 2010.

16 Trepel J, Mollapour M, Giaccone G and Neckers L: Targeting the dynamic HSP90 complex in cancer. Nat Rev Cancer 10, 537-549, 2010.

17 Wang X, Song X, Zhuo W, Fu Y, Shi H, Liang Y, Tong M, Chang G and Luo Y: The regulatory mechanism of HSP90alpha secretion and its function in tumor malignancy. Proc Natl Acad Sci USA *106*: 21288-21293, 2009.

18 Song X and Luo Y: The regulatory mechanism of HSP90alpha secretion from endothelial cells and its role in angiogenesis during wound healing. Biochem Biophys Res Commun *398*: 111-117, 2010.

19 Ullrich SJ, Robinson EA, Law LW, Willingham M and Appella E: A mouse tumor-specific transplantation antigen is a heat-shock-related protein. Proc Natl Acad Sci USA *83*: 3121-3125, 1986.

20 Becker B, Multhoff G, Farkas B, Wild PJ, Landthaler M, Stolz W and Vogt T: Induction of HSP90 protein expression in malignant melanomas and melanoma metastases. Exp Dermatol *13*: 27-32, 2004.
21 Ferrarini M, Heltai S, Zocchi MR and Rugarli C: Unusual expression and localization of heat-shock proteins in human tumor cells. Int J Cancer *51*: 613-619, 1992.
22 Eustace BK, Sakurai T, Stewart JK, Yimlamai D, Unger C, Zehetmeier C, Lain B, Torella C, Henning SW, Beste G, Scroggins BT, Neckers L, Ilag LL and Jay DG: Functional proteomic screens reveal an essential extracellular role for HSP90 alpha in cancer cell invasiveness. Nat Cell Biol *6*: 507-514, 2004.
23 Cid C, Regidor I, Poveda PD and Alcazar A: Expression of heat -shock protein 90 at the cell surface in human neuroblastoma cells. Cell Stress Chaperones *14*: 321-327, 2009.
24 Sapozhnikov AM, Ponomarev ED, Tarasenko TN and Telford WG: Spontaneous apoptosis and expression of cell surface heat-shock proteins in cultured EL-4 lymphoma cells. Cell Prolif *32*: 363-378, 1999.
25 Spisek R, Charalambous A, Mazumder A, Vesole DH, Jagannath S and Dhodapkar MV: Bortezomib enhances dendritic cell (DC)-mediated induction of immunity to human myeloma *via* exposure of cell surface heat-shock protein 90 on dying tumor cells: therapeutic implications. Blood *109*: 4839-4845, 2007.
26 Sidera K, Samiotaki M, Yfanti E, Panayotou G and Patsavoudi E: Involvement of cell surface HSP90 in cell migration reveals a novel role in the developing nervous system. J Biol Chem *279*: 45379-45388, 2004.
27 Picard D: HSP90 invades the outside. Nat Cell Biol *6*: 479-480, 2004.
28 McCready J, Sims JD, Chan D and Jay DG: Secretion of extracellular HSP90alpha *via* exosomes increases cancer cell motility: a role for plasminogen activation. BMC Cancer *10*: 294, 2010.
29 Stellas D, Karameris A and Patsavoudi E: Monoclonal antibody 4C5 immunostains human melanomas and inhibits melanoma cell invasion and metastasis. Clin Cancer Res *13*: 1831-1838, 2007.
30 Li W, Li Y, Guan S, Fan J, Cheng CF, Bright AM, Chinn C, Chen M and Woodley DT: Extracellular heat-shock protein-90alpha: linking hypoxia to skin cell motility and wound healing. EMBO J *26*: 1221-1233, 2007.
31 Basu S, Binder RJ, Ramalingam T and Srivastava PK: CD91 is a common receptor for heat-shock proteins gp96, HSP90, HSP70, and calreticulin. Immunity *14*: 303-313, 2001.
32 Triantafilou M and Triantafilou K: Heat-shock protein 70 and heat-shock protein 90 associate with Toll-like receptor 4 in response to bacterial lipopolysaccharide. Biochem Soc Trans *32*: 636-639, 2004.
33 Sidera K, Gaitanou M, Stellas D, Matsas R and Patsavoudi E: A critical role for HSP90 in cancer cell invasion involves interaction with the extracellular domain of HER-2. J Biol Chem *283*: 2031-2041, 2008.
34 Sidera K and Patsavoudi E: Extracellular HSP90: conquering the cell surface. Cell Cycle *7*: 1564-1568, 2008.
35 Stellas D, El Hamidieh A and Patsavoudi E: Monoclonal antibody 4C5 prevents activation of MMP2 and MMP9 by disrupting their interaction with extracellular HSP90 and inhibits formation of metastatic breast cancer cell deposits. BMC Cell Biol *11*: 51, 2010.
36 Tsutsumi S and Neckers L: Extracellular heat-shock protein 90: a role for a molecular chaperone in cell motility and cancer metastasis. Cancer Sci *98*: 1536-1539, 2007.
37 Li J and Lee AS: Stress induction of GRP78/BiP and its role in cancer. Curr Mol Med *6*: 45-54, 2006.
38 Lee AS and Hendershot LM: ER stress and cancer. Cancer Biol Ther *5*: 721-722, 2006.
39 Arap MA, Lahdenranta J, Mintz PJ, Hajitou A, Sarkis AS, Arap W and Pasqualini R: Cell surface expression of the stress response chaperone GRP78 enables tumor targeting by circulating ligands. Cancer Cell *6*: 275-284, 2004.
40 Rauschert N, Brändlein S, Holzinger E, Hensel F, Müller-Hermelink HK and Vollmers HP: A new tumor-specific variant of GRP78 as target for antibody-based therapy. Lab Invest *88*: 375-386, 2008.
41 Misra UK, Mowery Y, Kaczowka S and Pizzo SV: Ligation of cancer cell surface GRP78 with antibodies directed against its COOH-terminal domain up-regulates p53 activity and promotes apoptosis. Mol Cancer Ther *8*: 1350-1362, 2009.
42 Gonzalez-Gronow M, Misra UK, Gawdi G and Pizzo SV: Association of plasminogen with dipeptidyl peptidase IV and Na^+/H^+ exchanger isoform NHE3 regulates invasion of human 1-LN prostate tumor cells. J Biol Chem *280*: 27173-21178, 2005.
43 Gonzalez-Gronow M, Selim MA, Papalas J and Pizzo SV: GRP78: a multifunctional receptor on the cell surface. Antioxid Redox Signal *11*: 2299-2306, 2009.
44 Katanasaka Y, Ishii T, Asai T, Naitou H, Maeda N, Koizumi F, Miyagawa S, Ohashi N and Oku N: Cancer antineovascular therapy with liposome drug delivery systems targeted to BiP/GRP78. Int J Cancer *127*: 2685-2698, 2010.
45 Burikhanov R, Zhao Y, Goswami A, Qiu S, Schwarze SR and Rangnekar VM: The tumor suppressor Par-4 activates an extrinsic pathway for apoptosis. Cell *138*: 377-388, 2009.
46 El-Guendy N, Zhao Y, Gurumurthy S, Burikhanov R and Rangnekar VM: Identification of a unique core domain of par-4 sufficient for selective apoptosis induction in cancer cells. Mol Cell Biol *23*: 5516-5525, 2003.
47 Schwarze S and Rangnekar VM: Targeting plasma membrane GRP78 for cancer growth inhibition. Cancer Biol Ther *9*: 153-155, 2010.
48 Shrestha-Bhattarai T and Rangnekar VM: Cancer-selective apoptotic effects of extracellular and intracellular Par-4. Oncogene *29*: 3873-3880, 2010.
49 Zhao Y, Burikhanov R, Qiu S, Lele SM, Jennings CD, Bondada S, Spear B and Rangnekar VM: Cancer resistance in transgenic mice expressing the SAC module of Par-4. Cancer Res *67*: 9276-9285, 2007.
50 Pohle T, Brändlein S, Ruoff N, Müller-Hermelink HK and Vollmers HP: Lipoptosis: tumor-specific cell death by antibody-induced intracellular lipid accumulation. Cancer Res *64*: 3900-3906, 2004.
51 Brändlein S, Rauschert N, Rasche L, Dreykluft A, Hensel F, Conzelmann E, Müller-Hermelink HK and Vollmers HP: The human IgM antibody SAM-6 induces tumor-specific apoptosis with oxidized low-density lipoprotein. Mol Cancer Ther *6*: 326-333, 2007.
52 Fuchs E and Weber K: Intermediate filaments: structure, dynamics, function, and disease. Annu Rev Biochem *63*: 345-382, 1994.

53 Kokkinos MI, Wafai R, Wong MK, Newgreen DF, Thompson EW and Waltham M: Vimentin and epithelial–mesenchymal Transition in human breast cancer – observations *in vitro* and *in vivo*. Cells Tissues Organs *185*: 191-203, 2007.

54 Zhu QS, Rosenblatt K, Huang KL, Lahat G, Brobey R, Bolshakov S, Nguyen T, Ding Z, Belousov R, Bill K, Luo X, Lazar A, Dicker A, Mills GB, Hung MC and Lev D: Vimentin is a novel AKT1 target mediating motility and invasion. Oncogene *30*: 457-470, 2011

55 Bhattacharya R, Gonzalez AM, Debiase PJ, Trejo HE, Goldman RD, Flitney FW and Jones JC: Recruitment of vimentin to the cell surface by beta3 integrin and plectin mediates adhesion strength. J Cell Sci *122*: 1390-1400, 2009.

56 Glassy MC and Hagiwara H: Summary analysis of the pre-clinical and clinical results of brain tumor patients treated with pritumumab. Hum Antibodies *18*: 127-137, 2009.

57 Aotsuka Y and Hagiwara H: Identification of a malignant cell associated antigen recognized by a human monoclonal antibody. Eur J Cancer Clin Oncol *24*: 829-838, 1988.

58 Gires O, Mack B, Rauch J and Matthias C: CK8 correlates with malignancy in leukoplakia and carcinomas of the head and neck. Biochem Biophys Res Commun *343*: 252-259, 2006.

59 Hembrough TA, Vasudevan J, Allietta MM, Glass WF 2nd and Gonias SL: A cytokeratin 8-like protein with plasminogen-binding activity is present on the external surfaces of hepatocytes, HepG2 cells and breast carcinoma cell lines. J Cell Sci *108*: 1071-1082, 1995.

60 Hembrough TA, Li L and Gonias SL: Cell-surface cytokeratin 8 is the major plasminogen receptor on breast cancer cells and is required for the accelerated activation of cell-associated plasminogen by tissue-type plasminogen activator. J Biol Chem *271*: 25684-25691, 1996.

61 Borup-Christensen P, Erb K, Jensenius JC, Nielsen B and Svehag SE: Human–human hybridomas for the study of anti-tumor immune response in patients with colorectal cancer. Int J Cancer *37*: 683-688, 1986.

62 Ditzel HJ, Garrigues U, Andersen CB, Larsen MK, Garrigues HJ, Svejgaard A, Hellström I, Hellström KE and Jensenius JC: Modified cytokeratins expressed on the surface of carcinoma cells undergo endocytosis upon binding of human monoclonal antibody and its recombinant Fab fragment. Proc Natl Acad Sci USA *94*: 8110-8115, 1997.

63 Wells MJ, Hatton MW, Hewlett B, Podor TJ, Sheffield WP and Blajchman MA: Cytokeratin 18 is expressed on the hepatocyte plasma membrane surface and interacts with thrombin-antithrombin complexes. J Biol Chem *272*: 28574-28581, 1997.

64 Mahdi F, Shariat-Madar Z, Todd RF 3rd, Figueroa CD and Schmaier AH: Expression and colocalization of cytokeratin 1 and urokinase plasminogen activator receptor on endothelial cells. Blood *97*: 2342-2350, 2001.

65 Motta G, Shariat-Madar Z, Mahdi F, Sampaio CA and Schmaier AH: Assembly of high molecular weight kininogen and activation of prekallikrein on cell matrix. Thromb Haemost *86*: 840-847, 2001.

66 Furmaniak-Kazmierczak E, Nesheim ME and Côté GP: Coagulation factor Va is an actin filament binding and cross-linking protein. Biochem Cell Biol *73*: 105-112, 1995.

67 Moroianu J, Fett JW, Riordan JF and Vallee BL: Actin is a surface component of calf pulmonary artery endothelial cells in culture. Proc Natl Acad Sci USA *90*: 3815-3819, 1993.

68 Dudani AK and Ganz PR: Endothelial cell surface actin serves as a binding site for plasminogen, tissue plasminogen activator and lipoprotein(a). Br J Haematol *95*: 168-178, 1996.

69 Dubernard V, Faucher D, Launay JM and Legrand C: Identification of the cytoskeletal protein alpha-actinin as a platelet thrombospondin-binding protein. FEBS Lett *364*: 109-114, 1995.

70 Hajjar KA, Guevara CA, Lev E, Dowling K and Chacko J: Interaction of the fibrinolytic receptor, annexin II, with the endothelial cell surface. Essential role of endonexin repeat 2. J Biol Chem *271*: 21652-21659, 1996.

71 Srivastava M and Pollard HB: Molecular dissection of nucleolin's role in growth and cell proliferation: new insights. FASEB J *13*: 1911-1922, 1999.

72 Ginisty H, Sicard H, Roger B and Bouvet P: Structure and functions of nucleolin. J Cell Sci *112*: 761-772, 1999.

73 Christian S, Pilch J, Akerman ME, Porkka K, Laakkonen P and Ruoslahti E: Nucleolin expressed at the cell surface is a marker of endothelial cells in angiogenic blood vessels. J Cell Biol *163*: 871-878, 2003.

74 Shi H, Huang Y, Zhou H, Song X, Yuan S, Fu Y and Luo Y: Nucleolin is a receptor that mediates antiangiogenic and antitumor activity of endostatin. Blood *110*: 2899-2906, 2007.

75 Folkman J: Angiogenesis: an organizing principle for drug discovery? Nat Rev Drug Discov *6*: 273-286, 2007.

76 Ling Y, Yang Y, Lu N, You QD, Wang S, Gao Y, Chen Y and Guo QL: Endostar, a novel recombinant human endostatin, exerts antiangiogenic effect *via* blocking VEGF-induced tyrosine phosphorylation of KDR/Flk-1 of endothelial cells. Biochem Biophys Res Commun *361*: 79-84, 2007.

77 Huang Y, Shi H, Zhou H, Song X, Yuan S and Luo Y: The angiogenic function of nucleolin is mediated by vascular endothelial growth factor and nonmuscle myosin. Blood *107*: 3564-3571, 2006.

78 Yuan S, Fu Y, Wang X, Shi H, Huang Y, Song X, Li L, Song N and Luo Y: Voltage-dependent anion channel 1 is involved in endostatin-induced endothelial cell apoptosis. FASEB J *22*: 2809-2820, 2008.

79 Tjin Tham Sjin RM, Satchi-Fainaro R, Birsner AE, Ramanujam VM, Folkman J and Javaherian K: A 27-amino-acid synthetic peptide corresponding to the NH_2-terminal zinc-binding domain of endostatin is responsible for its antitumor activity. Cancer Res *65*: 3656-3663, 2005.

80 Weihua Z, Andersson S, Cheng G, Simpson ER, Warner M and Gustafsson JA: Update on estrogen signaling. FEBS Lett *546*: 17-24, 2003.

81 Kong EH, Pike AC and Hubbard RE: Structure and mechanism of the oestrogen receptor. Biochem Soc Trans *31*: 56-59, 2003.

82 Pietras RJ and Szego CM: Specific binding sites for oestrogen at the outer surfaces of isolated endometrial cells. Nature *265*: 69-72, 1977.

83 Wang Z, Zhang X, Shen P, Loggie BW, Chang Y and Deuel TF: Identification, cloning, and expression of human estrogen receptor-alpha36, a novel variant of human estrogen receptor-alpha66. Biochem Biophys Res Commun *336*: 1023-1027, 2005.

84 Wang Z, Zhang X, Shen P, Loggie BW, Chang Y and Deuel TF: A variant of estrogen receptor-{alpha}, hER-{alpha}36: transduction of estrogen- and antiestrogen-dependent membrane-initiated mitogenic signaling. Proc Natl Acad Sci USA *103*: 9063-9068, 2006.

85 Zou Y, Ding L, Coleman M and Wang Z: Estrogen receptor-alpha (ER-alpha) suppresses expression of its variant ER-alpha 36. FEBS Lett *583*: 1368-1374, 2009.
86 Shi L, Dong B, Li Z, Lu Y, Ouyang T, Li J, Wang T, Fan Z, Fan T, Lin B, Wang Z and Xie Y: Expression of ER-{alpha}36, a novel variant of estrogen receptor {alpha}, and resistance to tamoxifen treatment in breast cancer. J Clin Oncol *27*: 3423-3429, 2009.
87 Lee LM, Cao J, Deng H, Chen P, Gatalica Z and Wang ZY: ER-alpha36, a novel variant of ER-alpha, is expressed in ER-positive and -negative human breast carcinomas. Anticancer Res *28*: 479-483, 2008.
88 Lin SL, Yan LY, Liang XW, Wang ZB, Wang ZY, Qiao J, Schatten H and Sun QY: A novel variant of ER-alpha, ER-alpha36 mediates testosterone-stimulated ERK and Akt activation in endometrial cancer Hec1A cells. Reprod Biol Endocrinol *7*: 102, 2009.
89 Benkoël L, Bernard JP, Payan-Defais MJ, Crescence L, Franceschi C, Delmas M, Ouaissi M, Sastre B, Sahel J, Benoliel AM, Bongrand P, Silvy F, Gauthier L, Romagné F, Lombardo D and Mas E: Monoclonal antibody 16D10 to the COOH-terminal domain of the feto-acinar pancreatic protein targets pancreatic neoplastic tissues. Mol Cancer Ther *8*: 282-291, 2009.
90 Lombardo D, Fauvel J and Guy O: Studies on the substrate specificity of a carboxyl ester hydrolase from human pancreatic juice. I. Action on carboxyl esters, glycerides and phospholipids. Biochim Biophys Acta *611*: 136-146, 1980.
91 Lombardo D and Guy O: Studies on the substrate specificity of a carboxyl ester hydrolase from human pancreatic juice. II. Action on cholesterol esters and lipid-soluble vitamin esters. Biochim Biophys Acta *611*: 147-155, 1980.
92 Mas E, Abouakil N, Roudani S, Miralles F, Guy-Crotte O, Figarella C, Escribano MJ and Lombardo D: Human fetoacinar pancreatic protein: an oncofetal glycoform of the normally secreted pancreatic bile-salt-dependent lipase. Biochem J *289*: 609-615, 1993.
93 Albers GH, Escribano MJ, Gonzalez M, Mulliez N and Nap M: Fetoacinar pancreatic protein in the developing human pancreas. Differentiation *34*: 210-215, 1987.
94 Pasqualini E, Caillol N, Panicot L, Mas E, Lloubes R and Lombardo D: Molecular cloning of the oncofetal isoform of the human pancreatic bile salt-dependent lipase. J Biol Chem *273*: 28208-28218, 1998.
95 Escribano MJ and Imperial S: Purification and molecular characterization of FAP, a feto-acinar protein associated with the differentiation of human pancreas. J Biol Chem *264*: 21865-21871, 1989.
96 Sadoulet MO, Franceschi C, Aubert M, Silvy F, Bernard JP, Lombardo D and Mas E: Glycoengineering of alphaGal xenoantigen on recombinant peptide bearing the J28 pancreatic oncofetal glycotope. Glycobiology *17*: 620-630, 2007.
97 Panicot-Dubois L, Aubert M, Franceschi C, Mas E, Silvy F, Crotte C, Bernard JP, Lombardo D and Sadoulet MO: Monoclonal antibody 16D10 to the *C*-terminal domain of the feto-acinar pancreatic protein binds to membrane of human pancreatic tumoral SOJ-6 cells and inhibits the growth of tumor xenografts. Neoplasia *6*: 713-724, 2004.

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