Modulatory effects of T-cadherin on cell behavior and growth factor receptor activity in carcinoma cells

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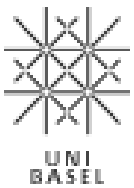

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ABSTRACT

Cancer is a major health problem. Epidermal growth factor receptor (EGFR) pathway plays an important role in cancer progression. EGFR-targeted anti-cancer drugs are being developed to improve anti-cancer therapy. These drugs can give good results especially when a disease is driven by a dominant oncogene. However, the malignant process is generally supported by multiple genetic alterations and a complex signaling network that can compensate for deactivation of signaling targets by increasing activity of other pathways. Deeper understanding of molecular mechanisms of growth factor signaling regulation in cancer will improve anti-cancer therapy and increase clinical benefits.

Classical cadherins are well recognized to be involved in cancer progression and regulation of receptor tyrosine kinase (RTK) signaling. Implication of T-cadherin, an atypical member of cadherin superfamily, in cancer progression has been documented in many cancers but mostly on genetic and epigenetic levels. Few studies have examined functional effects of T-cadherin in cancer, the molecular mechanisms of its effects are poorly understood, and whether T-cadherin regulates RTK signaling in tumor cells is unknown.

This thesis aimed at delineation of the functions of T-cadherin and molecular mechanisms of action in cutaneous squamous cell carcinoma (SCC). We found that T-cadherin loss promotes cell elongation, cell cluster disorganization, cell motility and invasive potential, while T-cadherin upregulation reduces malignant behavior of cells. T-cadherin loss increases, while T-cadherin upregulation blunts sensitivity to stimulation by EGF, manifest at the levels of ligand-induced EGFR phosphorylation/internalization, signal transduction, cell retraction and motility. Molecular mechanisms underlying functional effects of T-cadherin involve β1 integrin activation status and the Rho family of small GTPases. Effects of T-cadherin on EGFR activity are due to altered accumulation of EGFR within lipid raft domains; T-cadherin upregulation retains, while T-cadherin loss releases EGFR from these domains. Thus, T-cadherin acts as a negative auxiliary regulator

of EGFR. EGFR activation in SCC promotes T-cadherin redistribution to intercellular contacts, supporting a reciprocal nature of cross-talk between EGFR and T-cadherin. We postulated that modulation of EGFR activity by T-cadherin could be a regulatory mechanism common to other RTKs. Using prostate cancer cells DU145 (which express comparable levels of EGFR and IGF-1R) we found that T-cadherin regulates activity of both EGFR and IGF-1R and their cross talk. Therefore modulation of growth factor receptor tyrosine kinase activity and cross-talk may be a common mechanistic principle underlying T-cad-dependent control of carcinoma cell behavior. In summary, the findings of this thesis have advanced knowledge on the functional role of T-cadherin in cancer and the participating molecular mechanisms.

1 INTRODUCTION

1.1 Cancer – the current view

Cancer is a disease which kills each year more people than HIV/AIDS, tuberculosis, and malaria combined (8). Factors that cause cancer can be external (tobacco, infectious organisms, chemicals, and radiation, diet, environmental factors) or internal (inherited mutations, hormones, immune conditions, and mutations that occur from metabolism) and may act together or in sequence (9, 10).

Cancer is a multistep process which usually starts when a single once-normal cell acquires a number of gene mutations (10). Proliferating cancerous cells accumulate mutations and undergo changes which allow them to invade surrounding tissues, penetrate blood or lymphatic vessels and spread to other organs within a body (11). To spread within the tissues, tumor cells use similar mechanisms to those that non-neoplastic cells use during physiological processes such as embryonic morphogenesis, wound healing, and immune-cell trafficking (12). The difference lies in cancer cells lacking physiological “stop signals” that immobilize and anchor normal cells (13). Starting from a single mutated cell cancer can become a systemic disease in a relatively short time. Cancer is an enemy, which can make surrounding normal tissues work for its needs (14) and can acquire resistance to anti-cancer drugs (15). A better understanding of the mechanism of cancer progression will help to improve anti-cancer therapy.

1.2 Role of growth factors in cancer progression

Malignant and normal cells communicate through cell-cell contacts and secretion of signaling molecules activating surface receptors. The communication network between tumor and host cell populations is extensive and determines the fate of the growing tumor (16).

Included amongst the secreted signaling molecules are so-called growth factors which are a group of peptides, proteins and steroid hormones capable of stimulating cell proliferation (17). These molecules act through specific receptors on the cell surface which once activated trigger downstream pathways leading to the activation of transcription factors in the nucleus and increase in mRNA synthesis. Aberrations in the growth factor signaling pathways can lead to abnormal cell growth and contribute to malignant transformation in many types of cancer (18). Cancerous cells can regulate diverse processes such as proliferation, differentiation, survival and migration autonomously by autocrine secretion of growth factors (19) or by enhancing affinity and the number of growth factor receptors (20).

Many different families of growth factors and growth factor receptors have been shown to be implicated in carcinogenesis (21, 22). Among them are the epidermal growth factor receptor (EGFR) (23), the insulin-like growth factor receptor (IGF-1R) (24) and their cognate ligands.

1.3 Epidermal growth factor receptor (EGFR) signaling pathway

1.3.1 EGFR structure and its activation

Epidermal growth factor (EGF) binds to a specific receptor on the surface of responsive cells. EGFR is a trans-membrane receptor belonging to the ErbB family of receptor tyrosine kinases (RTK), which comprises four distinct receptors: the EGFR itself (also known as ErbB-1/HER1), ErbB-2 (HER2), ErbB-3 (HER3) and ErbB-4 (HER4) (25). These four proteins possess an extracellular ligand-binding domain, a single trans-membrane domain and a cytoplasmic tyrosine-containing domain (26) (Fig. 1). The extracellular ligand-binding domain is quite variable among the receptors, suggesting a difference in ligand binding specificity. In contrast, the intracellular tyrosine kinase domain of ErbB receptors is highly conserved. The extracellular domain of EGFR/ErbB monomers consists of two ligand-binding subdomains (L1 and L2) and two cysteine-rich domains (S1 and S2). S1 plays a crucial role in EGFR/ErbB dimerization. Protein tyrosine kinase domain, SH1, is located in the cytoplasmic domain, followed by six tyrosine residues available for trans-phosphorylation (5) (Fig. 1).

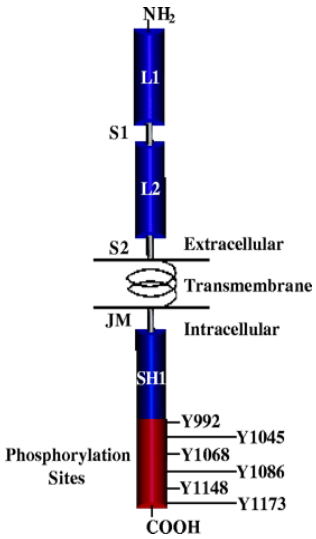

Figure 1. Schematic structure of EGFR monomer (5).

Binding of ligands to the extracellular domain of a single-chain EGFR, induces the formation of a homo- or heterodimers, which leads to phosphorylation of the kinase domain of the EGF receptor and subsequent receptor auto-phosphorylation within the cytoplasmic tail (27) (Fig. 2). This process triggers downstream activation by recruiting several other proteins that associate with the phosphorylated tyrosines through their Src homology 2 (SH2) and phosphotyrosine-binding (PTB) domains. Thus, the information from the extracellular environment transduces into the cell by recruiting a variety of cytoplasmic proteins involved in regulating cellular pathways.

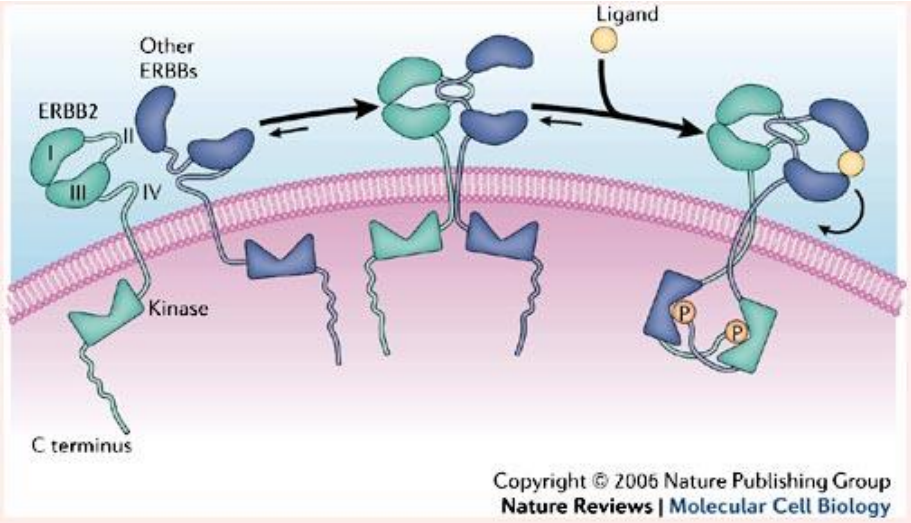

Figure 2. Structural basis for ERBB-receptor dimerization and activation. *An example of hetero-dimerization. Ligand binding stabilizes a dimer and by stabilizing a dimer and forcing a rotation in the vicinity of the membrane, ERBB ligands activate the kinase activity of the receptor (2).*

1.3.2 EGFR downstream signaling and its implication in cancer

Proteins which bind to the intracellular tail of the activated receptor include adapter proteins (e.g., p85, Shc, Grb2) which link the receptor to various downstream pathways, and enzymes such as: protein kinases (e.g., Src), protein phosphatases (e.g., SHP1), phospholipases (e.g., PLCγ1), and regulators of G proteins (e.g., p120-RasGAP, p85-RabGAP) (28). Thus, EGFR signaling activates two major pathways in normal and cancer cells: the RAS/RAF/MEK/MAPK and the PI3K/AKT/mTOR pathways that control cell proliferation, cell growth, migration, apoptosis, and angiogenesis.

The RAS/RAF/MEK/MAPK pathway. EGFR auto-phosphorylation facilitates recruitment of adapter proteins and results in cell proliferation through intermediate steps (Fig. 3).

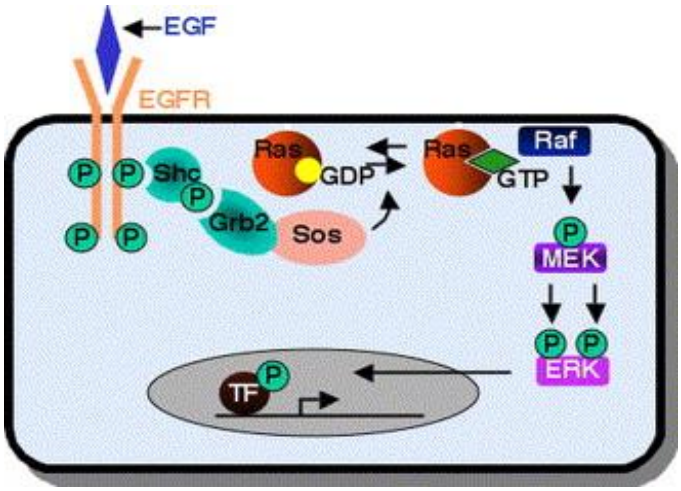

Figure 3. Schematic diagram showing the Ras/Raf/MEK/ERK pathway. *Ligand-induced EGFR activation leads to receptor auto-phosphorylation and binding of Grb2-Sos complex directly or through association with Shc. A change in Sos conformation allows Ras-GDP recruitment, resulting in Ras activation (Ras-GTP). Raf binds to the plasma membrane and to Ras–GTP facilitating activation of its serine/threonine kinase activity, which leads to MEK and ERK phosphorylation. Activated ERK translocates into the nucleus where it phosphorylates specific factors involved in cell proliferation (7).*

The PI3K/AKT/mTOR pathway. Ligand-induced EGFR activation leads to receptor auto-phosphorilation and recruitment of PI3K. It triggers downstream signaling, which modulates cell growth, cell proliferation and survival (Fig. 4).

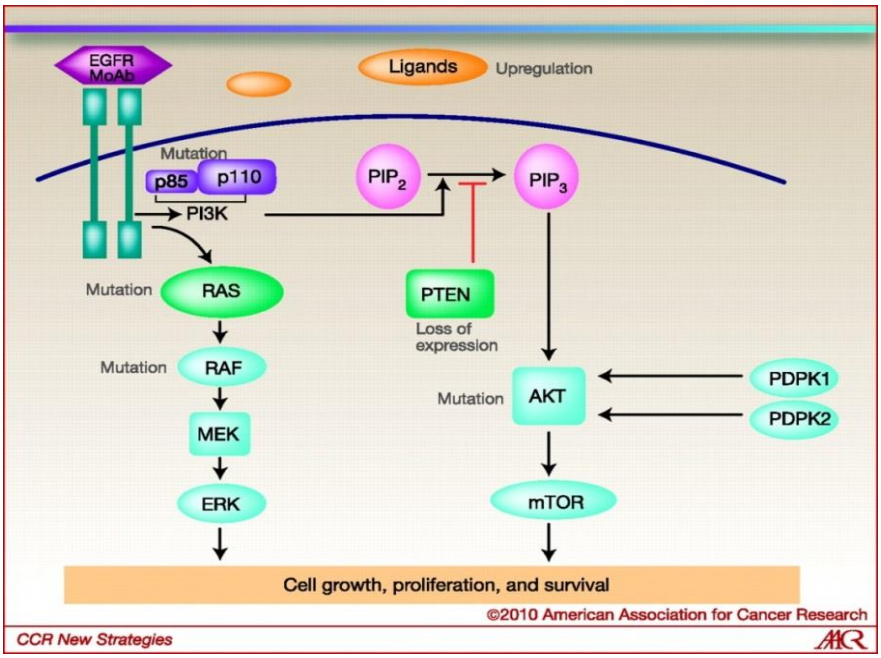

Figure 4. Schematic diagram showing the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. *Ligand-induced EGFR activation results in PI3K binding. PI3K is a dimeric enzyme composed of a regulatory p85 subunit, responsible for the anchorage to ErbB receptor-specific docking sites, and a catalytic p110 subunit. PI3K is a dimeric enzyme composed of a regulatory p85 subunit, responsible for the anchorage to ErbB receptor-specific docking sites, and a catalytic p110 subunit catalyzes the conversation of phosphatidylinositol 4,5-triphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3) in the membrane controlled by PTEN, that results in activation of a serine/threonine kinase Akt. Phosphorylated Akt regulates activity of several downstream targets involved in cellular growth (mTOR), proliferation (RAS-MAPK) and survival (4).*

Aberrant EGFR expression and its subsequent enhanced activity in tumors can be caused by amplification of the EGFR gene, receptor-activating mutations, or the loss of negative regulatory mechanisms (29, 30). Over-expressed EGFR is frequently detected in brain tumors, head and neck tumors, non-small-cell lung cancer, colorectal, prostate and ovarian carcinomas, and its elevated levels are often associated with poor prognosis (23, 31). Somatic mutations of the receptor vary depending on tumor type. For instance, somatic mutations in the kinase domain are usually found in non-small cell lung cancer (NSCLC) while being quite rare in others, such as glioblastoma multiforme (GBM) (32). Depending on the mutation type of EGFR, tumor cells can activate specific downstream signaling pathways, such as Ras/MAPK or PI3K/AKT; for example in NSCLC EGFR mutations more often activate Ras signaling than PI3K/AKT (33), while the opposite is true in GBM (34).

1.3.3 EGFR-targeted anti-cancer therapy

Targeted inhibition of specific receptors which are situated at the apex of complex signaling networks has become a dominant strategy in cancer treatment last years (35). Such inhibition of the EGFR activity suppresses signal transduction pathways and can lead to tumor regression. Small molecule tyrosine kinase inhibitors and monoclonal antibodies are among the most common EGFR-targeting agents and have been shown to be effective in treatment of human cancer (32).

Anti-EGFR monoclonal antibodies, such as cetuximab and panitumumab bind to the extracellular domain of EGFR when it is in inactive configuration and compete with other specific ligands for the ligand-binding region. As a result of this binding, monoclonal antibodies block ligand-induced EGFR tyrosine kinase activation by preventing receptor dimerization (36).

Most small-molecule EGFR tyrosine kinase inhibitors, such as erlotinib and gefitinib, are reversible inhibitors, which compete with adenosine-5’-triphosphate (ATP) to bind to the intercellular catalytic domain of EGFR tyrosine kinase, thereby inhibiting EGFR auto-phosphorylation and downstream signaling (37). Tyrosine kinase inhibitors may inhibit only one or all EGFR family receptors and some of them can also inhibit other growth factors, e.g. a dual EGFR/ErbB2 inhibitor lapatinib that is used clinically to treat HER-2 positive breast cancer patients (38).

Although the EGFR pathways have been extensively investigated, there are still many open questions concerning the function of different EGFR family oligomers and the impact of other molecules and signaling pathways which can modulate EGFR activity. To date, the results for EGFR directed therapeutics of cancer patients have been modest due to unresponsiveness or

compensation ability of targeted tumors and in some cases acquired resistance to EGFR-targeting drugs.

1.3.4 Complexity of the EGFR network and its balance

Activity of EGFR and other receptor tyrosine kinases pathways is regulated by a complex network of positive and negative feedback loops which ensures robustness of the system (39).

Positive feedback regulatory loops maintain appropriate strength and duration of the ErbB signaling by enhancing amplitude and prolonging the active state of pathways (40). One of the mechanisms regulating positive feedbacks is based on autocrine and paracrine secretion of EGF-like ligands and/or angiogenic factors in response to receptor activation; for example G-protein-coupled receptor-induced EGFR transactivation generates downstream signaling and leads to heparin binding (HB)-EGF secretion through the stimulation of surface proteinases (2). Other tyrosine kinase receptors can also play a positive regulatory role; for example ErbB2-containing heterodimers may avoid negative regulation (41).

Negative or inhibitory feedbacks attenuate signaling by multiple molecular mechanisms including receptor dephosphorylation by tyrosine phosphatases (42), endocytosis followed by degradation (43), as well as by molecules controlling membrane compartmentalization (44). Negative regulators of a receptor can either be constantly present or be synthesized after receptor stimulation, defining the window of active signaling (2, 45).

Several studies have shown that EGFR can be regulated by its lipid environment and activity of the receptor can depend on its localization in lipid rafts (46). Although, the data is contradictory and there is almost the same number of studies which show activating or inhibitory role of lipid rafts in EGFR activity, cumulatively the data suggest that lipid rafts play a role of signaling platforms which bring together different molecules to determine signaling preferences of a specific cell (47).

1.4 Insulin-Like Growth Factor-1receptor (IGF-1R) signaling pathway

1.4.1 IGF-1R structure and its activation

IGF-1R, IGF-2R and insulin receptor (IR) belong to the Insulin-like growth factor family. Apart from these receptors, the family contains IGF-1 and IGF-2 ligands, six high-affinity binding proteins (IGFBPs), low-affinity IGFBP-related proteins and a big group of IGFBP proteases.

IGFs are single-chain polypeptides derived from pre-propeptides like insulin and are highly homologous. IGF-1 is mainly produced by liver in responses to growth hormone but also, like IGF-2, can be synthesized by almost any tissue in the body. IGFs are involved in regulation of many processes such as cell proliferation, differentiation, apoptosis and transformation and can also be involved in autocrine, paracrine and endocrine signaling in normal and malignant tissues (48).

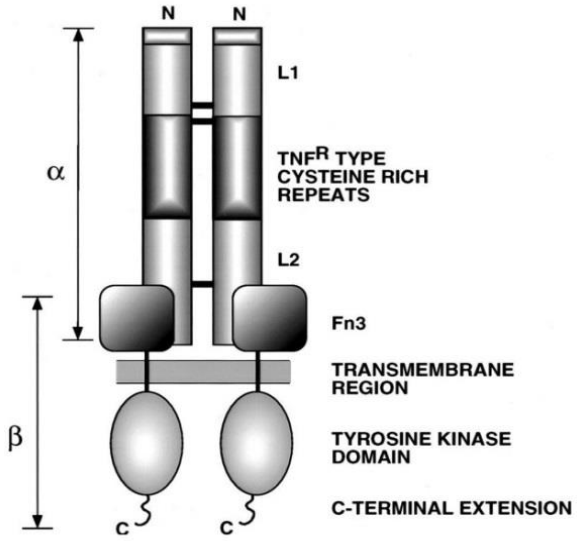

Figure 5. Schematic representation of the full-length IGF-I receptor (1).

IGF-1 binds to IGF-1R with 2-15 fold higher affinity than do IGF-2 and insulin. The IGF-1R is a glycoprotein found on the cell surface which belongs to the class of RTK and shares 60% homology at the amino acid sequence level with the IR. The IGF-1R consists of two extracellular α-subunits and two transmembrane β-subunits (Fig 5).

1.4.2 IGF-1R downstream signaling and its implication in cancer

The binding of ligands to the α-subunit of IGF-1R results in a conformational change of the β-subunits of the receptor that stimulates tyrosine kinase activity, leading to auto-phosphorylation of tyrosine residues of the receptor. Its phosphorylated tyrosine residues serve as a docking sites for insulin receptor substrates-1 (IRS-1) and adaptor proteins, such as Src- and Shc proteins, which induce two major signaling pathways involved in cellular processes such as cell proliferation, differentiation and survival: the metabolic and anti-apoptotic PI3K/Akt pathway and the mitogenic MAPK pathway (Fig 6).

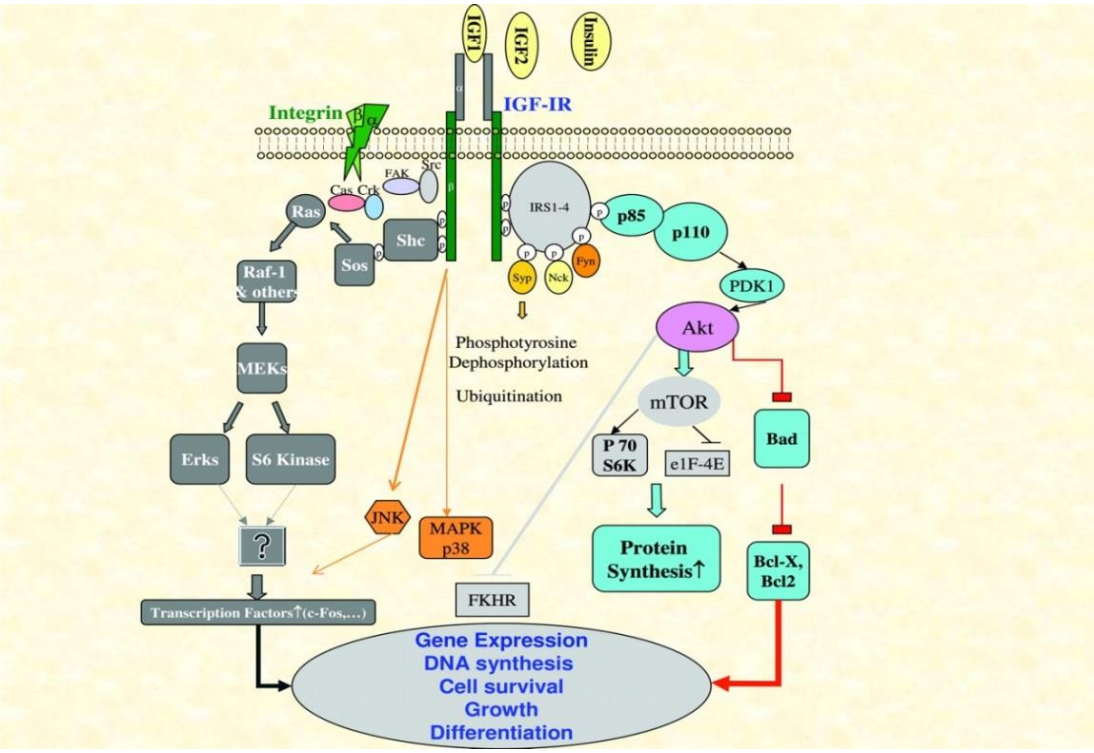

Figure 6. A schematic representation of the major signaling pathways that can be activated by the auto-phosphorylated IGF-IR. *Ligand-induced IGF-1R recruits and activates PI3K through tyrosine-phosphorylated IRS-1, which in turn activates Akt. Activated Akt has different substrates and thus can transmit signal to different downstream pathways. Akt can increase cell survival by inactivation of pro-apoptotic protein Bcl-2-associated death promoter (Bad) and caspase-9 as well as by activation of nuclear factor-kB (NF-kB) that results in transcription of pro-survival genes. Protein synthesis can be enhanced through activation of mTOR. Ligand-induced IGF-1R can also activate Ras, Raf, MEK1/2, and ERK1/2 through IRS-1 or Shc and Grb2/SOS complex and result in increased cell proliferation (3).*

These functions have made IGF-1R signaling a pivotal regulator of the normal tissue homeostasis and development as well as an important factor in cancer progression. Elevated levels of IGF-1, -2 and IGF-1R have been documented in breast, pancreatic and colon carcinomas (49). Higher levels of circulating IGF-1 have been also linked to an increased incidence of pre-cancerous colonic adenoma, suggesting a role of IGF pathway in the early stages of tissue transformation towards malignancy (50). It has been shown that IGF-1R expression level is elevated during progression from colonic adenocarcinoma to primary colorectal adenocarcinomas and metastasis (51). Other studies conducted on synovial sarcomas, uveal melanoma, gastric cancer and gallbladder carcinomas also linked IGF-1R overexpression with metastatic phenotype (3). A majority of metastatic prostate cancer specimens also express higher levels of IGF-1R compared to non-malignant tissues (52). There are also reports demonstrating an opposite role of IGF-1 in cancer progression. It has been shown that androgen-independent prostate tumor growth and metastasis was associated with decreased IGF-1R expression level (53). However, it is still unclear whether IGF-1R expression level is affected by changes in signaling pathway cross-talk or through other not yet identified functions of IGF-1R in tumor progression.

1.4.3 IGF-1R-targeted anti-cancer therapy

The findings that the IGF family is implicated in different cancers have led to the development of IGF-1R-targeting anti-cancer therapies, among them monoclonal antibodies and small molecule RTK inhibitors (54). Inhibition of the IGF-1 binding to IGF-1R or blocking the activation of IGF-1R at the ATP-binding domain leads to abrogation of downstream signaling that results in attenuation of signal transduction important for tumor cell growth and increases tumor cell responsiveness to other anti-cancer therapy (54). However, inactivation of the IGF-1R alone is often insufficient for complete inhibition of tumor cells expressing multiple receptors that interact with each other through a broad network of signaling molecules, downstream targets and positive/negative regulatory loops.

1.4.4 EGFR and IGF-1R cross-talk

One of the best known modalities of cross-talk between growth factor receptors implicated in cancer is that between EGFR and IGF-1R, which use several common adapter molecules to transmit signals within a cell. Transactivation of EGFR by activated IGF-1R *via* direct and indirect mechanisms has been shown in several studies. In normal human mammary epithelial cells IGF-1R can physically interact with EGFR and enhance IGF-1 dependent ERK activation through an increase in EGFR phosphorylation (55). Studies on COS-7 cells showed that IGF-1R activation induces phosphorylation of EGFR and Shc and the assembly of EGFR/Shc complexes that result

in increased ERK1/2 phosphorylation (56). Transactivation of EGFR by IGF-1 *via* metalloproteinase-mediated shedding of HB EGF was observed on a prostate cancer cell line, DU145 (57).

Several studies have observed that inhibition of EGFR signaling may lead to acquired drug resistance in some cancer cells by facilitating activation of other growth factor receptors, including IGF-1R. Signaling adaptation to abrogated EGFR activity by switching to IGF-1R signaling pathway and upregulation of its components has been observed in breast and prostate challenged by a prolonged treatment with gefitinib (58, 59).

Dual targeted therapy aimed at simultaneous inhibition of EGFR and IGF-1R by specific antibodies resulted in significantly better outcome *in vitro* and *in vivo* (60, 61).

1.5 The cadherin superfamily

1.5.1 Classical cadherins

The cadherin superfamily is a group of cell surface receptors that is comprised of more than 100 members and can be classified into several subfamilies including the type I and type II "classical" cadherins, the protocadherins, and the atypical cadherins (62) (Fig. 7). The biological functions of the family members are quite diverse, encompassing adhesive activity as well as modulation of signaling pathways. The first identified subfamily was the "classical" cadherins that are involved in establishing and maintaining cell-to-cell cohesion, cell-cell recognition during sorting and tissue reorganization, cell-upon-cell locomotion and epithelial polarity (63). Their effects on tissue organization and differentiation processes suggested relationships between the cadherin superfamily members and cancer progression. Tumor cells frequently show deregulated cadherin expression and inappropriate switching among family members, indicating cadherin dysfunction as a major contributor to cancer progression (64, 65).

The “classical” cadherins are transmembrane glycoproteins consisting of extracellular domain, a transmembrane sequence, and a cytoplasmic domain (which is lacking in non-classical cadherins) and are the most investigated members of the cadherin superfamily (66) (Fig. 7).

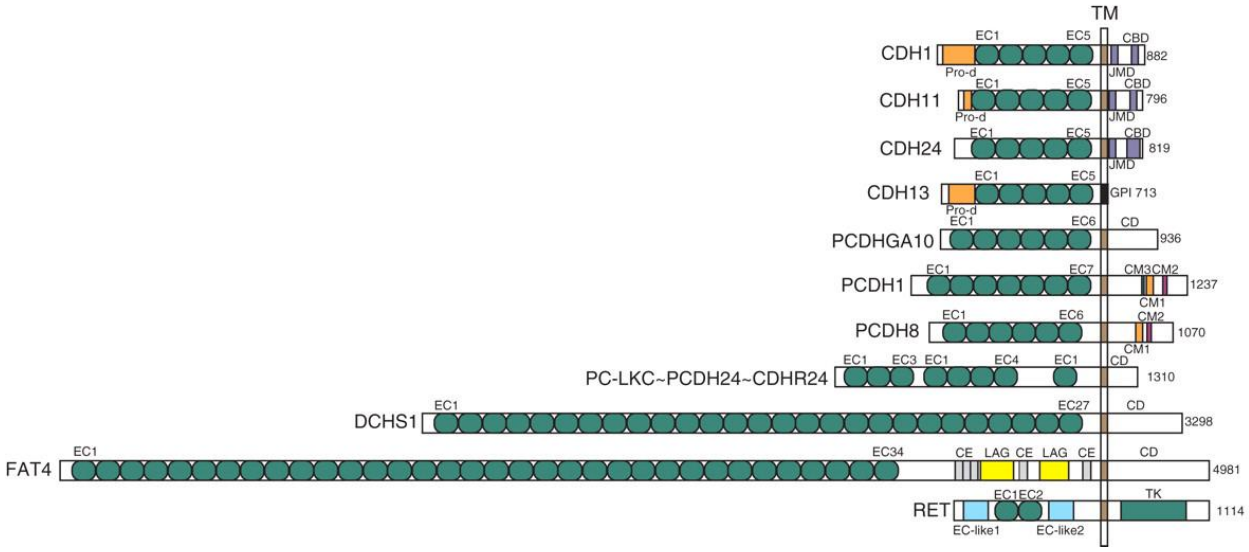

Figure 7. The cadherin superfamily. *All proteins are aligned at their transmembrane domain (TM). Their total sizes are indicated on the right (number of amino acid residues). The following protein domains are shown: CBD, (conserved cadherin-specific) catenin binding domain; CD, unique cytoplasmic domain; CE, Cysteine-rich EGF repeat-like domain; CM1 to CM3, conserved motifs in the CDs of δ-protocadherins; EC, extracellular cadherin repeat; GPI, glycosylphosphatidylinositol anchor; JMD, (conserved cadherin-specific) juxtamembrane domain; LAG, laminin A globular domain; Pro-d, prodomain; TK, tyrosine kinase domain (6).*

A common feature of all cadherins is the architecture of the extracellular domain which is structured into tandemly arrayed extracellular domains (ECs), called also cadherin repeats, that are highly variable in number and can contain from 1 to 34 ECs. The cadherin repeats contain conserved amino acid residues that are capable of binding calcium ions at the ends of each barrel to maintain the structural integrity of the cadherin ECs (67). It allows cell-cell adhesion through cis- or trans-interactions between the ectodomains of other cadherins presented on the same cell or on neighboring cells, respectively.

The mature type I classical cadherins mediate strong cell-cell adhesion and have five ECs (Fig. 7). E-cadherin (epithelial), N-cadherin (neuronal) and P-cadherin (placental) belong to the classical type I subfamily. The type II classical cadherins, such as VE-cadherin (vascular endothelial), also mediate strong adhesion, though the interaction is somewhat weaker than that mediated by type I cadherins.

Interactions of cadherins with each other or with other molecules at cell-cell surfaces generate different signals through their cytoplasmic tails. To date, the best understood proteins associated with classical cadherins are β-catenin and γ-catenin (plakoglobin), which bind to the same conserved region of the cytoplasmic tail of cadherins and are crucial for a proper function and stability of the mature protein. The α-catenin, which binds to β-catenin and γ-catenin, links the cadherin complex with the actin cytoskeleton.

Interaction of RTK with cadherins alters both cell-cell adhesion and RTK signaling. RTKs can phosphorylate E-cadherin, N-cadherin, β-catenin, γ-catenin and p120-catenin resulting in the disassembly of the cadherin-catenin complex and disruption of cell-cell adhesion. It has been observed that E-cadherin can activate EGFR in keratinocytes and in mammary epithelium even in the absence of a ligand, leading to a reduction in focal adhesion due to requirement of the extracellular domain of E-cadherin-receptor interaction. Other studies have shown that E-cadherin can inhibit cell responsiveness to EGF stimulation (68) and that E-cadherin ligation inhibits EGF-induced proliferation (69). N-cadherin. on the other hand, is incapable of interaction with EGFR (70) but was shown to interact with members of fibroblast growth factor receptors, inducing downstream signaling and promoting cell invasion, migration, and survival, thereby participating in cancer progression (71).

1.5.2 T-cadherin

T-cadherin (truncated cadherin) was first identified in chicken embryo brain in 1991 and was named for its atypical structure (72). It is the only known cadherin linked to the membrane *via* glycosylphosphatidylinositol (GPI) anchor and lacks the transmembrane and cytoplasmic domains typical for the classical cadherins. Human homologue of chicken T-cadherin was identified independently by two groups; in 1994 cadherin13 (CDH13) was reported by Tanihara et al. (73) following cloning analysis, and in 1996 Lee reported on his discovery of a candidate gene that appeared to be identical to CDH13 and expression of which was altered in breast cancer (74). Due to its strong expression in heart Lee named the protein H-cadherin.

T-cadherin gene is highly conserved and is localized on chromosome 16q24 along with other cadherins (E-cadherin, P-cadherin, VE-cadherin and CDH11) that makes possible the existence of common control elements of these closely localized genes (74).

T-cadherin shares sequence similarity of the extracellular domains with the classical cadherins. However, many amino acids important for the homophilic adhesive functions of E-,P- and N-cadherins such as HAV motif, are missing or replaced in T-cadherin EC1 structure, which results in

a significant weaker adhesion capacity of T-cadherin (75). T-cadherin is highly glycosylated protein, like all cadherins, and possesses eight potential N-glycosylation sites (76).

T-cadherin can be expressed on the cell membrane as either a mature protein or a partially processed T-cadherin precursor that retains the uncleaved propeptide (77). Many studies have detected more than one immunoreactive band on Western blots, with size ranging between 45 kDa and 130 kDa. This might be also explained by existents of isoforms or splice variants as well as differences in post-translational modifications such as degrees of glycosylation.

Localization of T-cadherin on the plasma membrane differs among cell types and depends on experimental conditions. In monolayer cultures of endothelial cells, smooth muscle cells and bladder carcinoma cell line T-cadherin was globally distributed over cell surface with low concentration at cell-cell contacts. Wounding of these monolayers resulted in T-cadherin redistribution to the leading edge of migrating cells (78). *In vivo* studies on chicken intestinal epithelium described the presence of T-cadherin on the apical, but not basolateral, cell surface (79). The same pattern was observed for T-cadherin transfected MDCK cell line (80).

In sucrose density gradient analysis performed on cardiac myocytes, T-cadherin was detected in the caveolin-enriched fraction insoluble in Triton. However, immunopurified caveolin-containing membranes did not contain T-cad. The authors suggested that T-cad can be localized in other membrane domains, lipid rafts, which can be isolated in complex with caveolae membrane fragments (81). T-cad was also isolated in a detergent insoluble low-density membrane domain co-distributing with caveolae markers in vascular smooth muscle cells (82).

1.6 T-cadherin in cancer

1.6.1 T-cadherin genetic, epigenetic status and protein expression levels in cancer

The recognized role played by classical cadherins in tumor progression and together with localization of T-cadherin gene at the chromosome region exhibiting loss of heterozygosity (LOH) in many solid tumors attracted initial interest to T-cadherin as a molecule potentially involved in carcinogenesis.

Aberrant methylation of T-cadherin gene, LOH and decreased expression of T-cadherin gene or protein has been documented in many cancers (Table 1).

Table 1. CDH13 methylation[1]/ LOH[2]/ lost or decreased[3] expression in cancer	
Breast cancer:	
Tissues	(83)[3], (84)[1,3], (85)[2], (86)[1], (87)[1], (88)[1], (89) (90)[3], (91)[1,3], (92)[1], (93) (94)[1], (95)[1], (96)[1], (97)[1], (98)[1]
MDAMB435 and other cell lines	(74)[3], (99)[3]
MDA-MB-435, MDA-MB-46B, BT-20, MDA-MB-231, MCF-7	(88)[1]
HCC1954	(86)[3]
Lung cancer:	
Tissues	(100)[1,2,3], (84)[1,3], (101)[3], (102)[1], (103)[1], (104)[1], (105)[1], (106)[1], (107)[1], (108)[1], (109)[1], (110)[1], (111)[1], (112)[1], (113)[1], (114)[1], (115)[1], (116)[1], (117)[1], (118)[1], (119)[1], (120)[1], (121)[1], (122)[1], (123)[1], (124)[1], (125)[1], (126)[1], (127)[1], (128)[1], (129)[1]
S-2[3], I-87[1,2,3], Sq-1[1,2,3], and EBC-1[1,2,3] cell lines	(100)
A549, H322, H520, H596, H661 cancer cell lines	(101)[3]
silica-induced multistep lung carcinogenesis model driven by chronic inflammation	(130)[1]
Esophageal and Gastric cancers:	
esophageal cancer tissues	(131)[1], (132)[1], (133)[1]
gastric cancer tissues	(131)[1], (134)[1]
gastric cancer cell lines AZ521, KatoIII, MKN7	(135)[3]
Sporadic Duodenal carcinoma:	
Tissues	(136)[1]
Colorectal cancer:	
Tissues	(137)[1,3], (138)[1], (139)[1], (140)[1], (141)[1], (141, 142)[1], (143)[1], (144)[1], (145)[1], (146)[1],
Colorectal cell lines LoVo, HCT116, SCC10, SW1417, RKOAU, COLO201	(137)[1,3]
Hct116 cells	(147)[1,3]

Ovarian cancer:	
Tissues	(148)[1,2,3], (149)[1], (150)[1], (151)[1], (152)[1], (153)[1], (154)[1], (155)[1], (156)[1], (157)[1], (158)[1]
Bladder cancer:	
Tissues	(159)[1], (160)[1], (161)[3]
Prostate cancer:	
Tissues	(162)[1], (163)[1], (164)[1], (165)[1], (166)[1], (167)[1], (168)[1]
LNCaP, DU145, CWR22Rv cancer cell lines: PC3M2, PC3M-LN4 sublines; LuCaP23, LuCaP35 xenograft prostate tumor models	(164)[3]
Cervical cancer	
Serum	(169)[1], (170)[1]
Tissues	(171)[1], (172)[1]
Cancer cell lines CAC-1, OMC-4, HeLa, TMCC-1, Caski, ME-180	(173)[3]
Tissues and cancer cell lines SiHa, HeLa, CaSki, 778, 808, 866, 879, 915	(174)[1]
Endometrial cancer:	
Vaginal secretion	(175)[1]
Tissues	(176)[1], (177)[1], (143)[1], (178)[1], (179)[1]
Hepatocellular carcinoma:	
Tissues	(180)[1]
Cancer cell lines HepG2[1,3], PLC/PRF/C[3], TONG[3], HA22TNGH[1,3], Hep3B[3], and PLC/PRF/5[3]	(181), (182)
Tissues and cancer cell line HepG2	(183)[1,3]
Tissues[3] and cancer cell lines PLC/PRF/5[3], HepG2[1], HUH71[1], SNU449[1,3], HLE[1,3], SNU182[3], SNU368[3]	(184)[1,3]
Gallbladder carcinoma:	
Tissues	(185)[1], (186)[1,3], (187)[1,3]
Cancer cell line NOZ	(186)[3]
Neuroblastoma:	
Tissues and neuroblastoma cell lines	(188)[1]

Neuroblastoma cell lines TGW and NH-12	(189)[3]
Pancreatic cancer:	
Tissues[1] and cancer cell lines[1,3] AsPC-1, BxPC-3, Capan-1, Capan-2, MIA PaCa-2, SW1990	(190)[1,3]
Tissues	(191)[1]
Nasopharyngeal carcinoma:	
Tissues[1], cell line C666-1[1,3] and C15, C17 xenografts[1,3].	(192)[1,3],
Tissues	(193)[1]
Pituitary adenoma:	
Tissues	(194)[1,3]
Blood cancers:	
Chronic myeloid leukemia (CML)	(195)[1,3], (196)[1,3], (197)[1]
diffuse large B cell lymphomas and some cell lines	(198)[1,2,3]
Acute lymphoblastic leukemia (ALL)	(199)[1], (200)[1]
Acute myeloid leukemia (AML) and in leukemia cell lines: Myeloid: BV173R, HL60, HEL, KG-1, KG-1a, ML-1, MV4:11, KBM5R, OCI-AML3, TF-1; Lymphoid: T-cells: CEM, JTAg, Jurkat, MOLT-4, T-ALL, Peer, B-cells: ALL1, BJAB, Raji, RS4;11	(201)[1], (197)[1]
Chronic lymphocytic leukemia (CLL)	(202)[1]
Glioblastoma:	
Tissues	(203)[1], (204)[3]
Head and neck squamous cell carcinoma:	
Squamous cell carcinoma line UMSCC-17A	(205)[1]
Tissues	(206)[1]
Thyroid carcinomas:	
Tissues	(207)[3]
Melanoma:	
Melanoma cell lines B16; B16F10	(208)[3]; (209)[3]
Tissues[3] and melanoma cell lines[1,3]	(210)
Melanoma cell lines WM115F, A375P, DM4,	(211)[1]

PAI4, MEWO, MEL 888, C8161	
Tissues[3] and melanoma cell lines[1,3]: 501mel, HMB2 and SKMel28	(212)
Cutaneous squamous cell cancer (SCC):	
Tissues and cancer cell line A431	(213)[1,2,3]
Cancer cell line HSC-1 and immortalized keratinocyte cell lines derived from SCC	(214)[3], (215)[3]

The majority of studies mentioned above have described association between T-cadherin hypermethylation and/or down-regulation with invasive type of cancer, bigger tumor size, advanced stage of the disease and poor overall prognosis.

Table 2 presents studies reporting that T-cadherin status was comparable between cancer and normal tissues.

Table 2. CDH13 without[1] or low[2] frequency methylation/ unchanged[3] or increased[4] mRNA/protein expression levels in cancer	
Testicular germ cell tumors:	
Tissues	(216)[2], (217)[2]
Primary pediatric tumors:	
Tissues	(218)[2]
Wilms' tumor[1] and adult renal[2] cell carcinoma:	
Tissues	(219)
Astrocytoma:	
Tissues	(220)[2], (221)[1], (203)[1]
Osteosarcoma:	
HOS and SAOS2 cell lines	(74)[3], (222)[4]

In yet other cancers, specifically *retinoblastoma, basal cell carcinoma (BCC) and hepatocellular carcinoma (HCC)*, the results have been controversial. It was shown that T-cadherin expression levels did not differ between retinoblastoma cell lines and normal retina (223). Also, no somatic mutations were detected in retinoblastoma samples (224). However, a recent study revealed decreased T-cadherin mRNA levels in five retinoblastoma cell lines (225). An initial study on T-cadherin expression in cutaneous BCC tissues specimens described loss of the protein expression (226). However, later T-cadherin was shown abundantly expressed in BCC tissues and especially concentrated at intercellular borders and invasive fronts of the tumors (227). Aberrant methylation, LOH and decreased T-cadherin expression were shown in hepatocellular carcinoma (181, 184). However, in some HCC areas T-cadherin was found upregulated, as well as highly expressed in aggressive HCC cell line Mahlavu (182). In addition, T-cadherin was not detected on hepatocytes in normal liver (182). The controversial data indicate that the relationship between T-cadherin and cancer progression is not as straightforward as in the case of other tumor suppressors or oncogenes such as: p53 or HER-2/neu.

1.6.2 Functional role of T-cadherin in cancer

From the large number of genetic and epigenetic studies mentioned above one might assume a relevant role for T-cadherin in cancer development. However, disproportionately few studies have addressed what the cellular functions and mechanisms of action of T-cadherin might be.

One of the first functional studies was conducted on breast cancer cell lines MDAMB231 and MDAMB435 which lack T-cadherin expression. Ectopic T-cadherin over-expression resulted in tumor cell growth inhibition and decreased tumor growth in nude mice, as well as transformation of invasive phenotype to the normal epithelial morphology in Matrigel assay (74, 99). Another study showed decreased MDAMB231 cell proliferation and invasion caused by cervastatin-induced T-cadherin expression (228).

In prostate cancer cell line DU145 re-expression of T-cadherin reduced cell growth, colony formation in soft-agar and ability to form subcutaneous tumors in nude mice (164).

In gallbladder carcinoma cell lines NOZ and OCUG T-cadherin over-expression promoted epithelial cell-like morphology, reduced collagen gel invasive activity, reduced soft-agar colony formation activity, suppressed Akt3 expression and overall phosphorylation of Akt, and upregulated SET7/9 expression without changing cell growth; silencing increased invasiveness without altering cell growth and down-regulated SET7/9 (186, 187).

In bladder transitional cell carcinoma cells 5637 T-cadherin silencing promoted migration, invasion and adhesion of the cells, as well as matrix metalloproteinase-2 (MMP2) expression (161).

In the aggressive HCC cell line Mahlavu T-cadherin silencing led to decreased invasive potential and motility but did not have any effect on cell proliferation (182). In HepG2 HCC cell line restoration of T-cadherin expression by promoter demethylation reduced cell proliferation (183). In a variety of human HCC cells and HCC cell lines enforced T-cadherin expression induced G (2)/M cell cycle arrest, reduced cell proliferation, anchorage-independent growth in soft agar, increased sensitivity to TNFα-mediated apoptosis and suppressed activity of a crucial oncoprotein c-Jun (184).

In melanoma cells T-cadherin over-expression decreased anchorage-independent growth, migration and invasion and resulted in reduced tumorigenicity *in vivo*, without changing cell proliferation; T-cadherin silencing increased invasive capacity of the cells (210). Expression of T-cadherin in B16F10 melanoma cells which were originally deficient in T-cadherin expression markedly reduced cell proliferation and invasiveness through Matrigel. The percentage of early apoptotic cells and cells in the G_2/M phase of the cell cycle was markedly increased compared with control cells, suggesting G_2/M arrest. (209)

In immortalized keratinocyte cell lines derived from SCC, T-cadherin over-expression reduced cell proliferation and induced a delay in the G (2)/M phase; no effect on cell-cell adhesiveness and cell

motility was detected (214). T-cadherin over-expression also resulted in enhanced cell–matrix adhesiveness, increased expression of functional β1 integrin, suppressed caveolae-mediated endocytosis, and reduced tyrosine-phosphorylation of EGFR in SCC cell line HSC-1 (215).

Table 3 summarizes the above-described attributed functional roles of T-cadherin in cancer. The effects of T-cadherin silencing or overexpression on any given function are sometimes discrepant, probably reflecting multi-functionality of T-cadherin and cell type specific properties of T-cadherin.

Table 3. Effects of T-cadherin *in vitro* and *in vivo*

Assays	T-cadherin over-expression	T-cadherin silencing
Cell proliferation	↓↓↓0↓↓0↓↓	00
Cell invasion	↓↓↓↓↓	↑↓↑↑
Cell migration	↓	↑
Cell-cell adhesion	0	
Cell-matrix adhesion	↑	↑
Cell motility	0	↓
Colony formation in soft-agar	↓↓	
Anchorage-independent growth in soft agar	↓↓	
Apoptosis	↑↑	
Delay in the G_2/M phase	↑↑↑	
Tumorigenicity *in vivo*	↓↓↓	
↓ decrease; ↑ increase; 0 no effect		

1.6.3 T-cadherin and hormone receptors/ receptor tyrosine kinases

Several studies have suggested a possible regulation of T-cadherin expression by hormones and growth factors. It was shown that T-cadherin expression can be regulated by treatment with hormones: in smooth muscle cells, expression of T-cadherin was reduced under treatment with growth factors PDGF-BB, IGF, EGF, and bFGF (229); in cultured liver sinusoidal endothelial cells, expression of T-cadherin was induced by FGF-2 (230); in human osteosarcoma cells, progesterone and EGF increased T-cadherin transcription, and dexamethasone increased total T-cadherin expression (231). In glioblastoma multiforme cells, hepatocyte growth factor stimulation upregulated transcription factor Snail and N-cadherin and suppressed T-cadherin, suggesting a T-cadherin to N-cadherin switch and epithelial-to-mesenchymal transition (204). Hypermethylation of CDH13 was negatively associated with estrogen receptor, progesterone receptor, and HER2/neu

expression in breast cancer (88). Other studies in breast cancer revealed positive association between T-cadherin and HER2/neu expression levels (87, 98). A functional interaction between T-cadherin and EGFR was documented in SCC lines, where EGFR phosphorylation was shown to be reduced by T-cadherin over-expression (215). The data remain controversial, but nevertheless suggests a possible link between T-cadherin expression level and hormone receptors/RTKs.

1.6.4. Concluding statement

There have been a multitude of studies which have examined associations between cancer development and T-cadherin expression at the genetic level. Functional studies have yielded some controversial data. Molecular mechanisms whereby T-cadherin mediates its effects on tumor cell signaling and behavior are poorly understood.

2. THESIS THEMATIC

My thesis addresses cellular and molecular consequences of alterations in T-cadherin expression in squamous cell carcinoma (SCC) and the molecular mechanisms through which T-cadherin regulates EGFR activity in SCC. Ongoing studies address the role of T-cadherin in EGFR/IGF-1R signaling and their cross-talk in prostate cancer.

3. SPECIFIC OBJECTIVES, RESULTS AND CONTRIBUTION

3.1. Focus on examining functional responses to changes in T-cadherin expression in cutaneous SCC cell line A431.

The results have been published.

T-cadherin loss induces an invasive phenotype in human keratinocytes and squamous cell carcinoma (SCC) cells in vitro and is associated with malignant transformation of cutaneous SCC in vivo.

British Journal of Dermatology, 2010. 163. pp353-363.

Pfaff D, Philippova M, Buechner SA, **Maslova K**, Mathys T, Erne P, Resink TJ.

The paper is appended (thesis pp29-39).

I conducted the following experiments:

1. Expression of T-cadherin (WB, ICC) and E-, P- and N-cadherins (WB);
2. Influence of T-cadherin expression on random migration of A431 cells (time-lapse videomicroscopy);
3. Influence of T-cadherin expression on the invasive phenotype of A431 cells in 2D (fluorescence microscopy).

3.2. Focus on examining T-cadherin involvement in regulation of EGFR accessibility to cognate ligand EGF and its downstream signaling in cutaneous SCC cell line A431.

The results have been published.

T-cadherin is an auxiliary negative regulator of EGFR pathway activity in cutaneous squamous cell carcinoma: impact on cell motility.

Journal of Investigative Dermatology, 2012. 132, pp2275–2285

Kyriakakis E[1], **Maslova K[1]**, Philippova M, Pfaff D, Joshi MB, Buechner SA, Erne P, Resink TJ.

[1]*These authors contributed equally to this work.*

The paper is appended (thesis pp40-69)

I conducted the following experiments:

1 Co-localization of T-cadherin and EGFR in lipid raft domains isolated using detergent and non-detergent based methods;
2 Influence of T-cadherin expression on EGF-induced changes in cell retraction and cell motility (time-lapse microscopy);
3 Influence of T-cadherin expression on constitutive integrin β1 activity and EGF-induced integrin β1 activation;
4 Influence of T-cadherin expression on EGF-dependent activation of small Rho GTPases: Rac1 and Cdc42 was measured using pull-down assay, RhoA was determined using the G-Lisa RhoA activation assay kit.

3.3. Focus on examining reciprocity of interaction between EGFR and T-cadherin in cutaneous SCC cell line A431.

The results have been published.

Cross-talk between EGFR and T-cadherin: EGFR activation promotes T-cadherin localization to intercellular contacts.

Cellular Signalling, 2013. 25, pp1044-1053

Kyriakakis E, **Maslova K**, Frachet A, Ferri N, Contini A, Pfaff D, Erne P, Resink TJ, Philippova M.

I conducted the experiment presented in Supplemental Figure S3 (lipid rafts isolation from serum-deprived and EGF-stimulated cells) (thesis pp70-71).

3.4 Ongoing study. Focus on examining auxiliary regulation of EGFR and IGF-1R activation and cross-talk by T-cadherin in prostate cancer cell line DU145.

A possible title of the future manuscript: **Impact of altered T-cadherin expression on cell behavior and EGFR/IGF-1R pathway activity and functions in prostate carcinoma.**

The main results which will be a part of the future manuscript are described (thesis pp72-79).

4. ADDITIONAL RESEARCH PROJECT PARTICIPATION

In the following papers my contribution was restricted to performance of single experiments/protocols. These papers are not included with this dissertation.

4.1. Focus on effects of different levels of expressed T-cadherin on xenograft growth in vivo.

Paradoxical effects of T-cadherin on squamous cell carcinoma: up- and down-regulation increase xenograft growth by distinct mechanisms

The Journal of Pathology, 2011, 225 (4), pp512-524.

Pfaff D, Philippova M, Kyriakakis E, **Maslova K**, Rupp K, Buechner SA, Iezzi G, Spagnoli GC, Erne P, Resink TJ.

4.2. Focus on T-cadherin functional role in vascular endothelial cells.

T-cadherin attenuates insulin-dependent signalling, eNOS activation, and angiogenesis in vascular endothelial cells.

Cardiovascular Research, 2012. 93, pp498-507.

Philippova M, Joshi MB, Pfaff D, Kyriakakis E, **Maslova K**, Erne P, Resink TJ.

BJD
British Journal of Dermatology

T-cadherin loss induces an invasive phenotype in human keratinocytes and squamous cell carcinoma (SCC) cells *in vitro* and is associated with malignant transformation of cutaneous SCC *in vivo*

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None of the authors have any conflict of interest to disclose.

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Summary

Background Cadherins play important roles in controlling keratinocyte growth, differentiation and survival. Atypical glycosylphosphatidylinositol-anchored T-cadherin (T-cad) is highly expressed in the basal keratinocyte layer of skin. The role of T-cad in keratinocyte biology and pathology is unclear.

Objectives To define the role of T-cad in the pathogenesis of cutaneous squamous cell carcinoma (SCC) through gain-of-function and loss-of-function studies *in vitro* and through examination of T-cad expression patterns in human cutaneous SCC specimens in relation to histological classification of degree of tumour differentiation.

Methods *In vitro* studies employed lentiviral-mediated overexpression/silencing of T-cad in normal human keratinocyte (HaCaT) and SCC (A431) cell lines, monolayer and multicellular spheroid culture models, cell morphology analyses and assays of random motility and invasion. Immunohistochemistry was performed on skin specimens from patients with actinic keratosis, Bowen disease or SCC.

Results *In vitro*, silencing of T-cad induced a morphologically elongated and disorganized cell phenotype, increased random motility and markedly enhanced invasive potential. Overexpression of T-cad induced a morphologically spread and compact cell phenotype and blunted invasive potential. *In vivo*, regional loss of T-cad expression was more frequent and prominent in SCC classified as moderately-to-poorly differentiated than in SCC classified as well differentiated. However, in both categories aberrant and/or absence of T-cad expression was associated with histological features of a potentially more malignant and invasive phenotype of cutaneous SCC.

Conclusions T-cad is a controlling determinant of SCC phenotype and invasive behaviour and its loss is associated with the process of malignant transformation from noninvasive to invasive SCC.

Cutaneous squamous cell carcinoma (SCC) is a malignant neoplasm that may arise from the keratinocytes of the epidermis or its appendages. SCC may arise *de novo* or from preceding lesions such as actinic keratosis (AK) or Bowen disease. The majority of primary SCCs are low risk and usually easily treatable, but their potential to recur and metastasize leads to significant morbidity and mortality. Therefore it is important to identify those tumours that are more aggressive.

SCC was originally graded into four histological categories according to the degree of tumour differentiation.[1] Although various classification systems for SCC have since been proposed (reviewed by Cassarino *et al.*[2,3]), no definitive and comprehensive classification according to tumour aggressiveness has been widely accepted. SCC includes many subtypes with diverse clinical behaviours, ranging from indolent to aggressive tumours with significant metastatic potential. Poorly

differentiated SCCs usually exhibit a higher risk of recurrence and metastasis. However, biological behaviour can rarely be predicted by the degree of differentiation. Advancing knowledge of mechanisms regulating or dysregulating keratinocyte proliferation, motility and invasion has given rise to the opportunity of using biomarkers to make a prognosis or predict malignant cell behaviour in SCC.[4] A variety of molecular markers showing promising association with the aggressive, invasive and metastatic behaviour of cutaneous SCC have been identified. These include increased expression of phosphorylated STAT3 (p-STAT3),[5] transcription factor Ets-1,[6] CD44-9v/10v[7] and CD44-6v[8] variants, MMP-9, p53, Mib-1,[9] MMP-2,[10] MMP-1[8] and Ki-67.[11] Decreased expression of the adherens junction cadherin E-cadherin[12–15] and increased expression of the desmosomal junction cadherin desmoglein 2[16] have also been linked to invasive and metastatic potential of SCC.

Another cadherin family member relatively recently identified on keratinocytes is glycosylphosphatidylinositol-anchored T-cadherin (T-cad).[17] In healthy skin, T-cad expression is almost exclusively restricted to the basal keratinocyte layer.[18,19] Dermatopathological investigations on expression of T-cad in keratinocytic dysplasias/neoplasias are scant. T-cad was reported to be downregulated in psoriasis,[18] absent in 90% of SCC samples[20] and, conflictingly, either absent in 75%[21] or upregulated in 100%[19] of basal cell carcinomas (BCCs). While the roles of the classical and desmosomal cadherins have been extensively studied in relation to regulation of keratinocyte behaviour in the progression of benign and malignant skin diseases,[22–27] the function of T-cad in keratinocyte biology and pathobiology has been poorly explored. It has been proposed that the function of T-cadherin in the skin is to regulate keratinocyte proliferation negatively, and inactivation of T-cadherin is, conversely, the cause for keratinocyte hyperproliferation.[18,28] However, a categorical biological function for T-cad as a negative regulator of keratinocyte proliferation does not correlate with immunohistochemical findings that (i) in the healthy epidermis T-cad is strongly expressed in the basal layer keratinocytes,[17,19] which actively proliferate; (ii) T-cad is upregulated in BCC,[19] a hyperproliferative disorder of basaloid cells; and (iii) T-cad is commonly present in AK,[20] an *in situ* SCC also characterized by accentuated keratinocyte proliferation. These discrepancies, together with observations that T-cad overexpression in SCC cell line HSC-1 increased cell–matrix adhesiveness and surface β1 integrin levels and reduced epidermal growth factor receptor phosphorylation,[29] imply that the role of T-cad in SCC is more complicated and may involve other aspects of keratinocyte biology.

There are no studies that have addressed whether T-cad regulates SCC cell motility and invasive capacities, two crucial determinants in tumour malignancy. We aimed to define the role of T-cad in control of malignant transformation in SCC through both gain-of-function and loss-of-function studies *in vitro* and through immunohistochemical analysis of T-cad in lesional tissue. Using human keratinocyte (HaCaT) and epidermoid SCC (A431) cell lines stably transduced with respect to overexpression or deficiency of T-cad, we present data demonstrating for the first time that T-cad expression levels regulate cell spreading, motility and invasion, with loss of T-cad leading to acquisition of a malignant phenotype characterized by increased motility and invasive potential. Furthermore, immunohistochemical analysis of the patterns of T-cad expression in skin specimens from patients with AK (early SCC *in situ*), Bowen disease (SCC *in situ*) and SCC histopathologically classified as well differentiated or moderately-to-poorly differentiated revealed that loss/aberrant T-cad expression in tumours is not a ubiquitous phenomenon but occurs in association with the histological features of malignant transformation from noninvasive to invasive SCC.

Materials and methods

Cell lines and lentiviral vector transduction

A431 (epidermoid carcinoma of skin; ATTC, CRL-1555) and HaCaT cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and penicillin/streptomycin. T-cad overexpression in cells (Tcad+) was achieved using pLVX-puro vector carrying full-length human T-cad cDNA (Takara Bio USA, St Germain-en-Laye, France) with empty pLVX-puro vector as control (E).[19] T-cad-silencing in cells (shTcad) was achieved using Mission pLKO.1-puro vector (Sigma-Aldrich Chemie, Buchs, Switzerland) carrying T-cad short-hairpin RNA (shRNA) (product no. TRCN0000055546; Sigma-Aldrich) or nontarget shRNA (product no. SHC002V; Sigma-Aldrich) as control (shC).[19] Selection was by culture in the presence of puromycin (1 μg mL^{-1}). For some experiments (confocal microscopy) A431 cells were co-transduced with green fluorescent protein (GFP)-expressing pWPXL vector (Tronolab, Lausanne, Switzerland).

Immunofluorescence microscopy

Immunofluorescence techniques have been described in detail previously.[30,31] In experiments involving analysis of migrating cells confluent monolayers were scrape-wounded and cells were allowed to migrate into the wound area. After fixation (4% paraformaldehyde) and permeabilization (0·1% Triton X-100) cells were sequentially incubated with primary goat anti-T-cad IgG (R&D Systems Europe Ltd, Abingdon, U.K.) and secondary donkey antigoat Cy3-labelled IgG. For nonspecific controls, nonimmune goat IgG substituted primary antibodies. Actin cytoskeleton was visualized by staining with 0·5 μg mL^{-1} tetramethyl rhodamine isothiocyanate-conjugated (TRITC)-phalloidin (Sigma-Aldrich). Nuclei were counterstained using Hoechst (Molecular Probes, Leiden, the Netherlands). Coverslips were mounted upside-down on slides using FluorSave™ reagent (Calbiochem, Darmstadt, Germany). Samples were studied under an Olympus BX61 fluorescent microscope (Olympus Switzerland, Volketswil, Switzerland),

and images captured using a digital camera and AnalySIS 5.0 software (Soft Imaging System GmbH, Munich, Germany). Micrographs present typical images.

Immunoblotting

The following antibodies were used for Western blot analysis of whole cell lysates: goat anti-T-cad (R&D Systems), mouse anti-E-, anti-P- and anti-N-cadherin (BD Biosciences, Basel, Switzerland), rabbit anti-β-actin (Santa Cruz Biotechnology Inc., Heidelberg, Germany), rabbit anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Abcam, Cambridge, U.K.). Secondary horseradish peroxidase-conjugated antispecies IgG were from Southern Biotechnology (BioReba AG, Reinach, Switzerland). Amersham ECL (Amersham Biosciences, Little Chalfont, U.K.) was used for detection. Representative blots are shown.

Time-lapse videomicroscopy

Cells were plated at low density (10^4 cells per well) into 24-well plates and filmed at a rate of one frame per 30 min for up to 18 h using an Olympus IX-81 inverted time-lapse microscope equipped with a digital camera within a humidified incubation chamber with 5% CO_2 at 37 °C. Acquired images were processed and analysed using Cell® software (Soft Imaging System GmbH). Each experiment contained two parallel wells for every experimental condition. The migration trajectories of a total number of 200 cells of each type from four randomly selected fields of each well were traced and for each cell the total path length covered over 18 h was calculated.

Transwell migration/invasion assay

Transmigration assays were performed in Costar transwell chambers (8-μm pore size, 6·5-mm diameter, 24-well format) with modifications of published procedures.[32] Inserts were washed with DMEM, coated with 50 μL of Matrigel (BD Biosciences; diluted to 2·5 mg mL^{-1} in DMEM) and incubated for 20 min at 37 °C to allow solidification. Trypsinized suspensions of A431 or HaCaT in DMEM/0·1% FCS were plated (200 μL, 1×10^5 cells per well) onto the Matrigel matrix. The lower chamber contained DMEM/10% FCS as chemoattractant. After 36 h in culture, cells were fixed by placing inserts in 4% paraformaldehyde/phosphate-buffered saline (PBS) for 10 min at room temperature. Inserts were washed with H_2O and stained with 0·5% toluidine blue in 2% Na_2CO_3 for 20 min. After washing with H_2O cells on the upper side of the insert were removed with cotton swabs. Migration and invasion of cells across the matrix to the filter underside was evaluated by microscopic determination (Olympus IX50 and Cell® software (Soft Imaging System GmbH), of stained areas within five visual fields per well and two wells per transductant. Results from each experiment were averaged and the mean of three independent experiments was calculated. Migration capacity was expressed as the area stained by T-cad+-, shC- and shTcad-transduced cells relative to the area stained by E-transduced cells.

Three-dimensional spheroid invasion assay

Spheroids composed of A431 or HaCaT were prepared using the 'hanging drop' method[33] and embedded within collagen 1 (3 mg mL^{-1}; BD Biosciences) or Matrigel (2·5 mg mL^{-1}).[34] Gels were overlaid with 200 μL normal growth medium and incubated for up to 5 days with regular medium changes. Area of invasion was measured morphometrically using AnalySiS 5.0 software (Soft Imaging System). Invasion capacity was calculated as the difference between the circumference of the outer invasion border and the circumference of the original spheroid. All experiments were performed with duplicate wells for each transductant; every spheroid was analysed and the results averaged. At least three independent experiments were performed and the mean of average invasion capacities from all independent experiments was calculated.

Confocal microscopy of A431 spheroids embedded in three-dimensional gels

Spheroids of A431 cells co-transduced with T-cad constructs and GFP were prepared and embedded in collagen I gel in eight-well glass-bottom chamber slides (Lab-Tek™ II; Thermo Fisher Scientific – Nunc GmbH, Langenselbold, Germany). After 48 h samples were rinsed with PBS, fixed in 4% paraformaldehyde for 2 h, washed with PBS, permeabilized with 0·1% Triton X-100 in PBS and stained with 0·5 μg mL^{-1} TRITC-phalloidin (Sigma-Aldrich) followed by counterstaining of nuclei with Hoechst (Molecular Probes). After extensive washes with PBS samples were mounted in Mowiol 4.88 reagent (Calbiochem) and analysed using an LSM-710 laser scanning microscope (Zeiss, Feldbach, Switzerland).

Tissues and immunohistochemistry

Formalin-fixed, paraffin-embedded skin biopsies from 30 patients with SCC, 20 patients with AK and 20 patients with Bowen disease and from a control group consisting of 10 normal skin specimens were studied. All tissue specimens were obtained from patients presenting at local dermatology practices. SCCs were classified as well or moderately-to-poorly differentiated according to a modified Broders grading.[1] The World Health Organization histological classification of keratinocytic skin tumours was used for the histopathological diagnosis of SCC variants.[35] Sections (4 μm) of formalin-fixed, paraffin-embedded tissue were stained for the presence of human T-cad using goat anti-recombinant human T-cad IgG (R&D Systems) as previously described.[19] Any absence of T-cad expression was considered specific as endothelial cells of the dermal blood vessels

and basal layer of the epidermis were always strongly immunoreactive.

Statistical analysis

All *in vitro* experiments were performed on at least three separate occasions, and unless otherwise stated all results are given as mean + SD. Differences were determined using one way ANOVA followed by Bonferroni's post hoc multiple comparison when appropriate using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, U.S.A.). A P value of $< 0{\cdot}05$ was considered significant.

Results

Expression of T-cadherin in normal keratinocytes and squamous cell carcinoma cells

In order to examine the specific functional contribution of T cad in keratinocyte and SCC cell behaviour we established gain of function and loss of function experimental models using the immortalized human keratinocyte cell line HaCaT, which is known to retain all the functional differentiation properties of normal keratinocytes,[36,37] and the long established human epidermal SCC cell line, A431.[38] Forced overexpression of T cad protein for gain of function studies was achieved using lentiviral delivery of full length human T cad cDNA (lanes labelled Tcad+, Fig. 1a). Highly effective knockdown of T cad expression for loss of function studies was achieved using lentiviral delivery of T cad shRNA (lanes labelled shTcad, Fig. 1a). Immunofluorescence microscopy (Fig. 1b) showed that T cad in HaCaT and A431 cells was localized both to cell–cell boundaries and on the cell surface, staining being more weak and diffuse in A431 cells. Visual examination of the immunofluorescence micrographs, acquired at a fixed exposure period, confirm overexpression and knockdown of T cad, and also illustrate that the general pattern of T cad distribution in cell monolayers was not changed by overexpression.

Molecular weights of transduced T cad proteins in HaCaT and A431 cells are the same as those expressed endogenously. The endogenous expression of T cad in control vector transduced HaCaT and A431 cell lines is illustrated in Figure 1a (lanes labelled E and shC). HaCaT cells express predominantly the mature T cad protein (105 kDa band), while A431 express mature and precursor T cad proteins at comparable levels. In A431 the molecular weights of both forms are slightly lower (90 kDa and 120 kDa) than those in HaCaT (105 kDa and 130 kDa). With equivalent loading of whole cell lysates (10 μg per lane) it is evident that HaCaT cells exhibit a higher level of endogenous T cad protein expression than A431. HaCaT also expressed higher levels of E, N and P cadherin (Fig. 1c). Total levels of E, N and P cadherin proteins in HaCaT and A431 cells were not affected by overexpression or knockdown of T cad (Fig. 1c).

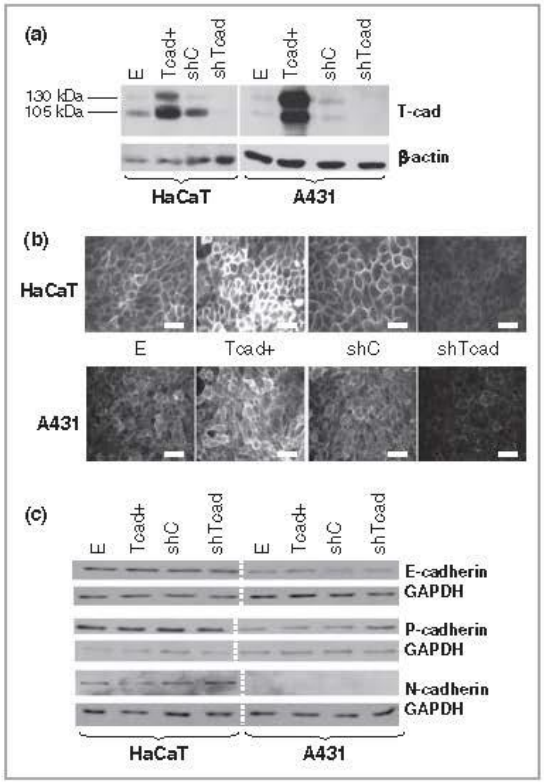

Fig 1. Expression of T-, E-, P- and N-cadherins in HaCaT and A431 cell lines. T-cadherin (T-cad) expression in confluent monolayers of E-, Tcad+-, shC- and shTcad-transduced HaCaT and A431 cells was evaluated by immunoblotting of whole cell lysates (a) and by immunofluorescence microscopy (b). For immunoblots, equivalence of protein loading (10 μg per lane) was checked by probing blots with anti-β-actin antibodies. Blots of A431 cells were exposed longer than those of HaCaT in order to detect their endogenous expression of T-cad protein. Mature (105 kDa) and precursor (130 kDa) T-cad proteins in HaCaT are indicated. Mature and precursor T-cad proteins expressed by A431 have molecular weights of ~90 kDa and ~120 kDa, respectively. For immunofluorescence microscopy anti-T-cad antibody was detected using Cy3-conjugated secondary antibodies. For any given cell line, images were captured with equal exposure (500 ms) to compare truthfully fluorescence intensities of the transductants. Scale bar = 50 μm. Expression of E-, P- and N-cadherin in whole cell lysates of confluent monolayers of transduced HaCaT and A431 cells was evaluated by immunoblotting (c). Blots here were probed with anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal loading control.

Influence of T-cadherin expression on random migration of HaCaT and A431 cells

Random motility rates in low density cultures of HaCaT and A431 on a two dimensional (2D) surface were measured using time lapse videomicroscopy. Motility rates of HaCaT were too low for reliable quantitation and therefore only

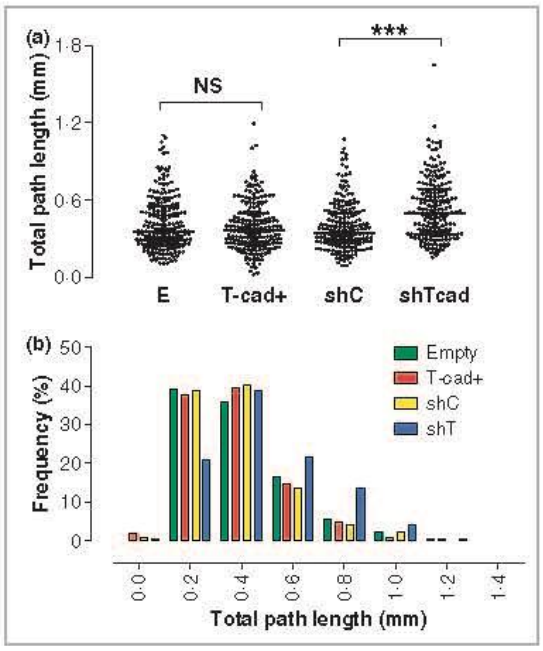

Fig 2. Influence of T-cadherin (T-cad) expression on random migration of A431 cells. Random motility of E-, Tcad+-, shC- and shTcad-transduced A431 seeded at low density was calculated after 18 h. (a) Results from a single experiment are presented. Each data point in the dot plot represents the total path length covered by any given cell over 18 h. Error bars represent the median and interquartile range. ***$p < 0.001$, NS, not significant. (b) The histogram presents a relative frequency distribution analysis of all data acquired in three separate experiments.

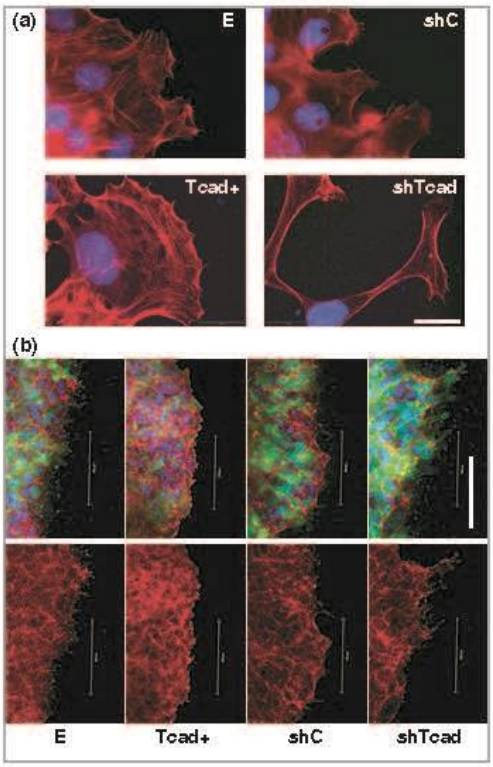

Fig 3. Influence of T-cadherin (T-cad) expression on the invasive phenotype of A431 in 2D and 3D *in vitro* systems. (a) Scrape-wounded monolayers of E-, Tcad+-, shC- and shTcad-transduced A431 were allowed to migrate into the wound area for 18 h. Actin cytoskeleton is stained with tetramethyl rhodamine isothiocyanate-conjugated (TRITC)-phalloidin (red), nuclei are counterstained using Hoechst (blue). Scale bar = 20 μm. (b) Confocal microscopy of spheroids. Spheroids made from E-, Tcad+-, shC- and shTcad-A431 co-transduced with green fluorescent protein (GFP) (green) were embedded in collagen gel and after 48 h fixed, stained with TRITC-phalloidin (red) and Hoechst (blue) and analysed by confocal microscopy. Scale bar = 100 μm. Note the compactness of the invading front of the Tcad+ spheroid as opposed to the protruding disorganized single cells within the invading front of the shTcad spheroid.

data for A431 are presented. The dot plots (Fig. 2a) and frequency distribution histogram (Fig. 2b) demonstrate that silencing of T cad (shTcad) in A431 significantly increased cell motility. ANOVA did not reveal significant differences between T cad+ A431 and control E vector transduced A431.

Influence of T-cadherin expression on invasive phenotype of A431 in two-dimensional and three-dimensional *in vitro* systems

While analysing images acquired with time lapse videomicroscopy for the above described random motility experiments we discerned effects of T cad overexpression or silencing on the shape of migrating cells. We performed wound assays on cover slips and examined morphology of cells migrating into the wound by immunofluorescence analysis of the actin cytoskeleton with TRITC phalloidin (Fig. 3a). T cad overexpressing A431 cells migrating out from the edge of the cell monolayer into the wound area were clearly more flattened and spread than control vector transduced cells and remained in tight contact with neighbouring cells. In stark contrast, migratory T cad silenced A431 cells were elongated, less spread and frequently exhibited lamellipodia and multiple leading edges. Similar observations were made following confocal microscopy analysis of the patterns of invasion from multicellular A431 spheroids embedded in collagen gels (Fig. 3b). For T cad silenced A431 spheroids, cells invading the gel were elongated and appeared to protrude out from the spheroid surface as disorganized single cells, while for T cad overexpressing spheroids, cells preferentially invaded the gel as a tight and compact well organized cell sheet. Control vector transduced spheroids exhibited invasive patterns intermediate between T cad overexpressing and T cad silenced cells.

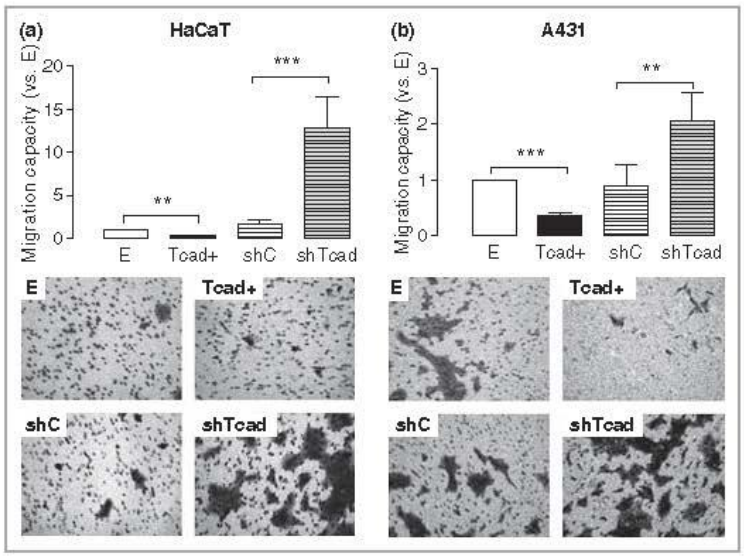

Fig 4. Modulation of T-cadherin (T-cad) expression in HaCaT and A431 cell lines influences their potential to migrate and invade: single-cell suspension transmigration assay. Migration of single-cell suspensions of E-, Tcad+-, shC- and shTcad-transduced HaCaT (a) and A431 (b) cells across transwell inserts containing Matrigel as the matrix barrier was evaluated after a 36-h culture period. Representative images after toluidine-blue staining of insert undersides are shown. Histograms present quantitative analysis of migration capacity as determined after digital computation of stained areas. Migration capacity of transduced HaCaT (a) and A431 (b) cells is expressed relative to that of their respective E-transduced cells. Data are given as mean + SD of three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$.

Influence of T-cadherin expression on directed matrix invasion of HaCaT and A431 cells

Single cell suspensions of Tcad+ , shTcad and control vector transduced cells were plated into transwell inserts containing Matrigel as the matrix barrier and their migration/invasion through to the underside of the insert was determined after 36 h in culture. Images of stained insert undersides in Figure 4 illustrate the markedly greater inherent migration/invasion potential of control transduced A431 cells compared with control transduced HaCaT cells. E (or shC) transduced A431 and HaCaT cells invaded ≈20·4% and ≈2·5% of the total transwell area, respectively. Overexpression of T cad diminished migration/invasion for both A431 and HaCaT cells; compared with their controls, invaded areas represented ≈7% and ≈1% of the total transwell area (Fig. 4). Silencing of T cad increased migration/invasion capacities of A431 and HaCaT; compared with their controls, invaded areas represented ≈42% and ≈31% of the total transwell area (Fig. 4). It is noteworthy that, whereas T cad overexpression inhibited the migration/invasion potential of A431 and HaCaT to a comparable degree (~ 0·3 fold vs. E controls), the effects of T cad silencing on migration/invasion potential were markedly more prominent in HaCaT than in A431 (respectively, ≈12 fold and ≈2 fold differences vs. shC controls).

Influence of T-cadherin expression on invasion properties of spheroid cultures of HaCaT and A431

To analyse the effect of T cad on invasive potential in the context of the multicellular organization of tumours, spheroids composed of transduced cells were embedded within matrices composed of either collagen I or Matrigel. Invasion through the matrices was determined after culture periods of 36 h for A431 spheroids and 4–5 days for HaCaT spheroids. In the case of the latter, the invasion rate was too slow to be quantifiable after a 36 h culture period. For A431 spheroids, and compared with their respective control vector transduced counterparts, overexpression of T cad significantly diminished invasion within collagen and Matrigel matrices whereas silencing of T cad significantly increased invasion (Fig. 5a,c). For HaCaT spheroids embedded in collagen, invasive capacities were unaffected by T cad overexpression and significantly increased by silencing of T cad (Fig. 5b). HaCaT spheroids embedded in Matrigel remained remarkably compact irrespective of the cellular level of T cad expression (Fig. 5d). Invasion was manifest only as the occasional presence of cells 'budding' out from the spheroid (see shTcad image in Fig. 5d). Invasion capacity of HaCaT spheroids in Matrigel, expressed as the percentage of spheroids exhibiting evidence of 'budding' at the outer edges, was unaffected by T cad overexpression but significantly increased by silencing of T cad.

Taken together our *in vitro* observations strongly support that loss of T cad induces an invasive phenotype and invasive behaviour of SCC. The next step was to investigate whether these observations would translate into a clinical setting. To this end we analysed T cad expression in relation to a histological classification of degree of SCC tumour differentiation; specimens from patients with AK (early SCC *in situ*), Bowen disease (SCC *in situ*) and SCCs histopathologically classified as well differentiated or moderately to poorly differentiated were included.

Expression of T-cadherin in actinic keratosis and Bowen disease

Figure 6 presents some representative patterns of immunostaining for T cad in AK and Bowen disease. In all 20 cases of AK, which is considered a low risk precursor or *in situ* SCC,[3]

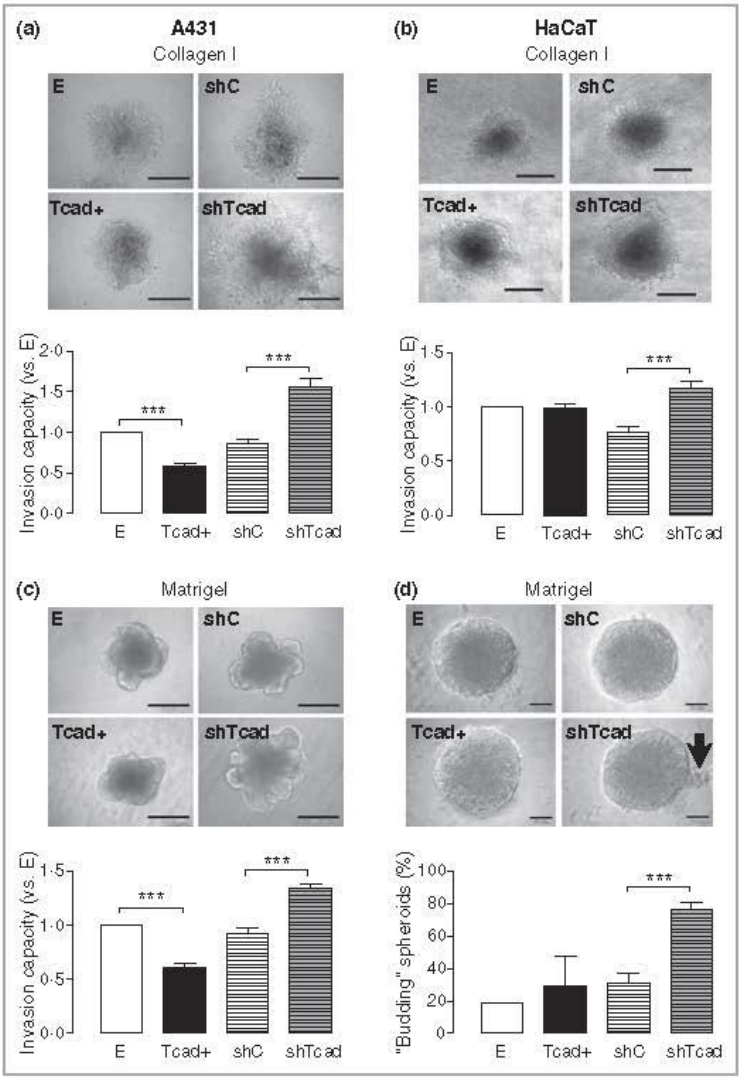

Fig 5. Modulation of T-cadherin (T-cad) expression influences the invasive potential of spheroids composed of A431 cells, but not of spheroids composed of HaCaT cells. Spheroids composed of E-, Tcad+-, shC- and shTcad-transduced A431 (a, c) or HaCaT (b, d) cells were embedded within collagen gel (a, b) or Matrigel (c, d) and invasion into the matrices was evaluated after culture periods of 36 h for A431 and 96–120 h for HaCaT. Representative images are shown (scale bars = 200 μm in a–c and 50 μm in d). Histograms in a–c present quantitative analysis of invasion capacity as determined following morphometric computation of the original spheroid circumference and outer invasion border circumference. Invasion capacity of transduced cells is expressed relative to that of their respective E-transduced cells. For Matrigel-embedded HaCaT-spheroids, which did not exhibit a clear outward invasiveness even after 5 days in culture (d), invasive capacity was evaluated by determining the percentage of spheroids on which outward 'budding' of cells/cell aggregates was evident. Data are given as mean + SD of three independent experiments. ***$P < 0.001$.

T cad was expressed on the atypical basal keratinocytes with pronounced expression on the basal side of the cells, although the levels varied between cases (a–b and c–d in Figure 6 represent two different specimens). In some cases, atypical keratinocytes in the suprabasal layer also expressed T cad (e.g. Fig. 6a,b) although the intensity of immunostaining was usually weaker than in the basal layer. Focal downward prolif erations and buds of atypical squamous cells with marked nuclear pleomorphism showed an absent or weak membra nous expression of T cad (e.g. Fig. 6c,d).

In Bowen disease, considered a high risk and potentially malignant *in situ* SCC,[2] the pattern of T cad expression was more variable than in AK (Fig. 6). In all 20 cases of Bowen disease there was a markedly weakened expression of T cad on the basal cell layer compared with the adjacent normal epidermis [e.g. Fig. 6e; compare regions on either side of the line demarcating normal (n) and diseased (d) tissue]. The atypical keratinocytes in the suprabasal layers of the epidermis were almost negative for T cad.

Expression of T-cadherin in squamous cell carcinoma

Figure 7a–e illustrates representative patterns of immunostain ing for T cad in well differentiated SCC. T cad was expressed by 18 of 20 (90%) of well differentiated primary SCCs arising in AK. Figure 7a illustrates the transition from AK to invasive SCC in which there was a loss of expression in some parts of the tumour. Generally there was a striking, strong membra nous T cad expression on the basaloid cells at the periphery of tumour nests invading the dermis (e.g. Fig. 7b,c). However,

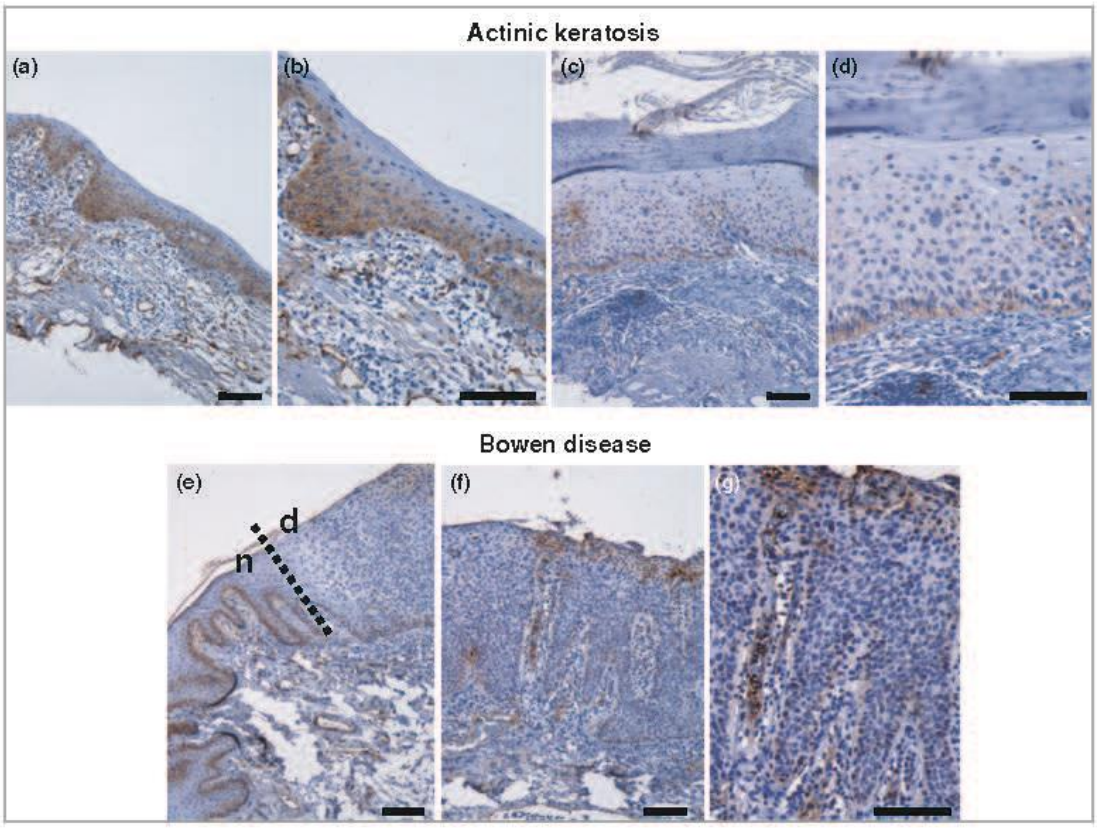

Fig 6. T-cadherin (T-cad) immunostaining in actinic keratosis (AK) and Bowen disease. Immunohistochemical staining for T-cad in skin specimens from two cases of AK (a–b and c–d, respectively) show that it was strongly present on the basal side of basal keratinocytes and, albeit at variable levels (e.g. staining intensity is strong in a–b, but very weak in c–d), in atypical keratinocytes in the suprabasal layer. In Bowen disease (e–g) immunostaining for T-cad was weak on the basal layer and almost negative in the suprabasal layers. Dashed line in (e) demarcates adjacent normal (n) and diseased (d) epidermal regions. Scale bars = 100 μm.

different patterns of T cad expression were observed on the cells forming the central parts of tumours (e.g. Fig. 7b–e). Atypical central squamous cells exhibited either a weak cell membrane and/or a diffuse cytoplasmic staining of T cad, and some areas of tumour nests were even completely immuno negative (e.g. Fig. 7b,d). SCCs exhibiting basaloid cell differ entiation showed a bright staining at the cell borders as well as a weak diffuse cytoplasmic staining for T cad at the centres of tumours (e.g. Fig. 7e).

Figure 7f–k presents representative patterns of T cad immunostaining in moderately to poorly differentiated inva sive SCCs. In four of 10 invasive SCCs there was membranous and cytoplasmic staining for T cad both in the superficial areas of tumours (Fig. 7f) and also on the tumour nests invading the deep dermis (e.g. Fig. 7f–h). In the other six cases of the moderately to poorly differentiated invasive SCCs a total or near total loss of T cad expression was noted (e.g. Fig. 7i,j). Some isolated tumour nests or individual cells showed a weak membranous and cytoplasmic staining for T cad. Acantholytic and adenosquamous SCCs did not express T cad.

Taken together the above observations suggest that loss or aberrant expression of T cad in SCC is variously associated not only with hyperproliferation, but also with the acquisi tion of invasive potential and malignant transformation of SCC.

Discussion

Our gain of function and loss of function studies in A431 demonstrate for the first time that T cad expression levels profoundly affect their phenotype and invasive behaviour both on a 2D surface and within a 3D matrix, thus revealing a mechanism of T cad involvement in SCC progression that is alternative to growth.[18,28] The distinct morphological appear ances of migratory T cad overexpressing and T cad silenced A431 cells in 2D (spread and elongated, respectively) and the nature of the invasive patterns in 3D (compact and dis organized, respectively) indicate that loss of T cad may facili tate signal transduction regulating cytoskeletal and focal adhesion rearrangements, promoting cell motility. This is in

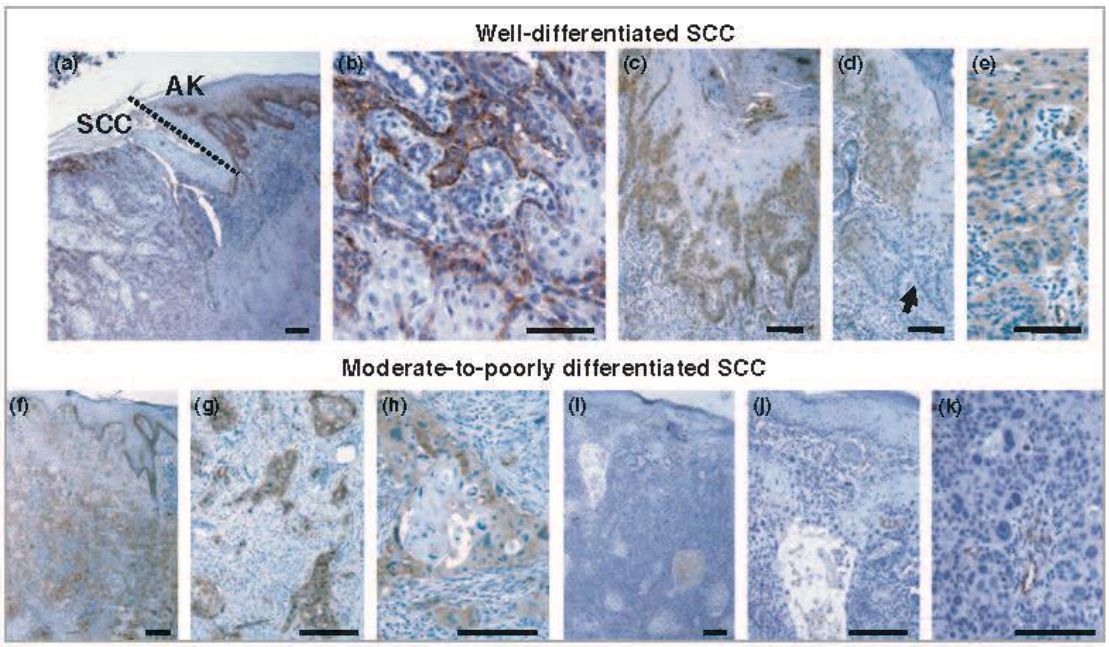

Fig 7. T-cadherin (T-cad) immunostaining in well-differentiated and moderately-to-poorly differentiated squamous cell carcinoma (SCC). (a,b) and (c–e) represent T-cad immunostaining patterns in two different cases of well-differentiated cutaneous SCC. Dashed line in (a) demarcates adjacent epidermal regions presenting with SCC or actinic keratosis (AK). T-cad was generally [but not always, as indicated by arrowhead in (d)] present on basaloid cells at the periphery of invading tumour nests, but was frequently weaker than that on basal keratinocytes in either normal epidermis or zones of AK. Within central regions of the tumours T-cad staining was generally weakened; note the variety of staining patterns (membranous, cytoplasmic or absence) for T-cad in central parts of the tumours. (f–h) and (i,j) represent T-cad immunostaining patterns in two different cases of moderately-to-poorly differentiated cutaneous SCC. One case (f–h, representing four of 10 cases) illustrates the presence of T-cad in superficial tumour regions and on tumour nests invading the deep dermis. The second case (i,j, representing six of 10 cases) illustrates a total or near total loss of T-cad expression. Scale bars = 100 μm.

line with previous observations made by ourselves and other groups that T cad is involved in regulation of migration and guidance of other cell types including endothelial cells,[30,31,34,39] neuronal cells,[40,41] astrocytes[42] and hepatocel lular carcinoma cells.[43]

Important distinctions between A431 and HaCaT cell lines emerged with respect to their invasive potential in 3D (i.e. spheroid model). Firstly, A431 were more invasive than HaCaT spheroids. This may, in part, reflect the inherently lower expres sion of classical adherens junction cadherins and increased migratory potential of A431 cells; these properties together may give rise to a greater overall destabilization of spheroid architec ture and thereby facilitate invasion into the surrounding matrix. Secondly, and in spite of the enormous impact of T cad silenc ing on the transmigration/invasion potential of single cell sus pensions of HaCaT (≈12 fold increase), stimulatory effects of T cad silencing on the invasion capacity of HaCaT spheroids were weak, albeit significant. This might imply that *in vivo* a loss of T cad from keratinocytes within the basal layer is unlikely to be the initiating event in malignant transformation. However, secondarily to initial priming events, which can signal detach ment from neighbouring cells (e.g. dysregulation of integrin mediated cell matrix adhesion or classic cadherin mediated cell–cell adhesion[23,44–46]), a reduction or loss of T cad might have significant consequences for conversion to malignant cancer.

Support that loss of T cad is biologically relevant to malig nant transformation of keratinocytes in SCC can be derived from our immunohistochemical observations that aber rant/loss of T cad expression in SCC tumour samples was not a universal phenomenon as previously suggested,[20] but rather occurs in association with the histological features of malignant transformation from noninvasive to invasive SCC. Regional loss of T cad expression occurred more frequently and to a greater extent in SCC classified as moderately to poorly differentiated than in SCC classified as well differenti ated. However, in both categories aberrant and/or absence of T cad expression was associated with histological features of a potentially more malignant and invasive phenotype of cuta neous SCC. In addition, highly proliferative regions of atypi cal keratinocytes with loss of polarity and nuclear pleomorphism in the lower epidermis of AK showed a loss of T cad expression, which contrasts markedly with the strong expression of T cad of basal keratinocytes showing

only a mild atypia. These findings are suggestive of the occurrence of an initially discrete, but subsequently progressive loss of T-cad from the cell surface during the process of malignant transformation from noninvasive to invasive SCC. High expression of T-cad in AK may explain the low risk of progression (between 0·025% and 16% per year) of AK to an invasive SCC.[47]

Importantly, and in contrast to AK, all 20 cases of Bowen disease investigated in this study exhibited a broadly weakened or absence of T-cad expression. The risk for development of metastatic disease in well-differentiated SCC is very low, accounting for < 0·5% of the cases.[2,3,48,49] Although the majority of SCC arising in AKs shows an indolent behaviour, some tumours may transform to an invasive, more aggressive variant with a high risk for development of metastatic behaviour. This may explain why some (10%) of the well-differentiated SCC examined in this study exhibited loss of T-cad within the deeper zones of the tumour. The patterns of T-cad expression within moderately-to-poorly differentiated SCCs variously encompassed a strong membrane staining, a cytoplasmic staining, or a complete absence. Remarkably, a complete loss of T-cad was found in acantholytic and adenosquamous SCCs, which are considered to be intermediate-risk and high-risk tumours, respectively. However, irrespective of the grade of differentiation, depth of invasion is an important index for metastatic behaviour, and even so-called well-differentiated SCC may metastasize when achieving the critical threshold invasion depth. Although this study did not investigate the direct correlation between lymph node metastasis and loss of T-cad expression, it is possible that T-cad downregulation promotes both invasive and metastatic capacities of SCC.

Our immunohistochemical analysis does not confirm the high frequency of complete absence of T-cad expression in SCC and its precursors as reported in a previous immunohistochemical assessment of T-cad expression in SCC. A loss of T-cad expression in 50% of AK specimens, in 23% of Bowen disease specimens and in 90% of invasive SCC was reported.[20] On the other hand, and in contrast with the reported absence of T-cad protein expression in 90% of invasive SCCs, a loss of heterozygosity and/or aberrant methylation was reported for only 28·5% and 36·8%, respectively, of SCCs, but not in any of the cases of AK and Bowen disease.[20] Therefore, and as considered in a previous study on T-cad expression in BCC, where we found a generally high expression[19] rather than the absent expression described by these authors,[21] discrepancies in immunohistochemical results may relate to antibody sensitivities; antibodies raised against peptides (as used by Takeuchi *et al.*[21]) are known to be generally less sensitive than those raised against polypeptide/holoprotein (Buechner *et al.*[19] and this study), possibly yielding an artificially high negative staining outcome.

In conclusion, this study supports that T-cad is a controlling determinant of the phenotype and invasive behaviour of SCC and that its loss is associated with the process of malignant transformation from noninvasive to invasive SCC.

What's already known about this topic?

- Immunohistochemical studies to date suggest some involvement of T-cadherin (T-cad) in keratinocytic epidermal dysplasias and neoplasias; T-cad was downregulated in psoriasis, lost in invasive cutaneous squamous cell carcinoma (SCC) and, conflictingly, either lost or upregulated in basal cell carcinoma.
- It was proposed that the function of T-cad in the skin is to regulate keratinocyte proliferation negatively, and inactivation of T-cad is, conversely, the cause for keratinocyte hyperproliferation.

What does this study add?

- A mechanism of T-cad involvement in SCC pathogenesis alternative to growth regulation is identified.
- We demonstrate that T-cad expression levels regulate cell spreading, motility and invasion, with loss of T-cad prompting acquisition of a malignant phenotype characterized by increased motility and invasiveness.
- Further, we show that loss of T-cad expression in cutaneous SCC tumours is not ubiquitous but occurs locally in association with the process of malignant transformation from noninvasive to invasive SCC.

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T-Cadherin Is an Auxiliary Negative Regulator of EGFR Pathway Activity in Cutaneous Squamous Cell Carcinoma: Impact on Cell Motility

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Genetic and epigenetic studies in different cancers, including cutaneous carcinomas, have implicated T-cadherin (T-cad) as a tumor suppressor. Immunohistochemical and *in vitro* studies have suggested that T-cad loss promotes incipient invasiveness in cutaneous squamous cell carcinoma (SCC). Molecular mechanisms are unknown. This study found that the main consequence of T-cad silencing in SCC is facilitation of ligand-dependent EGFR activation, whereas T-cad overexpression impedes EGFR activation. Gain- and loss-of-function studies in A431 SCC cells demonstrate T-cad-controlled responsiveness to EGF with respect to pharmacological inhibition of EGFR and to diverse signaling and functional events of the EGFR activation cascade (EGFR phosphorylation, internalization, nuclear translocation, cell retraction/de-adhesion, motility, invasion, integrin β1, and Rho small GTPases such as RhoA, Rac1, and Cdc42 activation). Further, T-cad modulates the EGFR pathway activity by influencing membrane compartmentalization of EGFR; T-cad upregulation promotes retention of EGFR in lipid rafts, whereas T-cad silencing releases EGFR from this compartment, rendering EGFR more accessible to ligand stimulation. This study reveals a mechanism for fine-tuning of EGFR activity in SCC, whereby T-cad represents an auxiliary "negative" regulator of the EGFR pathway, which impacts invasion-associated behavioral responses of SCC to EGF. This action of T-cad in SCC may serve as a paradigm explaining other malignancies displaying concomitant T-cad loss and enhanced EGFR activity.

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INTRODUCTION

Cutaneous squamous cell carcinoma (SCC), the second most common form of nonmelanoma skin cancer, exhibits variable degrees of atypia, invasiveness, and risk of metastasis (Alam and Ratner, 2001). Cellular events participating in transformation from noninvasive to invasive and potentially metastatic SCC are not well understood. Strict orchestration of cell–cell and cell–matrix adhesion interactions is vital to appropriate development and maintenance of all tissues, including the epidermis (Barker and McGrath, 2001). Tumor cells frequently show deregulated cadherin expression and inappropriate switching among family members (Yilmaz and Christofori, 2010). Immunohistochemical analyses of human cutaneous SCC specimens have identified gain of N-cadherin (Nguyen *et al.*, 2011) and desmoglein 2 (Kurzen *et al.*, 2003), and loss of E-cadherin (Lyakhovitsky *et al.*, 2004) and glycosylphosphatidylinositol-anchored T-cadherin (T-cad) (Takeuchi *et al.*, 2002; Pfaff *et al.*, 2010) as candidate participants in the development of incipient invasive SCC.

Knowledge regarding molecular mechanisms whereby T-cad participates in SCC progression is scant. Loss of T-cad expression through allelic loss or aberrant gene methylation in invasive cutaneous SCC was reported (Takeuchi *et al.*, 2002). Analysis of T-cad expression in relation to histological classification of degree of differentiation revealed that T-cad loss in cutaneous SCC was not ubiquitous but occurred locally in association with histological features of a potentially more malignant and invasive phenotype (Pfaff *et al.*, 2010). *In vitro* studies using HSC-1 or A431 SCC cell lines collectively demonstrated that T-cad silencing increased migratory, invasive, and proliferation potential, whereas the converse was true for T-cad overexpression (Mukoyama *et al.*, 2005; Pfaff *et al.*, 2010, 2011). However, in a murine xenograft model, either gain or loss of T-cad in A431 increased tumor expansion *in vivo* (Pfaff *et al.*, 2011). This paradox was attributed to enhanced vascular endothelial cell growth factor–mediated enhancement of angio/lymphangiogenesis for T-cad overexpression, and to enhanced

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Abbreviations: A431, human epidermoid SCC cell line; EMT, epithelial-to-mesenchymal transition; p-, phosphorylated; SCC, squamous cell carcinoma; sT, T-cad-silenced cell; T+, T-cad-overexpressing cell; T-cad, T-cadherin

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EGF/extracellular signal–regulated kinase-mediated proliferation for T-cad silencing.

Potential linkage between EGF and T-cad warrants attention. More than 90% of SCCs express elevated levels of EGFR. EGFR has a crucial role in SCC proliferation, invasion, and metastasis, and gathering evidence suggests that EGFR-directed therapies may be useful adjuncts to surgical treatment for SCC (Fung and Grandis, 2010). Understanding mechanisms underlying enhanced EGFR expression and/or activity is important for optimization of EGFR-directed therapies. This study investigated whether and how T-cad expression impacts invasive and motogenic responses of A431 to EGF. We report that T-cad modulates responsiveness of the EGFR pathway to EGF by influencing membrane compartmentalization of the receptor. Silencing or overexpression of T-cad, respectively, facilitates or impedes ligand-dependent EGFR activation, with similar significance for adhesion, motility, and invasion responses of SCC to EGF.

RESULTS

Ectopic alterations of T-cad expression in A431 cells are not accompanied by changes in markers of EMT but alter sensitivity of cells to EGFR inhibitors

This study used SCC cell line A431 stably transduced with respect to either T-cad overexpression (T+; using pLVX-puro vector carrying human T-cad complementary DNA) or T-cad silencing (sT; using pLKO.1-puro vector carrying T-cad short hairpin RNA), and stably transduced with empty pLVX-puro vector (E) or pLKO.1-puro vector carrying random short hairpin RNA (sT) as the respective controls (Pfaff *et al.*, 2010). Supplementary Figure S1 online shows levels of T-cad protein and transcript expression in the transductants. We previously reported that motile and invasive capacities of T+ and sT were decreased and increased, respectively (Pfaff *et al.*, 2010), but did not investigate mechanisms underlying these phenomena.

The occurrence of epithelial-to-mesenchymal transition (EMT) is commonly associated with migration and invasion of tumor cells (Yilmaz and Christofori, 2010). Immunofluorescence microscopy (Supplementary Figure S2 online), immunoblotting (Pfaff *et al.*, 2010), and quantitative PCR analysis (data not shown) revealed no influence of T-cad expression on either levels or cellular distribution of E-cadherin, P-cadherin, or vimentin. N-cadherin was not detectable with any analytical method applied (data not shown). As T-cad silencing in normal keratinocytes also increases migratory-invasive capacities and fails to influence epithelial and mesenchymal marker levels (Pfaff *et al.*, 2010), loss of T-cad in SCC likely affects migration and invasive behavior by mechanisms different from those typically associated with EMT (Yilmaz and Christofori, 2010).

As an alternative to EMT-based mechanisms, we considered whether effects of T-cad on A431 motile-invasive behavior involve EGFR pathway activity. A431 expresses high levels of EGFR, which, although making cell proliferation less dependent on exogenous EGF (Fan *et al.*, 1994), enhances EGF-induced motogenic responses (Malliri *et al.*, 1998). Assays of three-dimensional–spheroid invasion (Figure 1a) and of transwell migration (Supplementary Figure S3 online) confirmed (Pfaff *et al.*, 2010) decreased and increased invasiveness, respectively, for T+ and sT, and further demonstrated that invasion (Figure 1b) and transmigration (Supplementary Figure S3 online) by T+ was less sensitive than sT to EGFR tyrosine kinase inhibitors gefitinib or lapatinib. These data infer EGFR pathway inhibition in T+, and raise the possibility that T-cad expression levels regulate EGFR-dependent motile-invasive responses in A431.

T-cad expression levels modulate EGFR activity and internalization

To determine whether T-cad expression might influence EGFR activity, we examined indices of EGFR activation at the level of EGFR itself, namely EGFR phosphorylation and internalization (Schlessinger, 2000; Sorkin and Goh, 2009). T-cad overexpression in the SCC cell line HSC-1 was reported to result in constitutive reduction of total and phosphorylated EGFR (p-EGFR) levels (Mukoyama *et al.*, 2007). We found that neither T-cad overexpression nor T-cad silencing in A431 affected constitutive levels of total or p-EGFR (Figure 1c). However, upon EGF stimulation sT exhibited more prompt and amplified EGFR phosphorylation, whereas this was blunted in T+ (Figure 1c). EGF-induced loss of total EGFR occurred faster in sT and was delayed in T+ (Figure 1c). We next applied an immunofluorescence-based method (Francavilla *et al.*, 2009) that allows monitoring of receptor internalization. As expected, EGFR staining at cell–cell contacts was prominent at baseline and weakened upon EGF stimulation in association with increased intracellular staining (Figure 2a, Supplementary Figure S4 online, no acid wash). EGF-induced internalization occurred faster in sT and was delayed in T+ (Figure 2a, Supplementary Figure S4 online, with acid wash). Even at baseline, sT exhibited greater intracellular staining than T+. To obtain biochemical evidence for differences in plasma membrane-to-intracellular translocation of EGFR, we performed immunoblotting using lysates from cells collected without trypsinization (total EGFR) or after trypsinization (internalized EGFR). Intracellular EGFR levels under basal and EGF-stimulated conditions were elevated in sT and lowered in T+ (Figure 2b). Nuclear EGFR and p-EGFR levels were examined by immunoblotting of nuclear extracts. Purity of nuclear extracts was checked by immunoblotting for nuclear marker lamin A/C (positive), plasma membrane markers E-cadherin and ZO-1 (negative), and cytoplasm markers glyceraldehyde-3-phosphate dehydrogenase and Grp78 (negative) (Supplementary Figure S5 online). At baseline, EGFR and p-EGFR levels were higher in sT with a trend toward decrease in T+ (Figure 2c). Upon EGF stimulation, nuclear EGFR and p-EGFR levels increased in all transductants, but the increase was augmented for sT and blunted for T+ (Figure 2c). Thus, T-cad silencing or T-cad overexpression, respectively, facilitates or impedes EGFR activation.

T-cad promotes retention of EGFR in lipid raft domains

Changes in EGFR membrane compartmentalization have a major role in its activation: in the basal state EGFR partitions

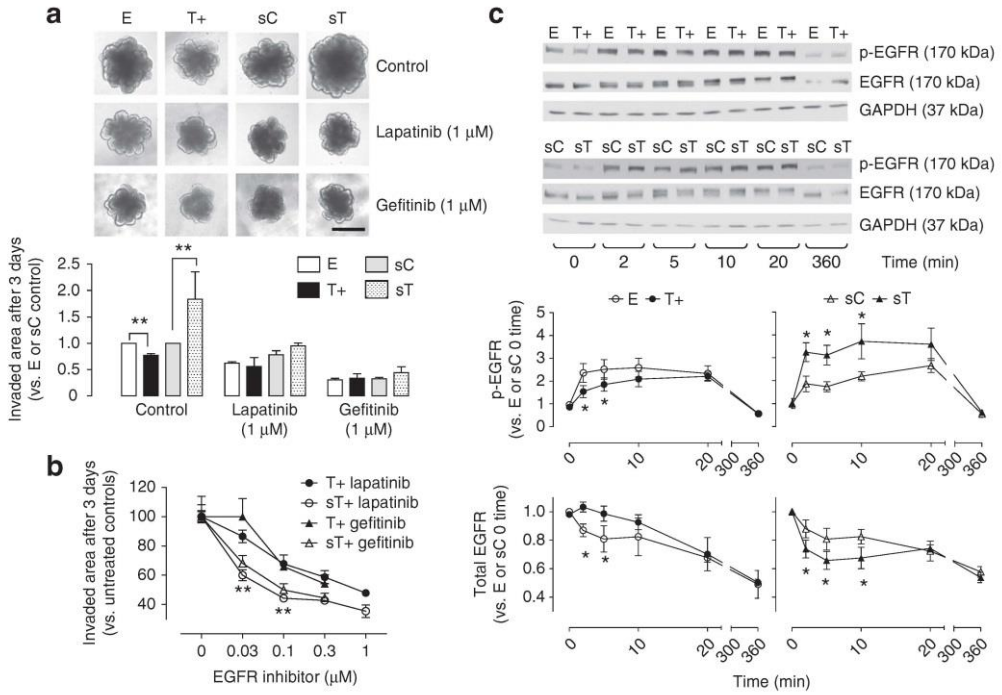

Figure 1. T-cad expression levels in A431 alter sensitivity to EGFR inhibition and EGFR activity. (**a**, **b**) Matrigel-embedded spheroids were cultured for 3 days without (control) or with inclusion of lapatinib or gefitinib (1 μM in **a**, 0–1 μM in **b**). (**a**) Representative images of the spheroids are shown (bar, 200 μm applicable to all images), $^{**}P<0.01$. (**b**) $^{**}P<0.01$ compares T+ and sT for either gefitinib or lapatinib. (**c**) Cells were stimulated with EGF (10 ng ml^{-1}) for the indicated times. Whole-cell lysates were analyzed for p-EGFR and total EGFR by immunoblotting. GAPDH served as internal loading control. Alterations in levels in E or T+ and sC or sT are expressed relative to levels in E and sC controls, respectively. $^{*}P$ at least <0.01. E, empty vector cell; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; p-EGFR, phosphorylated EGFR; sC, non-target short hairpin RNA cell; sT, T-cad-silenced cell; T+, T-cad-overexpressing cell; T-cad, T-cadherin.

into lipid raft domains of the plasma membrane, and sequestration of EGFR within the rafts exerts inhibitory effects on EGFR signaling (Pike, 2005; Balbis and Posner, 2010). As T-cad is also resident within lipid raft domains (Philippova *et al.*, 1998), we considered whether its expression level might influence EGFR localization and/or retention. Confocal microscopy images of empty vector and non-target short hairpin RNA–transduced cells double-stained for EGFR/T-cad depicted similar labeling patterns, with prominent co-staining at cell–cell contacts (Figure 3a). Co-staining signal intensities were enhanced in T+ and negligible in sT (Figure 3a). Isolation of membrane rafts using detergent and non-detergent protocols (Figure 3b and c) revealed higher and lesser co-fractionation of EGFR in T-cad- and caveolin-1-enriched, clathrin-negative raft domains from T+ and sT, respectively. Reciprocal co-immunoprecipitation experiments using a variety of detergent-based lysis buffer conditions (Supplementary information online) failed to reveal co-precipitation of EGFR with T-cad (Supplementary Figure S6 online).

To ensure that our buffer conditions maintain protein–protein interactions that occur within lipid rafts, we probed anti-EGFR immunoprecipitates for the presence of uPAR (Supplementary Figure S6a online), a raft-associated protein (Staubach and Hanisch, 2011) reported to complex with EGFR (Liu *et al.*, 2002; Mazzieri *et al.*, 2006). We have previously demonstrated association of T-cad with Grp78 and integrin-linked kinase in endothelial cells (Joshi *et al.*, 2007; Philippova *et al.*, 2008) but found no such associations in A431 (data not shown), suggesting that T-cad may have different membrane partners in different cell types. However, we reconfirmed formation of T-cad–Grp78 and T-cad IL-8 complexes in endothelial cells (Supplementary Figure S7 online), thus validating our buffer conditions also in the case of T-cad. Thus, EGFR and T-cad co-localize and T-cad expression levels modulate retention of EGFR in raft domains, but EGFR and T-cad appear not to be physically associated.

T-cad expression levels influence EGF-induced changes in cell retraction and motility

Retractile and motile responses to EGF were examined using time-lapse videomicroscopy and morphometric analysis. As expected (Chinkers *et al.*, 1981), EGF induces rapid cell rounding and retraction from the substrate (Figure 4a). sT exhibited more pronounced rounding/retraction, whereas the response was reduced in T+ (Figure 4a). Supplementary videos S1 and S3 online illustrate distinct retraction capacities of sT and T+. TRITC-phalloidin staining of cells

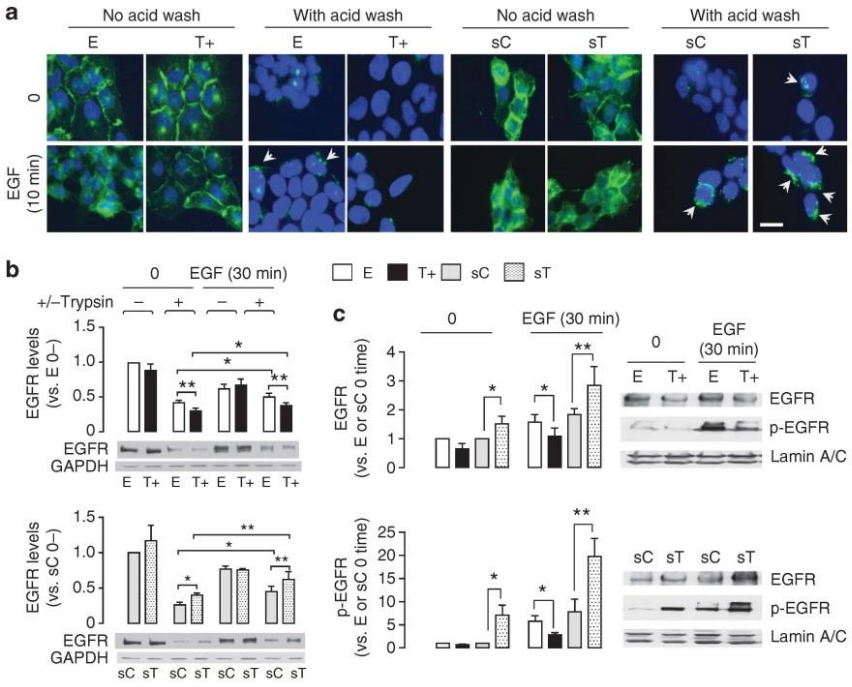

Figure 2. T-cad expression levels modulate EGFR internalization. (**a**) Alexa Fluor 488 IgG was used to visualize internalized EGFR (with acid wash) and total EGFR (no acid wash) after live-cell incubation with anti-EGFR antibodies (bar, 20 μm for all photomicrographs). (**b**) Immunoblotting for EGFR using lysates from cells harvested without trypsinization (total EGFR) or after trypsinization (internalized EGFR). (**c**) Immunoblotting for EGFR in nuclear extracts. EGFR and p-EGFR levels in T+ and sT are expressed relative to those in their respective E and sC controls. *$P<0.05$, **$P<0.01$. E, empty vector cell; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; p-EGFR, phosphorylated EGFR; sC, non-target short hairpin RNA cell; sT, T-cad-silenced cell; T+, T-cad-overexpressing cell; T-cad, T-cadherin.

cultured on gelatin-coated glass coverslips showed partial reattachment to the substratum following prolonged (24 hours) EGF stimulation. sT exhibited a retracted, poorly spread morphology, and extensions were thread-like and spiky, superficially resembling neurite-like protrusions, whereas protrusions in T+ were more broad and flattened (Supplementary Figure S8 online).

To monitor the effects of T-cad on motility responses to EGF, cells were seeded at lower density to minimize cell clustering. EGF was added to the medium (Figure 4b) or presented within a drop of polymerized fibrin (Figure 4c). In both cases, movement speeds were increased and decreased in sT and T+, respectively. With EGF added to the medium, cell movement was associated with dynamic extension and retraction of lamellipodia and filopodia, and, in accordance with absence of a chemotactic gradient, the direction of motility was random (Figure 4b). With fibrin-embedded EGF, cells moved toward the source of EGF and with amoeboid morphodynamics (Figure 4c).

T-cad expression levels influence basal and EGF-induced changes in the activity of integrin β1 and Rho small GTPases

To investigate the molecular mechanisms underlying T-cad effects on cell retraction and motility, we addressed activity levels of integrin β1 and the Rho family of small GTPases, which are crucial regulators of cell adhesion and migration (Hood and Cheresh, 2002; Lozano *et al.*, 2003). HUTS-4 antibodies, which specifically recognize the active conformation of β1 integrins (Monaghan-Benson and McKeown-Longo, 2006), were used to measure the levels of activated integrin β1 on the cell surface. Under normal culture conditions, sT contained higher levels of total integrin β1 and bound less HUTS-4 (Figure 5a). There were trends toward reduced total integrin β1 and increased HUTS-4 binding in T+. Expression of data as an activity ratio (active integrin β1/total integrin β1) revealed a significantly increased integrin β1 activation in T+ and confirmed the decreased integrin β1 activation in sT. EGF-induced integrin β1 activation was increased in T+ but decreased in sT (Figure 5b). EGF-induced activation of Rac1 and Cdc42 was increased in T+ but decreased in sT (Figure 6a and b). Conversely, RhoA activation was decreased in T+ and increased in sT (Figure 6c). EGF-induced phosphorylation of a 20-kDa myosin light chain (MLC_{20}) on threonine 20/serine 19, mediated by ROCK a downstream effector of Rho, was higher in sT (Figure 6d). To confirm the involvement of RhoA in T-cad-dependent effects on cell retraction, sT and T+ were co-transduced with adenovector expressing dominant-negative mutant RhoA (Adv-N19RhoA). Videomicroscopy (Supplementary videos S1, S2, S3 and S4 online) and analysis

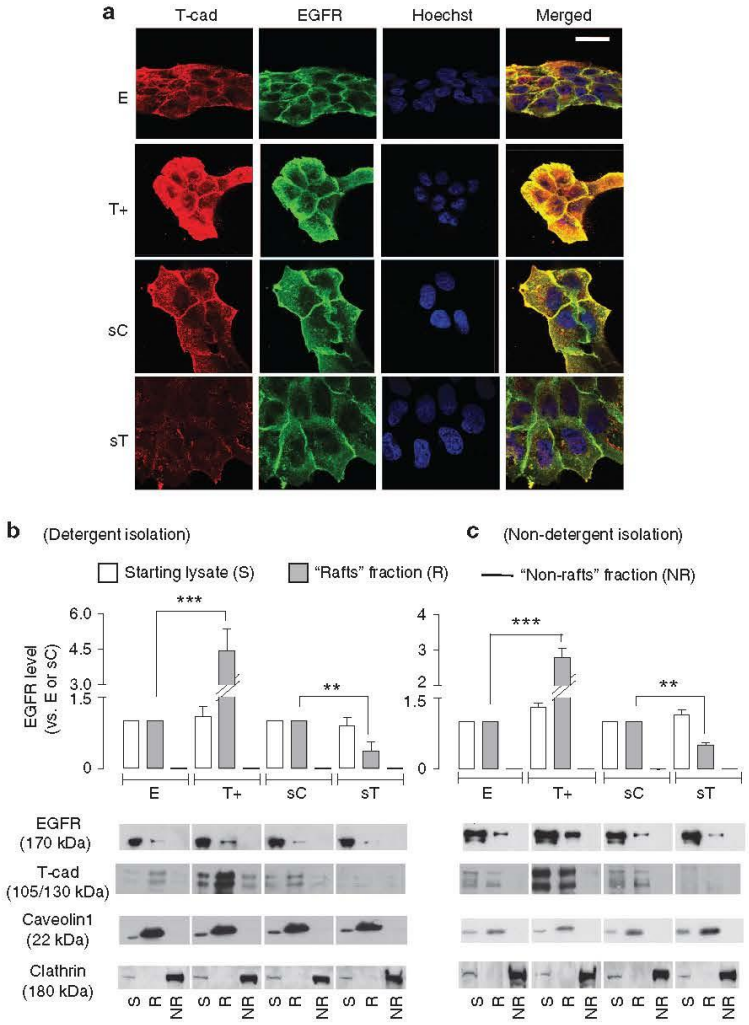

Figure 3. Co-localization of T-cad and EGFR. (**a**) Fixed and permeabilized cells were co-stained for T-cad (red), EGFR (green) with nuclear counterstaining using Hoechst (blue), and analyzed using an LSM-710 laser scanning microscope. Representative images of single and merged stainings are shown (bar, 20 μm applicable to all photomicrographs). Raft domains were isolated and fractionated using detergent- (Triton X-100; **b**) and non-detergent- (Optiprep; **c**) based protocols. Starting lysates (S), raft (R), and bottom non-raft (NR) fractions were analyzed by immunoblotting for EGFR, T-cad, caveolin 1, and clathrin heavy chain. Transduction sets (separated with white lines) were run on the same gel; representative blots are shown. EGFR levels in R isolated from T+ or sT cells are expressed relative to those in their respective E or sC controls. $^{**}P<0.01$, $^{***}P<0.001$. E, empty vector cell; sC, non-target short hairpin RNA cell; sT, T-cad-silenced cell; T+, T-cad-overexpressing cell; T-cad, T-cadherin.

of surface area coverage (Figure 6e) showed that N19RhoA reduced the enhanced retraction of sT.

DISCUSSION

This study has identified T-cad as a negative auxiliary regulator of EGFR pathway activation in SCC, with consequences for functional responses of SCC to EGF. First, spheroid invasion assay revealed that sTs were highly sensitive to inhibition by EGFR inhibitors lapatinib and gefitinib, indicating the contribution of EGFR pathway activity to their increased invasive potential. Second, greater responsiveness to EGF in sT was evident for many signaling and functional events of the EGFR activation cascade (e.g., EGFR phosphorylation, internalization, nuclear translocation, cell retraction/de-adhesion). The converse state of attenuated responsiveness to EGF was true for T+. These data might be interpreted to suggest that when T-cad is present in the cell membrane it acts as a "brake" on EGFR signaling, whereas loss of T-cad facilitates EGFR responsiveness and pathway activation.

We explored mechanisms underlying T-cad-dependent regulation of EGFR activity. Confocal microscopy revealed

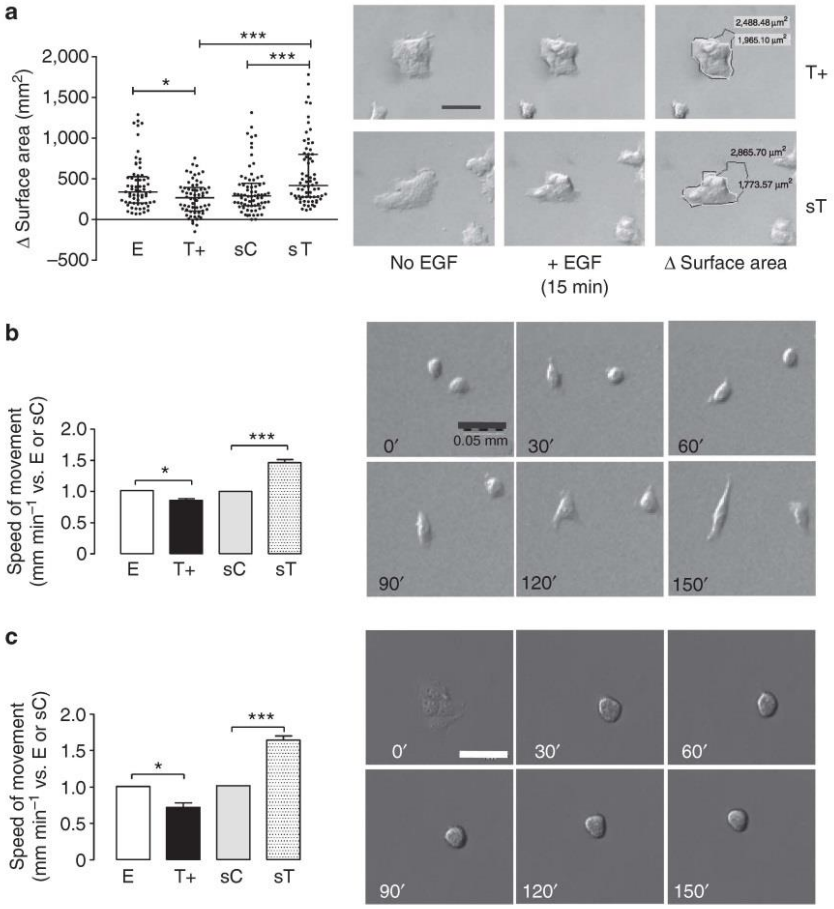

Figure 4. T-cad expression influences EGF-induced changes in cell retraction and motility. (**a**) Change (Δ) in surface area was calculated as the difference in cluster areas before and after EGF stimulation (10 ng ml^{-1}), as illustrated in still-frames acquired from T+ and sT. Dot plots present data for each cluster analyzed, with median and interquartile range; *P<0.05, ***P<0.001. (**b**, **c**) EGF was included in medium (10 ng ml^{-1}; **b**) or presented within a centrally positioned fibrin plug (70 μl containing 200 ng EGF per ml; **c**). Movement speeds (μm min^{-1}) of T+ or sT are expressed relative to speeds of their E (0.44 ± 0.12 for **b**; 0.5 ± 0.12 for **c**) or sC (0.39 ± 0.12 for **b**; 0.43 ± 0.11 for **c**) controls; *P<0.05, ***P<0.001. Images in **b** and **c** are still-frames (bars, 50 μm) extracted from videos of sT. E, empty vector cell; sC, non-target short hairpin RNA cell; sT, T-cad-silenced cell; T+, T-cad-overexpressing cell; T-cad, T-cadherin.

co-localization between T-cad and EGFR. However, based on our failed attempts to co-precipitate T-cad and EGFR from the plasma membrane, direct interaction between the two proteins would seem unlikely. Another possibility is that T-cad influences membrane distribution of EGFR. Several studies (reviewed in Pike, 2005; Balbis and Posner, 2010) have demonstrated that EGFR activity depends on its localization within lipid rafts, which are specialized domains of the plasma membrane that are enriched in sphingolipids and sterols and act as assembly platforms for receptors and their partner signaling molecules. Raft disruption resulted in loss of IgCAM-mediated EGFR activation in the olfactory system (Gibson *et al.*, 2009), suggesting that intactness of lipid raft platforms is prerequisite for EGFR function. On the other hand, evidence also supports the fact that lipid rafts negatively regulate EGFR activity by inhibiting lateral movement of EGFR and decreasing probability of EGFR dimerization. EGFR was inactivated when trapped in raft compartments (Mineo *et al.*, 1999), whereas binding of cognate ligands to EGFR caused its release out of lipid rafts, dimerization, and stimulation of tyrosine kinase activity (Lambert *et al.*, 2006). Raft destruction caused EGF-dependent hyperactivation of Erk1/2 (Furuchi and Anderson, 1998). It also induced spontaneous activation of EGFR even in the absence of ligand by causing its release into small confined domains of the plasma membrane where the receptors dimerize and autophosphorylate their cytoplasmic domains owing to their high density and/or separation from inhibitory molecules that associate with the receptors in rafts (Pike and Casey, 2002; Ringerike *et al.*, 2002; Lambert *et al.*, 2006). Our analysis of EGFR levels in lipid raft domains provides evidence that T-cad upregulation in A431 leads to retention

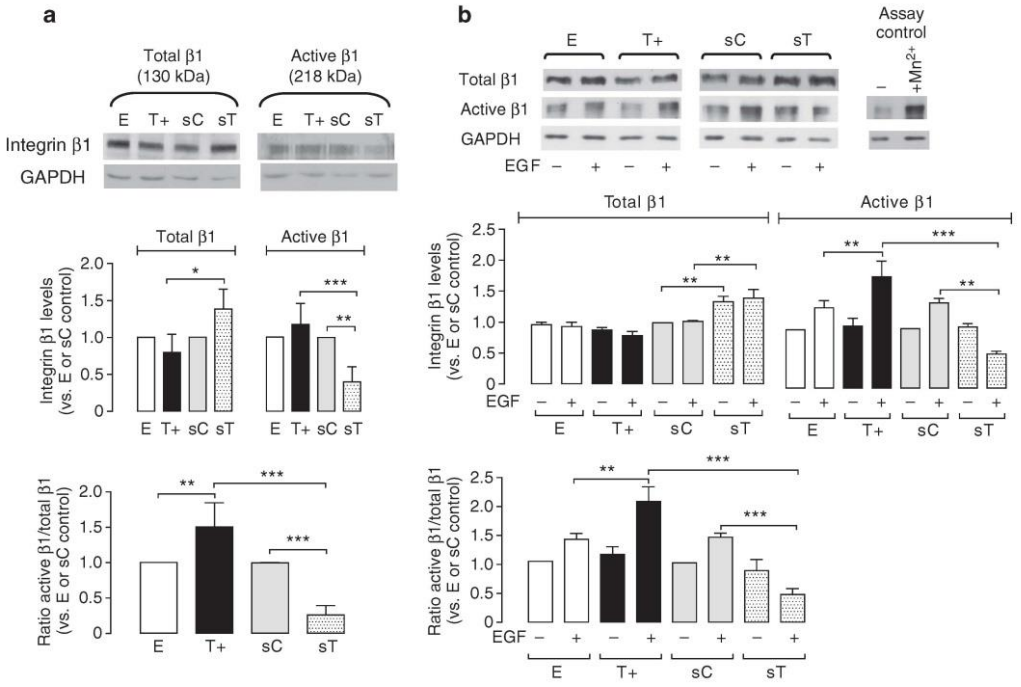

Figure 5. T-cad expression affects constitutive integrin β1 activity and EGF-induced integrin β1 activation. Integrin activation under normal culture conditions (**a**) or following EGF stimulation (10 ng ml^{-1}, 15 minutes; **b**) was measured using the HUTS-4 antibody specific for activated integrin β1. Cells were exposed to $MnCl_2$ (5 mM) as positive control. Typical blots are shown. The higher mass of active integrin β1 (218 kDa) derives from the complex of integrin β1 (130 kDa) and HUTS-4 antibody (88 kDa). Total and active integrin β1 levels are presented individually or as a ratio (active β1/total β1), and changes in T+ or sT are expressed relative to values in their respective E and sC controls; *$P<0.05$, **$P<0.01$, ***$P<0.001$. E, empty vector cell; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; sC, non-target short hairpin RNA cell; sT, T-cad-silenced cell; T+, T-cad-overexpressing cell; T-cad, T-cadherin.

of EGFR in lipid rafts, whereas T-cad silencing releases EGFR from this compartment. This, taken together with the differential EGF responsiveness of T+ (decreased) and sT (increased) would be concordant with the view that lipid raft localization negatively regulates EGFR activity.

As it is T-cad downregulation/loss that occurs *in vivo* in invasive SCC tumor specimens (Takeuchi *et al.*, 2002; Pfaff *et al.*, 2010), a central issue of the study concerns consequences of T-cad loss on EGF-dependent modulation of signaling systems controlling SCC migration and invasion. We found that sT exhibited increased total levels of integrin β1, whereas their integrin β1 activation status (active/total integrin β1 ratio) was decreased. The latter is consistent with increased EGFR activity in sT, which may promote integrin β1 internalization (Mukoyama *et al.*, 2007) and therefore inactivation. Many studies demonstrate associations between regulation of integrin expression and cancer. Changes in integrin patterns allow recognition of variable matrices by cancer cells and modulate signaling and gene expression (Hood and Cheresh, 2002). It is widely held that integrin β1 is required for cancer cell motility and invasion. Therefore, the finding that sT (which are characterized by enhanced invasive potential) display decreased integrin β1 activity was somewhat unexpected. However, SCC cancers are heterogeneous and may exhibit variable alterations in integrin expression and function (Janes and Watt, 2006). Integrin β1 is overexpressed in cervical SCC (Hughes *et al.*, 1994) and vulvar SCC particularly at the invading tumor borders (Brockbank *et al.*, 2005). In contrast, reduced expression of integrin β1 was reported in oral SCC (Jones *et al.*, 1993; Bagutti *et al.*, 1998). Lower expression of integrin β1 at the invasive front of oral SCC correlated with poor prognosis (Ohara *et al.*, 2009). There are also some ambiguities with respect to participation of integrin β1 in EGFR-modulated SCC growth and differentiation. Forced expression of integrin β1 in the suprabasal layer of the epidermis suppresses cell differentiation and cell cycle exit (Carroll *et al.*, 1995), possibly by promoting Erk1/2 activity (Zhu *et al.*, 1999). In contrast, in oral SCC cell line SCC4, which expresses an inactive mutant integrin β1 and is poorly differentiated, the introduction of wild-type integrin β1 stimulates differentiation (Evans *et al.*, 2003), whereas integrin β1 knockdown in A431 fails to affect either proliferation or Erk1/2 activity *in vitro* or *in vivo* (Brockbank *et al.*, 2005).

Decreased basal activity and EGF-dependent inactivation of integrin β1 in sT might be related to two phenomena. First, initiation of SCC motility by EGF involves cell morphology changes including cytoskeleton rearrangement, remodeling of cell–matrix contacts, loss of stress fibers, induction of

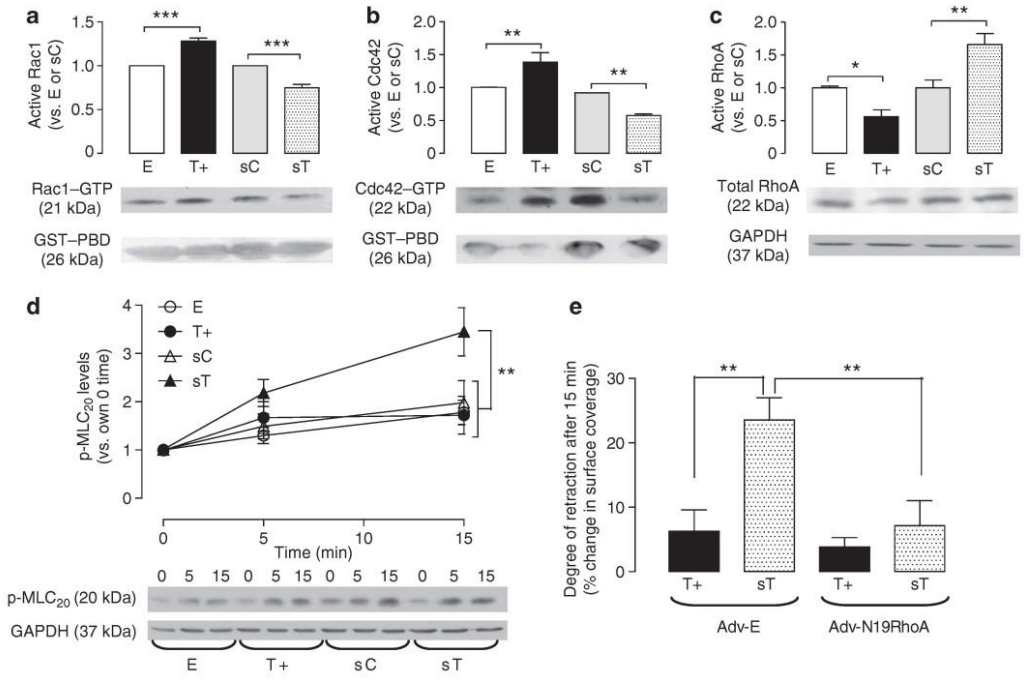

Figure 6. T-cad expression affects EGF-dependent activation of small Rho GTPases and myosin light-chain phosphorylation. (**a**–**c**) Transductants were stimulated with EGF (10 ng ml^{-1}) for 3 minutes (**a**–**c**), 5–15 minutes (**d**), and 15 minutes (**e**). Activation of small GTPases Rac1 (**a**) and Cdc42 (**b**) were measured by pull-down assay. Activation of RhoA (**c**) was measured by G-LISA. Activity levels in T+ or sT are expressed relative to those for E or sC controls. (**d**) Immunoblotting for p-MLC$_{20}$. (**e**) Time-lapse videomicroscopy (1 frame per 5 minutes) was performed on T+ and sT co-transduced with control empty adenovector (Adv-E) or adenovector expressing dominant-negative RhoA (Adv-N19RhoA). Retractile response was measured as percentage change in total surface area occupation. Illustrative videos and legends are provided in Supplementary information online; *P<0.05, **P<0.01, ***P<0.001. E, empty vector; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST-PBD, glutathione *S*-transferase-protein binding domain fusion protein; GTP, guanosine triphosphate; sC, non-target short hairpin RNA cell; sT, T-cad-silenced cell; T+, T-cad-overexpressing cell; T-cad, T-cadherin.

cortical actin polymerization, and cell rounding. Although integrins may be needed for later cell motility, the initial response to EGF is associated with loss of cell–matrix attachments (Hauck *et al.*, 2001). The fact that sT exhibit pronounced EGF-induced integrin β1 inactivation together with rapid disassembly of cell–matrix contacts, cell retraction, and rounding may reflect a generally enhanced EGFR activation status. Decreased basal integrin β1 activity in sT might be explained by residual EGFR activity even in the absence of high concentrations of exogenous EGF, invoking a possibility that *in vivo* T-cad loss may contribute to both ligand-dependent and -independent EGFR activation. Second, decreased integrin β1 activity may be associated with a specific type of cell motility. Single cancer cells may move using at least two distinct migration modes, namely mesenchymal or amoeboid migration (Lammermann and Sixt, 2009; Yamazaki *et al.*, 2009; Parri and Chiarugi, 2010; Yilmaz and Christofori, 2010). Mesenchymal migration is characterized by elongated morphology, clear front-to-rear end polarization, well-formed lamellipodia, dependence on integrins and matrix metalloproteinases, and increased Rac1 signaling. Cells moving in an amoeboid manner exhibit rounded morphology with bleb-like protrusions, decreased adhesion to the surface, integrin inactivation, and concomitant changes in small GTPases activity, namely, Rac1 inhibition and RhoA/ROCK activation. In accordance with literature (Malliri *et al.*, 1998; Sahai, 2007), our videomicroscopy experiments demonstrate that A431 may migrate in an amoeboid manner. Furthermore, the nature of changes in activities of integrin β1 (decreased) and small GTPases (Rac1 decreased, RhoA increased) in sT is precisely that expected for enhanced amoeboid motility (Lammermann and Sixt, 2009; Yamazaki *et al.*, 2009; Parri and Chiarugi, 2010). *In vivo*, such alterations in cell phenotype and integrin-dependent adhesion may contribute to more active migration and chemotaxis, leading to increased tumor cell dissemination (Kren *et al.*, 2007).

Literature evidence supports the existence of a broad spectrum of membrane molecules acting as auxiliary regulators (''co-receptors'') of growth factor receptor activity (Kirkbride *et al.*, 2005). Fine-tuning of receptor activation is often achieved by their rapid redistribution into various plasma membrane domains or internalization, allowing fast association of the receptor with various scaffolds and adaptors or signal attenuation. This enables prompt cell reactions to the changing environment, which is particularly important, for

example, for directed migration during angiogenesis, neurite outgrowth, or tumor front invasion. Examples of such paired regulation are interplay between neural cell adhesion molecule NCAM and fibroblast growth factor receptor FGFR-1 in tumor dissemination (Francavilla *et al.*, 2009; Zecchini *et al.*, 2011), IgCAM-mediated EGFR activation in the developing olfactory pathway (Gibson *et al.*, 2009), and others (Kirkbride *et al.*, 2005). Many of these ''co-receptors'', such as T-cad, are surface adhesion molecules localizing in lipid rafts that link signaling components together and define the mechanism of receptor complex activation, stabilization, or internalization. We invoke T-cad as a further example of a ''co-receptor'' involved in spatial and temporal adjustment of cell sensitivity to growth and chemotactic signals. Specific molecular mechanisms of T-cad-dependent regulation of EGFR compartmentalization and its consequences (e.g., ligand binding, receptor dimerization, interaction with adaptor proteins) are yet to be elucidated.

In conclusion, loss of T-cad in SCC may lead to ligand-dependent EGFR hyperactivation with resultant exaggeration of invasive and aggressive tumor behavior. Epigenetic studies (reviewed in Lochter *et al.*, 1997) have reported silencing of T-cad gene *CDH13* also in melanomas and other malignancies (e.g., breast, pancreatic, lung, ovarian, *inter alia*) in which enhanced EGFR activity frequently occurs. Therefore, it is pertinent to consider whether suppression of EGFR pathway activation might be a common tumor suppressor mechanism of T-cad.

MATERIALS AND METHODS

Cell culture

Lentivector-mediated generation of A431 (epidermoid carcinoma of skin; ATTC (Manassas, VA), CRL-1555) stably transduced with respect to T-cad overexpression (T+) or T-cad deficiency (sT), and with empty vector (E) or non-target short hairpin RNA (sC) cells as respective controls, has been described (Pfaff *et al.*, 2010). Cells were normally cultured in DMEM containing 10% fetal calf serum (DMEM/fetal calf serum) and were serum deprived culture for 40 hours in DMEM containing 0.1% BSA (DMEM/BSA) before EGF stimulation (10 ng ml $^{-1}$).

Invasion and transmigration assays

Spheroid invasion and transwell migration assays were performed as described (Pfaff *et al.*, 2010), with minor modifications (Supplementary information online).

Fluorescence microscopy

Fluorescence microscopy techniques have been described (Philippova *et al.*, 2003, 2005; Pfaff *et al.*, 2010). Staining protocols, antibody sources, and information on microscopes, cameras, and acquisition software are detailed in Supplementary information online.

Time-lapse videomicroscopy analysis of cell surface area and cell motility

Time-lapse videomicroscopy techniques have been detailed (Kyriakakis *et al.*, 2010). Seeding densities, acquisition, and morphometric analytical methods are detailed within Supplementary information online. Images were acquired at a rate of 1 frame per 5 minutes for 15 minutes to record early retraction/spreading or 1 frame per 5 or 10 minutes for 18–24 hours to record motility.

Assays for EGFR internalization and translocation

EGFR internalization was monitored using a previously described live-staining immunofluorescence-based method (Francavilla *et al.*, 2009) with minor modifications (Supplementary information online). Mouse anti-EGFR IgG (Ab-5, clone H11) and anti-mouse Alexa Fluor 488 IgG were used for EGFR detection. Plasma membrane–to-intracellular translocation of EGFR was determined using conventional nuclear fractionation and monolayer trypsinization approaches (Supplementary information online).

Lipid rafts isolation

Lipid rafts were isolated following exact protocols described for detergent (Triton X-100)-based isolation/fractionation through a 5–30% sucrose gradient (Philippova *et al.*, 1998) and non-detergent-based isolation/fractionation through a gradient of 0–20%-Optiprep (Nycomed Pharma AS, Oslo, Norway; Macdonald and Pike, 2005).

Assays for activity of Rho small GTPases

Pull-down assay for quantification of active guanosine triphosphate-bound Rac1 and Cdc42 was performed as described (Ren and Schwartz, 2000), with minor modifications (Supplementary information online). RhoA GTPase activity was determined using the G-LISA RhoA Activation Assay Kit (Cytoskeleton, Denver, CO).

Measurement of the occupied, active conformation of integrin β1

Activated integrin β1 was detected using HUTS-4 mAbs as described (Monaghan-Benson and McKeown-Longo, 2006), with minor modifications (Supplementary information online).

Immunoblotting

SDS-PAGE and immunoblotting protocols have been detailed (Kuzmenko *et al.*, 1998). Primary antibodies against the following proteins were used: T-cad, caveolin 1, clathrin heavy chain, RhoA, Rac1, Cdc42, EGFR, p-EGFR$^{(Tyr1068)}$, p-MLC$_{20}^{(Thr18/Ser19)}$, human integrin β1, glutathione *S*-transferase, ZO-1, lamin A/C, and glyceraldehyde-3-phosphate dehydrogenase. Sources of primary and secondary antibodies are given in Supplementary information online. Representative blots are shown.

Statistical analysis

All experiments were conducted on at least three separate occasions, and unless otherwise stated all results are given as mean ± SD. Differences were determined using one-way repeated measures analysis of variance with Tukey's multiple comparison using the GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA). $P<0.05$ was considered significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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T-cadherin is an auxiliary negative regulator of EGFR pathway activity in cutaneous squamous cell carcinoma: impact on cell motility. (Kyriakakis et al)

SUPPLEMENTAL INFORMATION

SUPPLEMENTAL METHODS

Spheroid invasion assay

Spheroid assay was performed as described (Pfaff *et al.*, 2010). In brief, spheroids composed of A431 transduced cells were prepared using the 'hanging drop' method (Timmins and Nielsen, 2007) and embedded (15-20 spheroids/gel) within Matrigel (3 mg/ml) (Philippova *et al.*, 2006). Gels were overlaid with 200 µl DMEM/FCS without or with inclusion of gefitinib or lapatinib (0 – 1 µM) and incubated for 3 days with regular medium changes. Images of spheroids were taken using an Olympus BX61 fluorescence microscope (Olympus Switzerland, Volketswil, Switzerland), LCPlan Fl 20x Ph1/0.4 NA objective (Olympus), Color-View camera (Olympus) and Cell^P software (Soft Imaging System GmbH, Munich, Germany). Area of invasion was measured morphometrically using AnalySIS 5.0 software (Soft Imaging System). Invasion capacity was calculated as the difference between the circumference of the outer invasion border and the circumference of the original spheroid. All experiments were performed with duplicate wells for each transductant; every spheroid was analyzed and the results averaged. At least three independent experiments were performed and the mean of average invasion capacities from all independent experiments was calculated.

Transwell migration assay

Transwell migration assays were performed in Costar transwell chambers (8 µm pore size, 6.5-mm diameter, 24-well format) following previously published procedures (Lochter *et al.*, 1997) with modification as described (Lochter *et al.*, 1997; Pfaff *et al.*, 2010). Inserts were washed with DMEM, coated with 50 µl of Matrigel (BD Biosciences, Allschwil, Switzerland; diluted to 2 mg/ml in DMEM)

and incubated for 30 min at 37°C to allow solidification. Trypsinized suspensions of A431 in DMEM (+0.1% FCS) were plated (200 µl, 1×10^5 cells per well) onto the Matrigel matrix. The lower chamber contained DMEM plus 10% FCS as chemoattractant. After 36 h in culture, cells were fixed by placing inserts in 4% paraformaldehyde/phosphate-buffered saline (PBS) for 10 min at room temperature. Inserts were washed with H_2O and stained with 0.5% toluidine blue in 2% Na_2CO_3 for 20 min. After washing with H_2O cells on the upper side of the insert were removed with cotton swabs. Migration and invasion of cells across the matrix to the filter underside was evaluated by microscopic determination (Olympus IX50 and Cell® software (Soft Imaging System GmbH), of stained areas within four visual fields per well and two wells per transductant. Results from each experiment were averaged and the mean of three independent experiments was calculated.

Fluorescence microscopy

Cells were cultured on 0.5% gelatine-precoated round 12-mm glass coverslips in 24-well plates.

Analysis of cell phenotype: Cells were plated at $2x10^4$ cells/well, allowed to adhere overnight and subjected to serum-deprivation. Cells (in DMEM/BSA) were further cultured for 24 h in the absence or presence of EGF (10 ng/ml), then fixed with 4% paraformaldehyde (PAF) and permeabilized with 0.1% Triton X-100 in PBS. After staining with 0.5 µg/ml TRITC-conjugated phalloidin (Sigma-Aldrich Chemie, Buchs, Switzerland) and counterstaining of nuclei with Hoechst (Molecular Probes, Leiden, Netherlands) cell phenotype was examined using an Olympus BX61 fluorescence microscope, UPlan Fl 20x Ph1/0.5 NA or UPlan Fl N 40x Ph2/0.75 NA objectives (Olympus), F-View camera and Cell^P software. Immunofluorescence microscopy techniques have been described in detail previously (Pfaff *et al.*, 2010; Philippova *et al.*, 2005; Philippova *et al.*, 2003).

A***nalysis of cadherins and vimentin***: Cells were plated at 2.5 $x10^5$ cells/well, grown to confluence and then either fixed with PAF immediately or scrape-wounded and fixed after a further 24 h culture period. After blocking with PBS containing 3% BSA cells were incubated for 2 h with primary mouse anti-E-cadherin, anti-P-cadherin, anti-N-cadherin IgG (all from BD Biosciences,), or mouse anti-vimentin IgG (Sigma-Aldrich Chemie). Incubation with secondary Cy3-conjugated goat

anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, Europe Ltd., Milan Analytica AG, Rheinfelden, Switzerland) was for 1 h.

Analysis of T-cad and EGFR co-localization: Cells were plated at $1.5x10^4$ cells/well, cultured for 48 h and then fixed with PAF. Blocking and permeabilization of cells was achieved by incubation with PBS containing 1% FCS, 1% BSA and 0.1% saponin. Cells were sequentially incubated (1 h each) with primary goat anti-T-cad IgG (R&D Systems Europe Ltd., Abingdon, UK) followed by secondary donkey Cy3-labelled anti-goat IgG. After extensive washing cells were further sequentially incubated (1 h each) with primary mouse anti-EGFR IgG (Ab-5, clone H11) (Thermo Scientific, Wohlen, Switzerland) followed by rabbit anti-mouse Alexa Fluor® 488 IgG (Invitrogen, Basel, Switzerland). For non-specific stainings, non-immune species IgG replaced the primary antibodies. Counterstaining of nuclei was achieved with Hoechst. Coverslips were mounted upside down on slides using Mowiol 4.88 reagent (Calbiochem, Läufelfingen, Switzerland). Single stained samples were analyzed using an Olympus BX61 fluorescence microscope, UPlan Fl N 40x Ph2/0.75 NA objective, F-View camera and Cell^P software. Double-stained samples were analyzed using an LSM-710 laser scanning microscope (Zeiss, Felbach, Switzerland), Pan Aprochromat 100x oil DIC M27/1.4 NA objective (Zeiss) and ZEN2010 software (Zeiss).

Analysis of EGFR internalization: See below under Assays for EGFR internalization and translocation: *Live-staining assessment of EGFR internalization.*

Assays for EGFR internalization and translocation

Live-staining assessment of EGFR internalization: The method described by Francavilla et al (Francavilla *et al.*, 2009) was slightly modified. Cells were plated at a density of $1.5x10^4$ cells/well onto 0.5% gelatine-precoated glass coverslips in 24-well plates, allowed to adhere overnight and then subjected to serum deprivation. Cells (in DMEM/BSA) were incubated for 50 min on ice with mouse anti-EGFR IgG (Ab-5, clone H11). Then cells were rinsed with DMEM/BSA and exposed to EGF (10ng/ml). At selected time intervals cells were fixed with 4% paraformaldehyde (PAF) for 10 min either immediately, or after a 15 min period of acid-wash on ice (using freshly prepared 0.2 N acetic acid/0.5 M NaCl). Blocking and permeabilization of cells was achieved using PBS containing 1%

FBS, 1% BSA and 0.1% saponin. Anti-mouse Alexa Fluor® 488 IgG was added for 45 min and nuclear staining was performed using Hoechst. Mounted (Mowiol 4.88) coverslips were analyzed using an Olympus BX61 fluorescence microscope, UPlan Fl N 100x oil/1.3 NA objective, F-View camera and Cell^P software.

Trypsinization procotol for estimation of plasma membrane-intracellular translocation of EGFR: For trypsinization protocols cells were seeded into 6-well plates ($2x10^5$ cells/well), allowed to adhere overnight and subjected to serum deprivation before exposure to EGF (10 ng/ml, 30 min). Cultures were rinsed with PBS, incubated for 20 min in the absence or presence of 0.25% trypsin/1mM EDTA, and total EGFR (no trypsin) and intracellular EGFR (+ trypsin) in whole cell lysates were measured by immunoblotting. GAPDH served as internal loading control.

Estimation of EGFR translocation to the nucleus: For nuclear fractionation cells were seeded into T75 flasks ($2x10^6$ cells/flask), allowed to adhere overnight and subjected to serum deprivation. Following EGF stimulation (10 ng/ml, 30 min) nuclear extracts were prepared using Chemicon® Nuclear Extraction Kit (Millipore, Billerica MA, USA) according to their specific protocol. Equal volumes of extracts prepared from identical starting cell numbers were diluted into Laemmli sample buffer and loaded at equal volumes for the immunoblotting of EGFR (170 kDa) and p-EGFR (170 kDa). Nuclear marker lamin A/C (74/65 kD) served as internal loading control. Purity of nuclear extracts was routinely checked by immunoblotting for nuclear marker lamin A/C (positive), plasma membrane markers E-cadherin and ZO-1 (negative), cytoplasm markers GAPDH and tubulin (negative).

Time-lapse videomicroscopy and analysis of retraction and motility responses

Cells were normally plated at $2\text{-}5x10^3$ cells/well into 24-well plates and subjected to serum-deprivation before addition of EGF to medium overlay. In some experiments cells were plated at $5x10^3$ cells/well into 12-well plates containing centrally positioned chambers into which 70 µl aliquots of fibrin (5 mg/ml) gel containing EGF (200 ng/ml) were placed after serum-deprivation protocols. Plates were placed under an Olympus IX-81 inverted time-lapse microscope equipped with a digital camera within a humidified incubation chamber with 5% CO_2 at 37°C, and filming was initiated immediately after

inclusion of EGF (in medium or within fibrin gel). Using a 10x CPlanFl RC1/0.3 NA objective (Olympus), F-View camera (Olympus) and Cell^P software images were captured at a rate of either 1 frame/5 min for up to 15 min to record early retraction/spreading responses or 1 frame/5 or 10 min for up to 18-24 h to record cell motility. Each experiment contained 2 parallel wells for every experimental condition. Acquired images were processed and analyzed for alterations in cell spreading/surface area or speed of movement using AnalySIS 5.0 software. Change in spreading/surface area was calculated as the difference in cluster area before and after 15 min EGF stimulation. For every transductant a total of 74 clusters composed of 2-5 cells were examined. Cell motility was determined by tracing migration trajectories of a total number of 50-100 cells of each transductant; for each cell the total path length and accordingly the speed of movement (μm/min) was calculated.

Detection of activated integrin β1

The method described by Monaghon-Benson and Mckeown-Longo (Monaghan-Benson and McKeown-Longo, 2006) was followed with slight modifications. Cells were plated at a density of $5x10^5$ cells/well into 6-well plates and allowed to adhere overnight. Cells under normal culture conditions were incubated with monoclonal antibodies (clone HUTS-4, Chemicon International Inc. Temecula, CA, USA) at a final concentration of 1 mg/ml for 30 min at 37°C. For EGF-stimulated activation of integrin β1 the cells were serum-deprived, incubated at 37°C with EGF (10 ng/ml) for 15 min, followed by addition of HUTS-4 antibodies and incubation at 4°C for 1 h. As a positive control for integrin β1 activation, $MnCl_2$ was included at a final concentration of 5 mM during the incubations at 37°C. Cells were rinsed with PBS and lysed in immunoblotting (IB) lysis buffer (PBS containing 1% SDS, protease inhibitor cocktail (Sigma-Aldrich), 1mM orthovanadate and 5 mM NaF). For immunblotting procedures whole cell lysates were subjected to electrophoresis under non-reducing for detection of bound HUTS-4 antibody using secondary HRP-conjugated goat anti-mouse IgG and under normal reducing conditions for detection of total β1 integrin.

Pull-down assay for Rac and Cdc42 GTPases

Glutathione *S*-transferase (GST)-PBD for Rac1 and Cdc42 activity assays was bacterially expressed, purified and immobilized on glutathione-agarose as described by Ren and Schwartz (Ren and Schwartz, 2000). Quantification of GTP-bound Rac1 and Cdc42 were performed as described by Ren and Schwartz (Ren and Schwartz, 2000) with slight modifications. Cells at 80% confluence were subjected to serum-deprivation, stimulated for 3 min with EGF (10 ng/ml), washed twice with ice-cold PBS and then scraped into 1 ml lysis buffer (50 mM Tris, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, protease inhibitor cocktail). Lysates were clarified by centrifugation at 13'000 rpm at 4°C for 10 min, protein concentrations determined by Lowry assay and normalized (0.5 mg/ml) in all samples using lysis buffer. After withdrawing an aliquot (100 µl) of the clarified lysates for total protein controls, equal volumes (800 µl) were incubated with GST-PBD (20-30 µg) glutathione-agarose at 4°C for 60 min on a rotation wheel. Incubates were transferred into Pierce Spin Cups-Paper filter tubes (Thermo Scientific), washed twice with washing buffer (50 mM Tris, pH 7.2, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 10 mM $MgCl_2$, protease inhibitor cocktail) and proteins were eluted from the beads by addition of 30 µl of IB lysis buffer. Eluates were analyzed by immunoblotting (IB) for Rac1 and Cdc42 and for GST as the loading control.

ELISA-based assay for RhoA GTPase activity

Cells at 80% confluence were subjected to serum-deprivation and incubated for 3 min without or with EGF (10 ng/ml). Cell lysates were prepared and assayed for RhoA GTPase activity using a commercially available G-LISA RhoA Activation Assay Kit (Cytoskeleton, Inc, Denver, CO). Exact protocols of the manufacturer were followed.

Immunoblotting

SDS-PAGE and immunoblotting procedures have been described before (Kuzmenko *et al.*, 1998). Immunoblotting was performed on (1) whole cell lysates, (2) "lipid raft" fractions, (3) pull-down eluates and (4) nuclear extracts. Protein concentrations in whole cell lysates, pull-down eluates and

"lipid raft" fractions were determined using the Lowry method and samples (diluted into Laemmli sample buffer) were loaded at 15 µg per lane. For nuclear extracts equal volumes of extracts prepared from identical starting cell numbers were diluted into Laemmli sample buffer and loaded at equal volumes. The following primary antibodies were used: goat anti-T-cad (R&D), goat anti-GAPDH (Abcam, Cambridge, UK), anti-E-cadherin, anti-caveolin 1, anti-clathrin heavy chain, anti-Rac1 and anti-Cdc42 (all from BD Biosciences, Allschwil, Switzerland), mouse anti-EGFR (Ab-5, clone H11) (Thermo Scientific), mouse anti-p-EGFR$^{(Tyr1068)}$ and anti-p-MLC$_{20}$$^{(Thr18/Ser19)}$ (Cell Signaling, Allschwil, Switzerland), mouse anti-human integrin β1 (Chemicon International Inc.), anti-RhoA (Millipore), mouse anti-GST (Sigma-Aldrich Chemie) rabbit anti-ZO-1 (Zymed, San Fransisco, USA) and mouse anti-lamin A/C (LaZ-1) (kind gift of Prof. Harald Herrmann, Department of Molecular Genetics, German Cancer Research Center, Heidelberg, Germany). Secondary HRP-conjugated goat anti-mouse IgG or anti-rabbit IgG (Southern Biotechnology, BioReba AG, Reinach, Switzerland) or donkey anti-goat IgG (Santa Cruz Biotechnology, Heidelberg, Germany) together with Amersham ECL (Amersham Biosciences, Little Chalfont, UK) were used for detection of immunoreactive proteins. Scanned images of autoradiograms were analyzed using AIDA Image or Scion (NIH) Image software.

Immunoprecipitation

Cells were grown in 10 cm dishes to 80% confluence. Immunoprecipitation protocols have been exactly described before (Philippova *et al.*, 2008). Here we used three different lysis buffers considered suitable for retaining protein-protein interactions in raft domains: Triton X-114 buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 5 mM $CaCl_2$, 1% Triton X-114) either without or with inclusion of 0.2% SDS, and NP-40 buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40). All buffers were supplemented with Complete Mini protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). For immunoprecipitation, mouse anti-c-*myc* IgG (Clontech/Takara Bio Europe, Saint-Germain-en-Laye, France), anti-EGFR (Ab5 clone H11, Thermo Scientific), anti-ILK (clone 65.1, Sigma) and non-immune mouse IgG (Sigma-Aldrich Chemie) were used (10 µg/dish). The following primary antibodies were used for immunoblot analysis: goat anti-T-cad (R&D), mouse anti c-*myc* (Clontech), Grp78 (BD Biosciences) and uPAR (American Diagnostica GmbH, Pfungstadt,

Germany), rabbit anti-EGFR (Cell Signaling) and anti-ILK (Merck Millipore AG, Zug, Switzerlann). Appropriate species HRP-conjugated light chain specific IgGs (Jackson Immunoresearch, Milan Analytica, Rheinfelden, Switzerland) were used for ECL detection of immunoprecipitated proteins.

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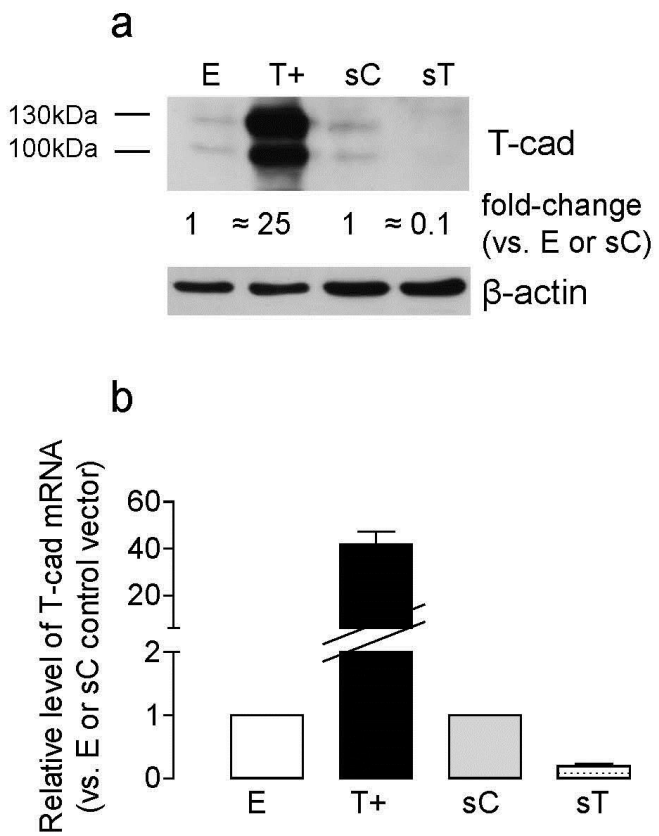
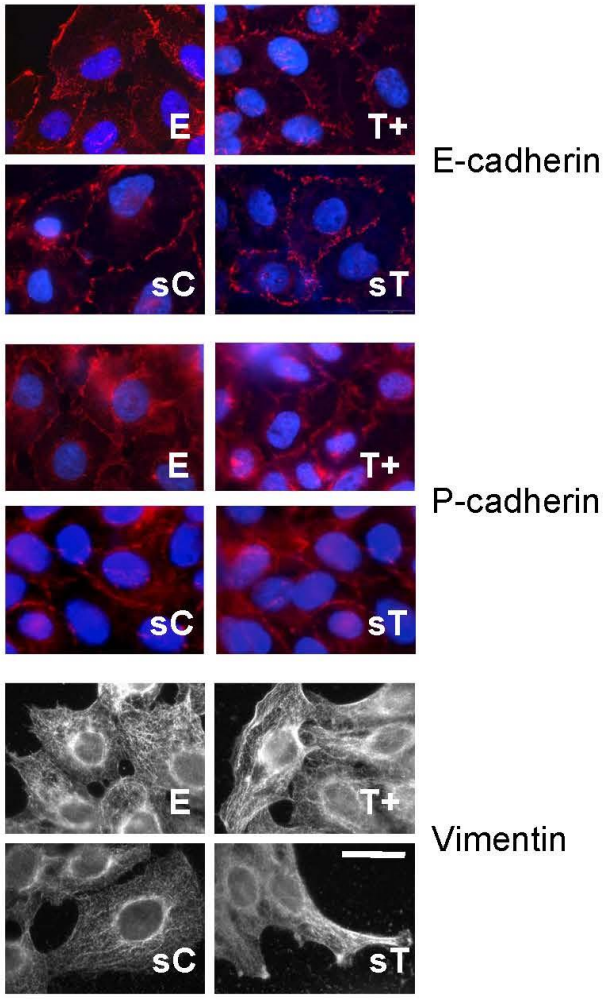
SUPPLEMENTAL DATA

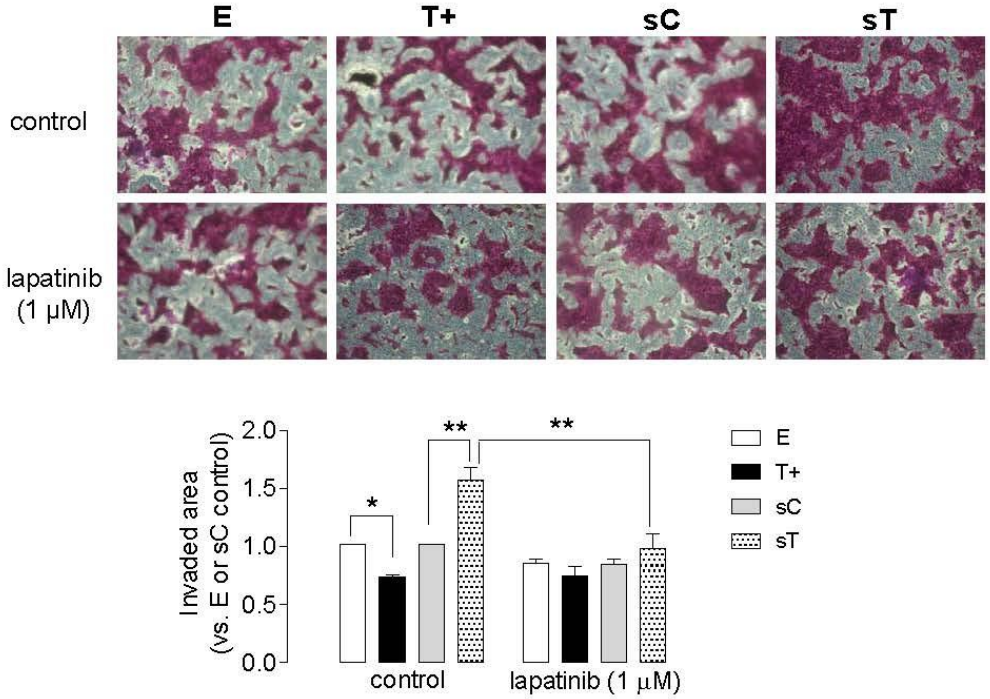


Supplemental Figure S1. T-cad protein and transcript expression in stable A431 T-cad transductants.

A431 were stably transduced with respect to T-cad-overexpression (T+) or T-cad-silencing (sT) and their respective empty vector- (E) or non-target shRNA (sC) controls using lentiviral vectors. (**a**) Whole cell lysates were analysed for T-cad protein (and β-actin as internal loading control) by immunoblotting. (**b**) T-cad transcript expression was determined by qPCR.



Supplemental Figure S2. T-cad expression does not change markers of EMT. A431 transductants were seeded at 2.5 x10^5 cells/well, grown to confluence and then either fixed with PAF immediately or scrape-wounded and fixed after a further 24 h culture period. Cultures were stained for E-cadherin or P-cadherin (cells at confluence) and for vimentin (cells at the healing edge of scrape-wounded cultures). The scale bar (20 μm) in the sT vimentin image is applicable to all photomicrographs.



Supplemental Figure S3. T-cad expression alters transwell migration and sensitivity to EGFR inhibition. Migration of single cell suspensions of A431 transductants across transwell inserts containing Matrigel as the matrix barrier was evaluated after a 36 h culture period without (control) or with inclusion of lapatinib (1 μM). Representative images after toluidine-blue staining of insert undersides are shown. Histograms present digital computation of invaded areas. Data for E or T+ and sC or sT are expressed relative to their respective E and sC under control conditions which were arbitrarily set as 1. Data are given as mean ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$.

Figure S4a

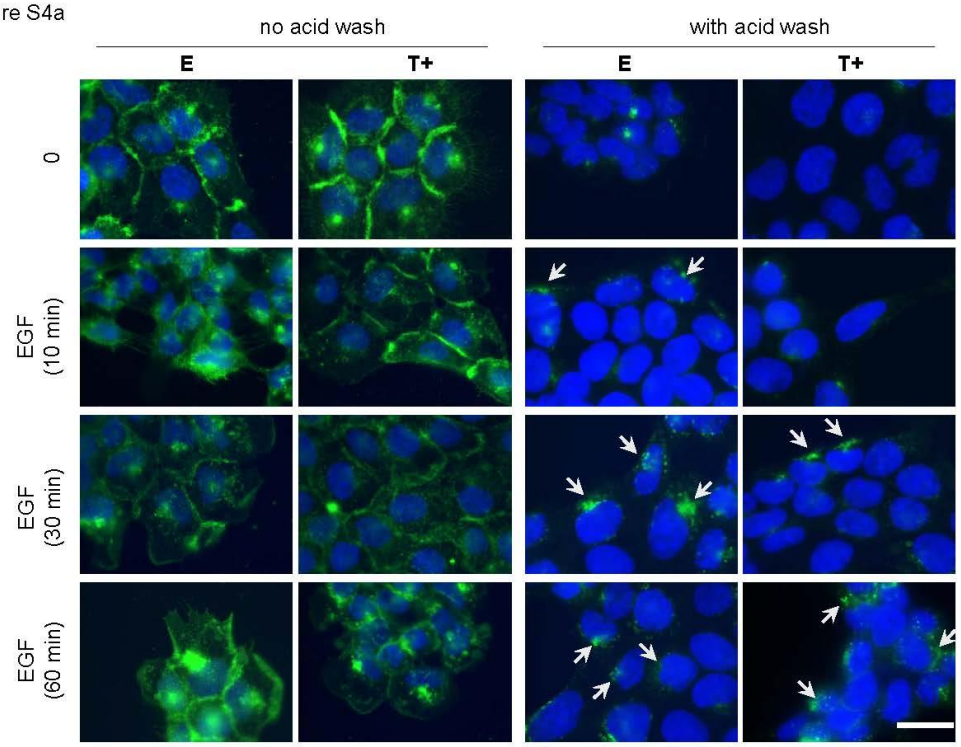
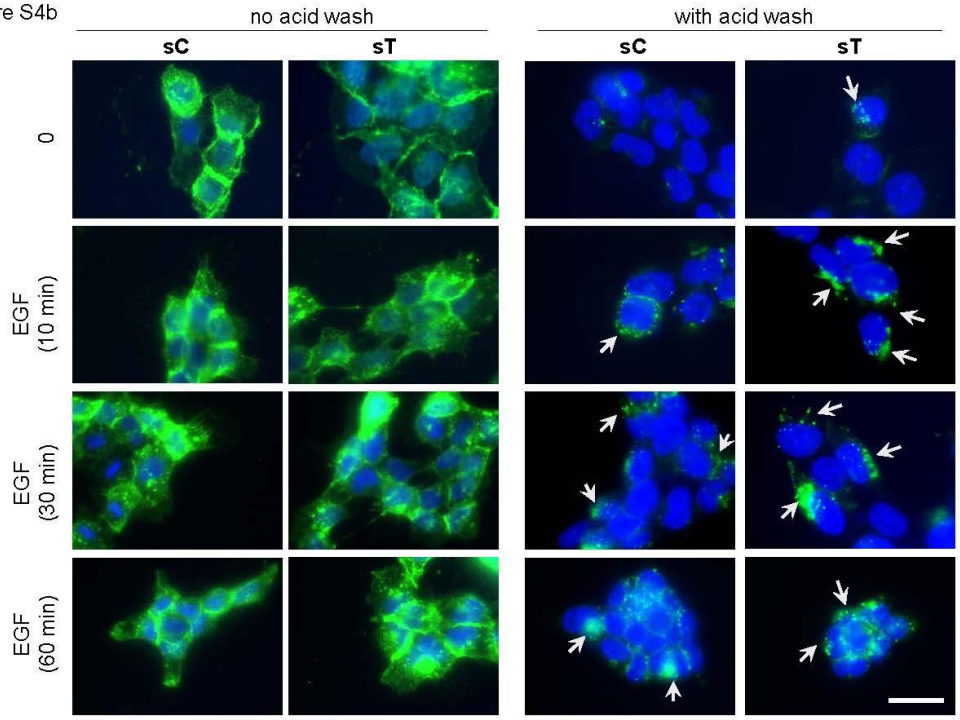
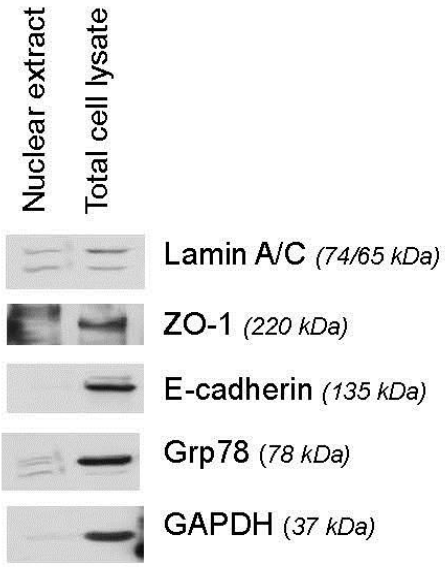

no acid wash
with acid wash
E
T+
E
T+
0
EGF (10 min)
EGF (30 min)
EGF (60 min)

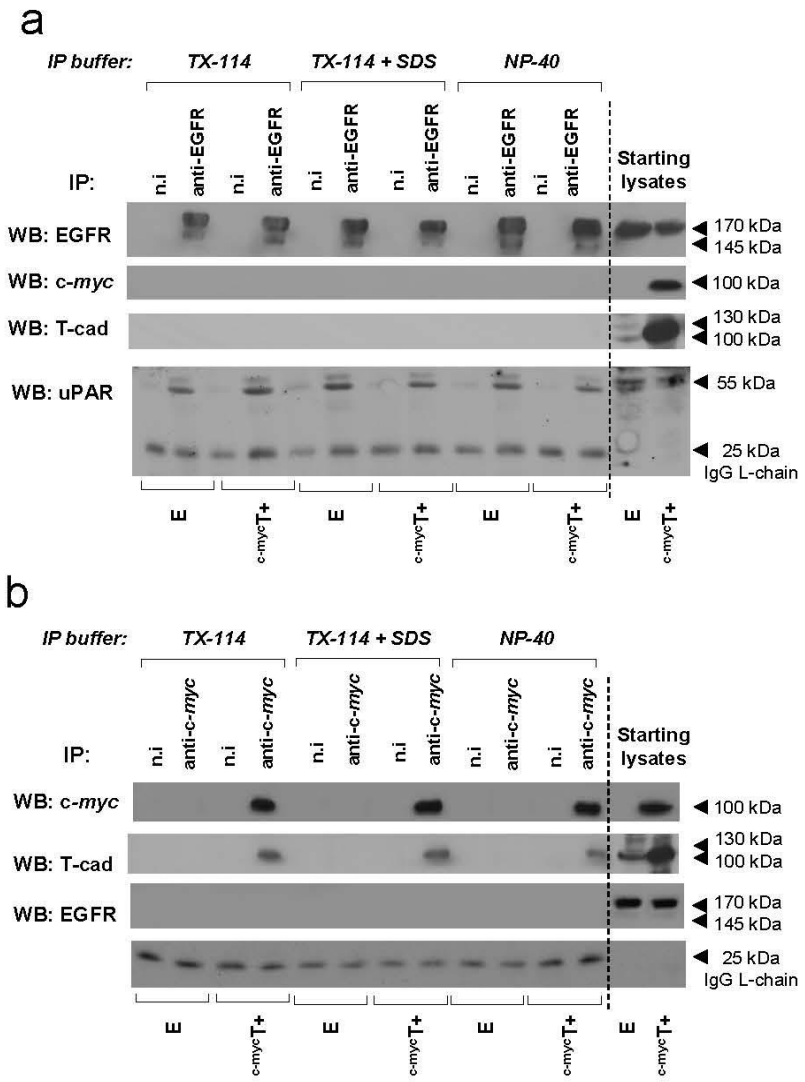
Figure S4b



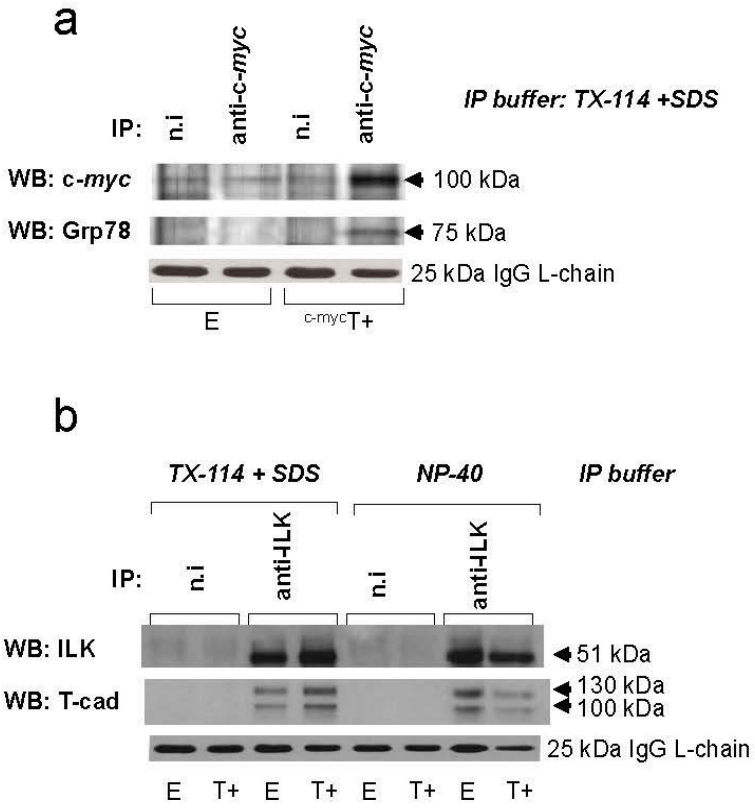
Supplemental Figure S4. T-cad expression levels modulate EGFR internalization. Alexa Fluor® 488 IgG was used to visualize internalized EGFR (with acid wash) and total EGFR (no acid wash) after live-cell incubation of E and T+ transductants (**a**) or sC and sT transductants (**b**) with anti-EGFR antibodies and stimulation with EGF (10 ng/ml). Scale bar, 20 µm for all photomicrographs.



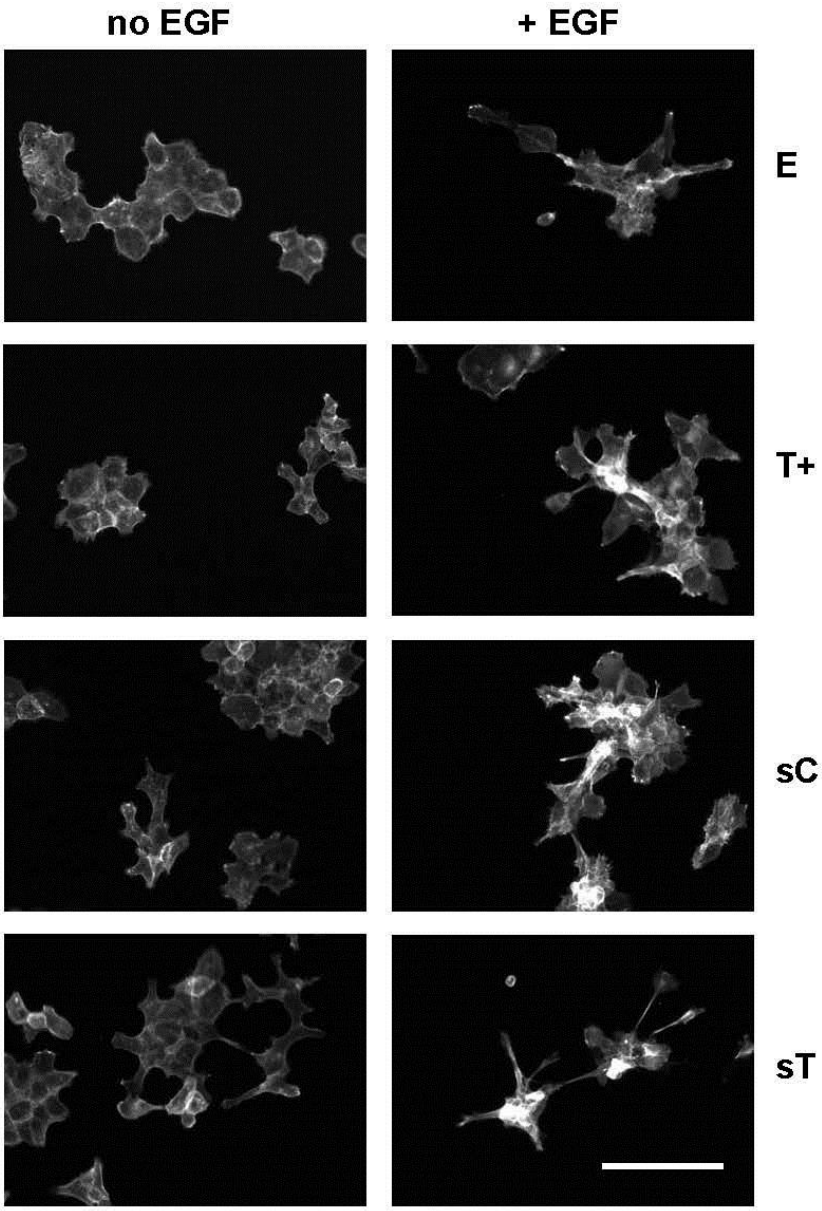
Supplemental Figure S5. Control for purity of nuclear extracts. Nuclear extracts prepared from the transductants using the Chemicon® Nuclear Extraction Kit were checked for purity by immunoblotting for nuclear marker lamin A/C (positive), plasma membrane markers ZO-1 and E-cadherin (negative), cytoplasm markers Grp78 and GAPDH (negative). Presence of all these proteins in total starting cell lysates is shown for comparison. Molecular masses of proteins are indicated. The blots shown here were obtained using T+ cells.



Supplemental Figure S6. T-cad and EGFR fail to co-precipitate. Immunoprecipitation (IP) was performed on A431 stably transduced with E (E) or *c-myc*-tagged T-cad ($^{c\text{-}myc}$T+) lentivectors. Mouse anti-c-*myc* IgG (**a**) anti-EGFR IgG (**b**) or non-immune mouse IgG (n.i.) (**a, b**) were used for IP. IP protocols used Triton X-114 buffer without or with inclusion of SDS or NP-40 buffer (components specified under "Immunoprecipitation" in Supplemental Methods). Goat anti-T-cad, rabbit anti-EGFR and mouse anti-c-*myc* and uPAR antibodies were used for immunoblot analysis (WB). Light chain specific HRP-conjugated IgG were used for ECL detection; the signal at 25 kDa serves as immunoprecipitate loading control.



Supplemental Figure S7. Validation of lysis buffers used for T-cad co-IP experiments. Immunoprecipitation (IP) was performed on endothelial cells transduced with control adenovector (E) or adenovectors expressing *c-myc*-tagged T-cad ($^{c\text{-}myc}$T+) or native T-cad (T+). Triton X-114 buffer with inclusion of SDS or NP-40 buffers were used for cell lysis. Mouse anti-c-*myc* (**a**), anti-ILK (**b**) and non-immune mouse (n.i.) IgG were used for IP. Mouse anti-c-*myc* and goat anti-Grp78 antibodies (**a**) or goat anti-T-cad and rabbit anti-ILK antibodies were used for immunoblot analysis (WB) of immunoprecipitates. Light chain specific HRP-conjugated IgG were used for ECL detection and the signal at 25 kDa serves as immunoprecipitate loading control.



Supplemental Figure S8. T-cad expression influences EGF-induced changes in cell morphology. Serum-deprived transductants ($2x10^4$ cells/well onto 0.5% gelatin-precoated round 12-mm glass coverslips in 24-well plates) were cultured for 24 h in the absence or presence of EGF (10 ng/ml). Actin filaments were stained with TRITC-conjugated phalloidin after fixation and permeabilization. The scale bar (50 μm) in the sT +EGF is applicable to all photomicrographs.

Supplemental Video S1 ***(Video of sT-Adv-E)*** **T-cad silencing in A431 cells enhances rounding/retraction responses to EGF.**

sT co-transduced with empty adenovector were serum-deprived and exposed to EGF (10 ng/ml). Images were acquired at 5 min intervals for 18 h. Bar, 200 μm. The video was used for the data in Fig. 6e. A comparison of this video with Video S3 (Video of T-Adv-E) illustrates the distinct retraction capacities of sT and T+ cells.

Supplemental Video S2 ***(Video of sT-Adv-N19RhoA)*** **Expression of dominant negative RhoA mutant in T-cad silenced A431 eliminates their enhanced rounding/retraction response to EGF.**

sT co-transduced with dominant negative RhoA mutant (N19RhoA) were serum-deprived and exposed to EGF (10 ng/ml). Images were acquired at 5 min intervals for 18 h. Bar, 200 μm. The video was used for the data in Fig. 6e. This video should be compared with Video S1 (Video of sT-Adv-E).

Supplemental Video S3 ***(Video of T-Adv-E)*** **T-cad overexpression in A431 cells reduces rounding/retraction responses to EGF.**

T+ co-transduced with empty adenovector were serum-deprived and exposed to EGF (10 ng/ml). Images were acquired at 5 min intervals for 18 h. Bar, 200 μm. The video was used for the data in Fig. 6e.

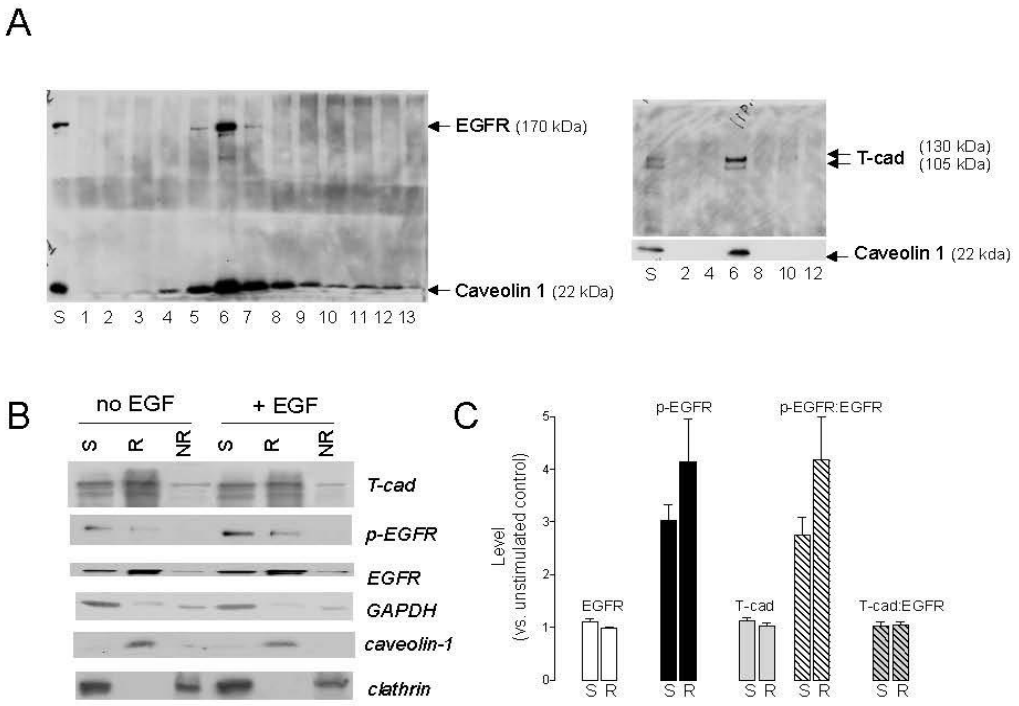
Supplemental Video S4 ***(Video of T-Adv-N19RhoA)*** **Expression of dominant negative RhoA mutant in T-cad overexpressing A431 does not markedly worsen their rounding/retraction response to EGF.**

T+ co-transduced with dominant negative RhoA mutant (N19RhoA) were serum-deprived and exposed to EGF (10 ng/ml). Images were acquired at 5 min intervals for 18 h. Bar, 200 μm. The video was used for the data in Fig. 6e.

3.3. Cross-talk between EGFR and T-cadherin: EGFR activation promotes T-cadherin localization to intercellular contacts.

As a consequence of our findings that T-cadherin can regulate EGFR activity we investigated whether EGFR might exert reciprocal effects on T-cadherin. We found that EGFR phosphorylation leads to T-cadherin re-distribution to cell-cell contacts that requires lipid rafts integrity, actin filament polymerization and intracellular mediators such as Rac1 and p38MAPK.

My contribution to this study was to demonstrate that the proportion of T-cadherin and EGFR in lipid rafts remains the same before and after EGFR activation, suggesting that T-cadherin re-localization does not change lipid rafts contents.



Supplemental Figure S3. EGF does not alter the relative proportionality of T-cad and EGFR/p-EGFR in lipid raft domains. (A) The immunoblots illustrate typical distribution profiles of EGFR, T-cad and caveolin 1 in normally growing cultures of A431 following non-detergent-based protocols isolation/fractionation of lipid rafts through a gradient of 0-20%-Optiprep [1]. S, starting lysate. Numbers indicate fractions from top to bottom of the gradient. (B) A431 cells were serum-deprived, stimulated or not with EGF (10 ng/ml, 30 min) and then raft domains were isolated and fractionated using Optiprep-based protocols. Starting lysates (S), raft (R = fraction 6) and bottom non-raft (NR = fraction 12) fractions were analyzed by immunoblotting for p-EGFR, EGFR, T-cad as well as internal loading controls for the different fractions (GAPDH, caveolin 1 and clathrin heavy chain). (C) EGF-stimulated alterations in levels of EGFR, p-EGFR and T-cad in S and R fractions were determined relative to their respective unstimulated levels. These data were further used to determine ratios of p-EGFR:EGFR and T-cad:EGFR.

3.4 Ongoing study: Impact of altered T-cadherin expression on cell behavior and EGFR/IGF-1R pathway activity in prostate carcinoma.

Having found that EGFR activity can be modulated by T-cadherin in SCC, we considered whether T-cadherin might commonly regulate EGFR activity in other cancer types. Moreover, since our recent study shows that in endothelial cells T-cadherin physically interacts with insulin receptor and modulates its activity, we hypothesized that regulation of growth factor receptor activity in the general principle underlying T-cadherin effects in different cell types. Many cancers acquire resistance to EGFR-targeted anti-tumor drug gefitinib and it has been shown on breast and prostate cancer cell lines that IGF-1R, a close "relative" of insulin receptor, plays an important role in this process (232). There is evidence for bidirectional cross-talk between EGFR and IGF-1R pathways. For example, IGF-1 stimulation may indirectly induce EGFR phosphorylation *via* the autocrine release of HB-EGF (57), or may enhance ERK-dependent tumor cell proliferation through EGFR activation by means of a direct physical association between EGFR and IGF-1R (55). On the other hand, EGFR may influence IGF-1R activity by modulating ubiquitination and degradation rates of IGF-1R protein (54). We hypothesized that T-cadherin might be involved not only in regulation of EGFR activity but also other RTKs, in particular IGF-1R, and might modulate EGFR/IGF-1R cross-talk.

1. The first objective of this study is to see whether the modulatory effect of T-cadherin on EGFR, shown by us previously, is a phenomenon shared by different cancer types.
2. The second objective of the study is to examine whether T-cadherin modulates IGF-1R activity and is involved in the cross-talk between EGFR and IGF-1R.

We have chosen prostate cancer cells DU145 as a model for this study, based on comparable expression levels of EGFR and IGF-1R in these cells.

RESULTS

The generation of stably transduced DU145 with respect to T-cadherin-overexpression (Tcad+) or T-cadherin-deficiency using T-cadherin-targeted shRNA (shTcad), and empty vector (E) or non-target shRNA (sC) as respective controls, was performed using lentivector methods described in our previous papers. Expression of T-cadherin was controlled by immunoblotting (Fig. 1)

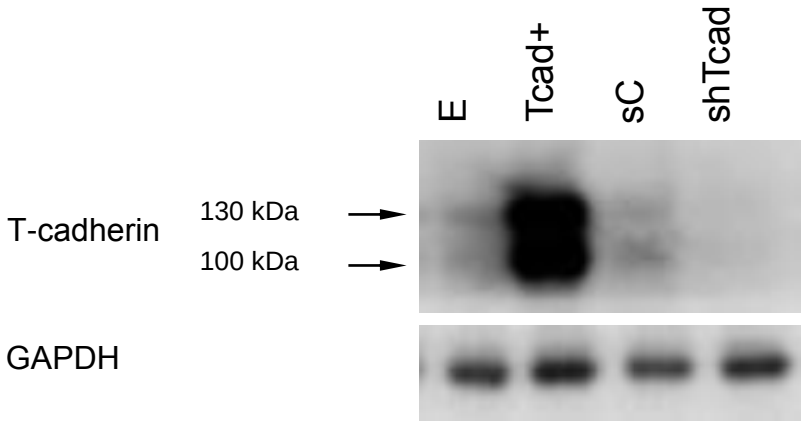


Figure 1. T-cadherin expression. *T-cadherin (T-cad) expression in subconfluent monolayers of E-, Tcad+-, sC- and shTcad-transduced DU145 cells was evaluated by immunoblotting of whole cell lysates.*

T-cadherin expression levels influence phenotype of DU145 cells.

Figure 2 illustrates the morphology of the different DU145 transductants as examined by phase contrast microscopy (Fig. 2A) as well as by fluorescence microscopy after staining for actin (TRITC-phalloidin, Fig. 2B) or golgi complex (anti-giantin, Fig. 2C). Compared with control E or sC cells T-cadherin overexpressing cells (Tcad+) exhibited a more disseminated and poorly polarized morphology whereas T-cadherin silencing (shTcad) promoted the formation of more compact and polarized colonies.

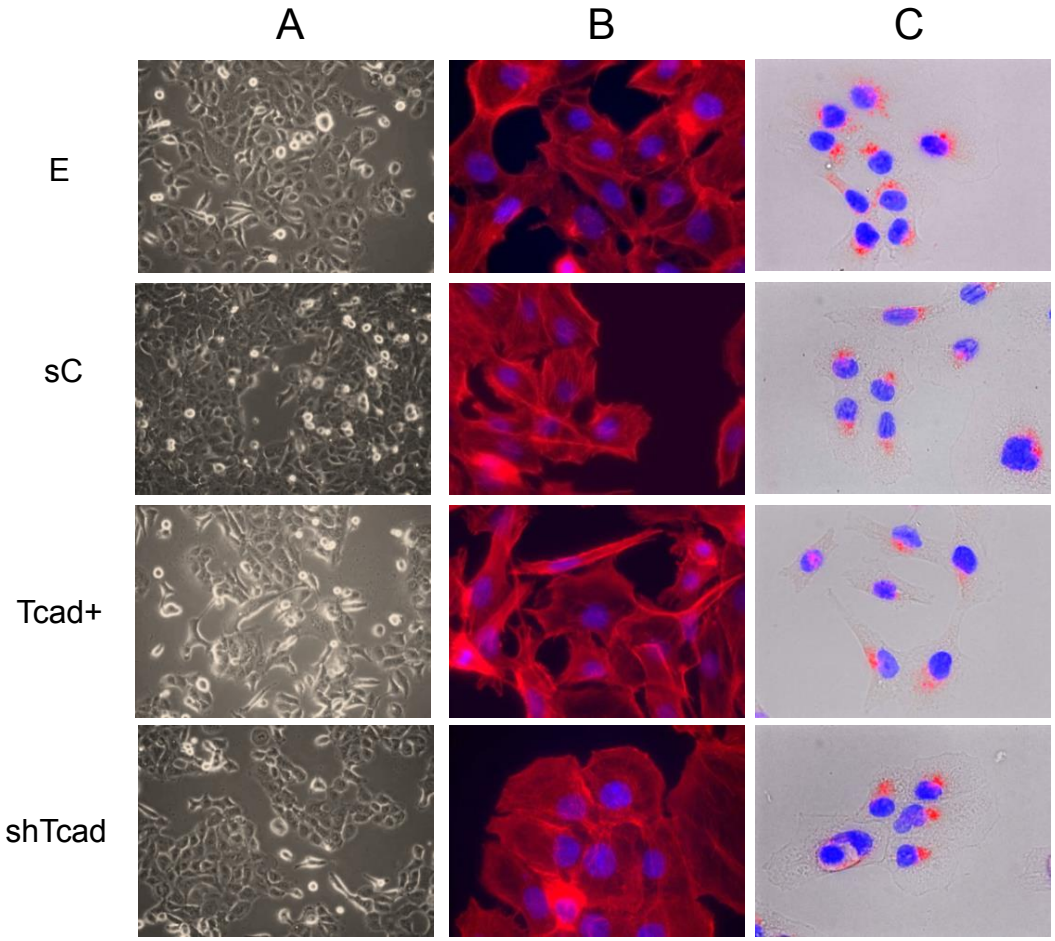


Figure 2. T-cadherin expression levels in DU145 alters cell morphology. *Control (E, sC), T-cadherin overexpressing (Tcad+) and T-cadherin silenced (shTcad) DU145 transductants suspended in normal growth medium were seeded (1.5x10^3 cells/well) onto gelatin (0.5%) precoated coverslips in 24-well plates. After 24h cells were examined for morphological differences by phase contrast microscopy (A, 10x magnification), or by epifluorescence microscopy after staining for actin (B, 60x magnification) or golgi complex (C, 60x magnification) with use of Hoechst for nuclear counterstaining.*

T-cadherin-dependent changes in cell morphology are linked to EGFR/IGF-1R activities.

To examine whether effects of T-cadherin on cell morphology might involve EGFR and/or IGF-1R the transductants were incubated for 2 days under control serum-containing conditions without or with inclusion of IGF-1R inhibitor (NVP-AEW541) or EGFR inhibitor (gefitinib). Cells were fixed and examined for golgi positioning by staining with anti-giantin and Hoechst. As illustrated in Figure 3, the disseminated, poorly polarized morphology characteristic of T-cadherin over-expressing cells was induced in all transductants by IGF-1R inhibition, whereas the tight, compact morphology typical for T-cadherin silenced cells was induced in all transductants by EGFR inhibition. These data suggest that 1) EGFR and IGF-1R have opposite effects on DU145 phenotype, EGFR causing cell dissemination and IGF-1 on the contrary reverting cells to a more epithelial-like morphology; 2) T-cadherin effects on DU145 cell morphology indeed closely resemble changes caused by modulation of EGFR and IGF-1R pathway activity; and 3) the T-cadherin-upregulated phenotype is characteristic for cells with low IGF-1R or high EGFR activity, while the T-cadherin-deficient phenotype corresponds to the morphology of cells displaying high IGF-1R or low EGFR activity.

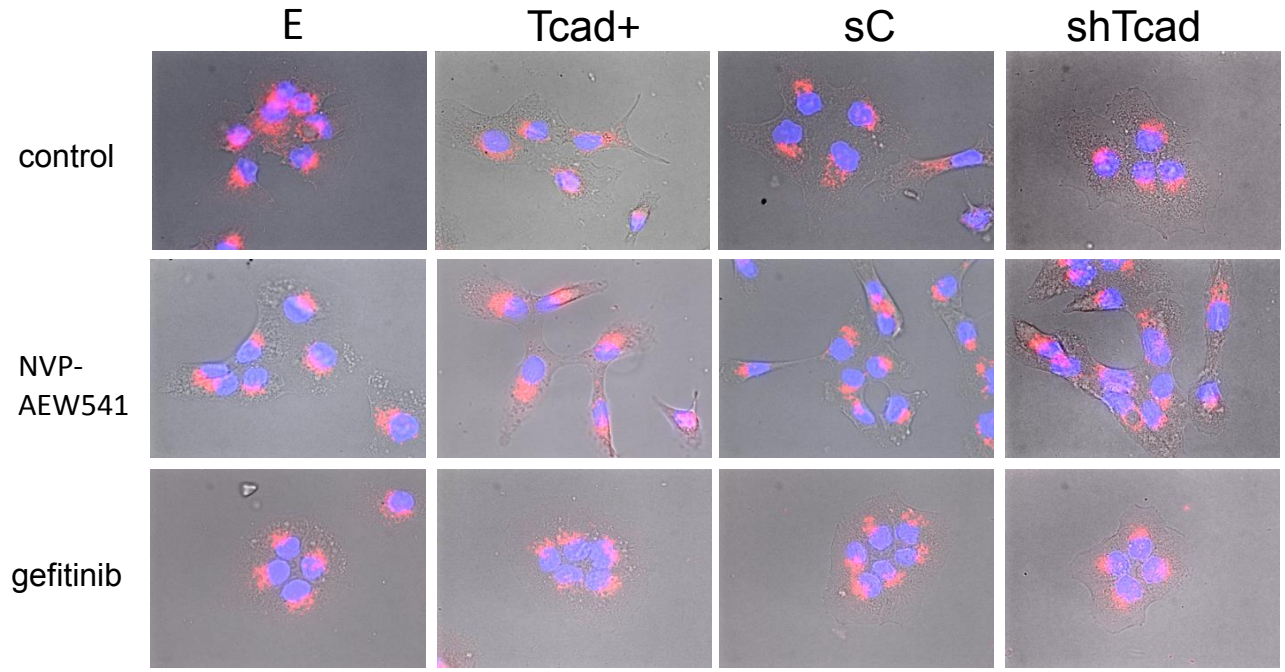


Figure 3. Inhibition of EGFR or IFGR-1R induces morphological interconversion on DU145 cells in 2D culture. *$2x10^4$ DU145 transduced cells were seeded on coverslips coated with gelatin. Next day cells were subjected to IGF-IR or EGFR pharmacological inhibition for 2 days with the use of NVP-AEW541 (5 μM) or genitinib (1 μM) respectively, fixed with 4% PAF and stained with anti-giantin antibody (red) and Hoechst (blue). Images were captured with the use of an epifluorescence microscope.*

T-cadherin expression levels modulate effects of EGF/IGF-1 on cell morphology.

Next we examined the effects of EGF and IGF-1 stimulation on the morphology of the DU145 transductants. For these experiments cells were subjected to a one day period of serum-deprivation and then further cultured for 2 days either without or with inclusion of EGF or IGF. Morphology was examined by phase contrast microscopy (Fig. 4). In the presence of EGF cell clusters appeared more disorganized with many cells exhibiting a disseminated, poorly polarized morphology; this effect of EGF was evident for all transductants, albeit most prominent in shTcad cells. IGF-1, in contrast, enforced cell clustering and polarization at the borders of colonies, also an effect evident in all transductants. The effects of EGF and IGF-1 (Fig. 4) are in accordance with the respective effects of IGF-1R and EGFR inhibition (Fig. 3). Also, in accordance with inhibition studies these data support that in DU145 cells T-cadherin dually modulates growth factor receptor pathways: T-cadherin gain favors "asocial" disorganized cell phenotype typical for high EGFR and low IGF-1R activity, while T-cadherin silencing promotes "prosocial" epithelial-like morphology characterized by low EGFR and high IGF-1R activity.

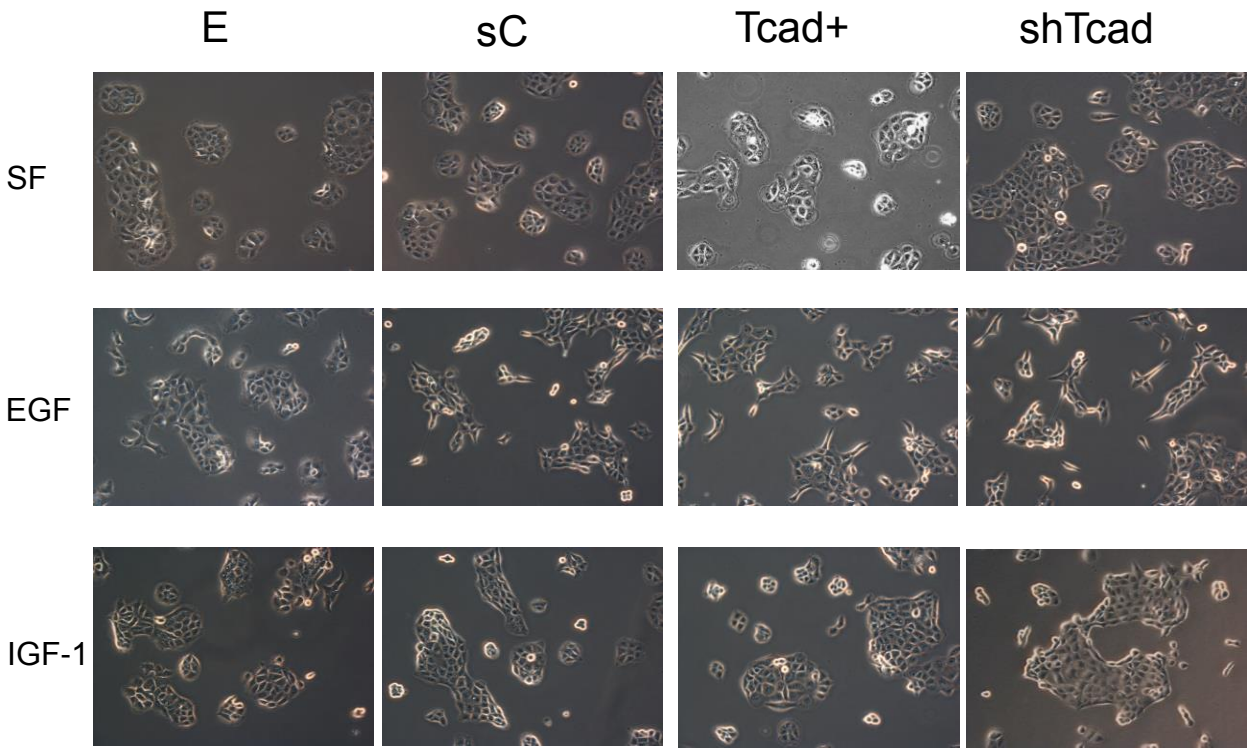


Figure 4. EGF and IGF-1 exert differential effects on morphology of DU145 cells in 2D culture. *DU145 transductants were seeded (2x10^4 cells/well in 12-well plates) and allowed overnight attachment. Cells were then serum-deprived for 24h before further culture for 48h under serum-free conditions (SF) without or with inclusion of EGF (10ng/ml) or IGF-1 (10ng/ml). Morphology was examined at 48h by phase contrast microscopy.*

T-cadherin expression levels influence EGFR and IGF-1R phosphorylation status

Next we examined the effects of T-cadherin expression on EGFR and IGF-1R phosphorylation status. Experiments were performed both under serum-containing conditions without or with inclusion of EGFR or IGF-1R inhibitors and under serum-free conditions without or with inclusion of EGF or IGF-1 (Fig 5.).

Immunoblot analysis of whole cell lysates revealed increased levels of pEGFR and pIGF-1R in shTcad cells under normal serum-containing culture conditions (Fig. 5A). Inhibition of EGFR (gefitnib) significantly decreased pEGFR and slightly increased pIGF-1R levels in all transductants. Inhibition of IGF-1R (NVP-AEW541) decreased pIGF-1R to an equivalent level in all transductants. Interestingly, IGF-1R inhibition also markedly decreased pEGFR levels.

ShTcad cells also exhibited increased levels of pEGFR and pIGF-1R under serum free culture conditions (Fig. 5B). EGF and IGF stimulated phosphorylation of their cognate receptors in all transductants, with shTcad cells achieving the highest levels of pEGFR and pIGF-1R. Stimulation with IGF-1 also increased the levels of pEGFR, which is in accordance with the inhibitory effects of NVP-AEW541 on pEGFR under serum-containing culture conditions.

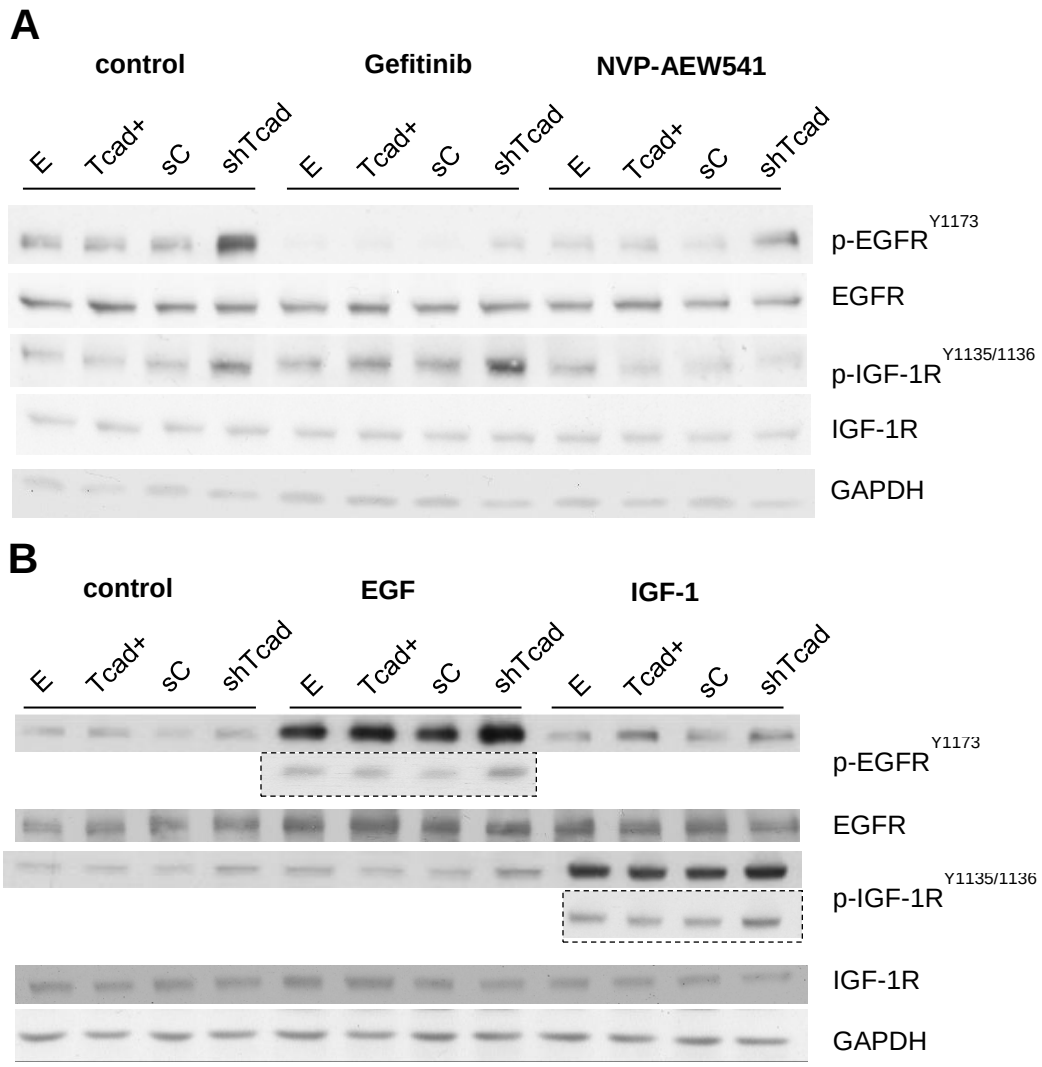


Figure 5. T-cadherin expression alters phosphorylation status of EGFR and IGF-1R. *(A) DU145 transductants were seeded (2 x10^5 cells/well in 6-well plates) and allowed overnight attachment. Medium was then refreshed in all wells with or without inclusion of Gefitinib (1 μM) and NVP-AEW541 (10 μM). Cells were lysed after 48h. (B) DU145 transductants were seeded (3 x10^5 cells/well in 6-well plates) and allowed overnight attachment. Cells were serum-deprived for 24h, stimulated for 10 min with EGF (10ng/ml) or IGF-1 (10ng/ml) and then lysed. Lysates were immunoblotted for total and phosphorylated EGFR and IGF-1R with GAPDH as internal protein loading control.*

Taken together these data suggest several interesting and somewhat unexpected conclusions.

First, we have identified a completely novel aspect of IGF-1R pathway activity in DU145 cells. Most studies show that IGF-1 acts as a mitogen for prostate cancer cells and therefore would be expected to have a pro-oncogenic effect in prostate tumors. Our data, however, show that IGF-1 may also be needed for differentiation of prostate cancer cells, promoting an epithelial-like phenotype and formation of organized cell clusters, while reduction of IGF-1R activity results in cell dissemination and loss of epithelial polarity (which is directly opposite to EGFR effects). This means that high IGF-1R activity in tumors could be beneficial through maintenance of a higher differentiation status, while the use of IGF-1R inhibitors might potentially present some risk due to promotion of tumor cell dissemination. Our observations may explain some controversial and as yet unexplained literature data demonstrating higher metastatic rates of prostate tumors in patients with low IGF-1 and/IGF-1R levels and activity.

Second, our signaling data show that the relationship between activities of the two growth factor receptor pathways, T-cadherin expression and prostate cancer cell phenotype is not straightforward. Although T-cadherin loss results in enhanced phosphorylation of both EGFR and IGF-1R receptors, T-cadherin silenced cells exhibit a morphology that is typical for high IGF-1R and not high EGFR activity. The situation is further complicated by existence of a cross-talk between EGFR and IGF-1R (transactivation of the first pathway by the second, as shown by ligand activation or inhibitory signaling experiments), with T-cadherin being able to affect not only activity of each given receptor but their cross-talk as well. Our preliminary conclusion is that T-cadherin loss in prostate cancer cells shifts this delicate balance in favor of IGF-1R dominance thus promoting maintenance of epithelial-like differentiation status.

5. CONCLUSIONS

Aberrant signal transduction is a major driving force in cancer progression. My dissertation has focused on functional role and molecular mechanisms of action of T-cadherin in cancer progression. SCC served as the principal cancer model. Ongoing studies use prostate carcinoma as an alternative model. The key findings are as follows:

1) *T-cadherin expression levels in SCC modulate cancer cell phenotype and invasive behavior*.
Immunohistochemical analysis of human cutaneous biopsies showed that a decrease or loss of T-cadherin expression occurs in human cutaneous SCC tumors locally at the sites of tumor invasion, and not ubiquitously as described earlier*. In vitro* studies on SCC cells revealed that loss of T-cadherin promoted cell elongation, motility and invasiveness, these being characteristics of the malignant phenotype. Functional effects of T-cadherin suggest involvement of signaling pathways in regulation of cytoskeleton and focal adhesion.

2) *T-cadherin expression levels in squamous cell carcinoma regulate EGFR pathway activity.*
We demonstrated that inhibition of EGFR activity in T-cadherin-deficient SCC cells resulted in reduction of their invasive capacity to a level comparable with control cells. This suggests that EGFR signaling pathway contributes to the enhanced invasive potential of cells with depleted level of T-cadherin. T-cadherin-deficient SCC cells were more responsive to EGF stimulation, manifest as enhanced ligand-induced EGFR phosphorylation, receptor internalization, cell retraction and motility. Over-expression of T-cadherin exhibited a converse blunted responsiveness to EGF.

3) *Regulation of EGFR activity by T-cadherin involves receptor sequestration within plasma membrane lipid raft domains*.
The enhanced EGFR pathway activation in T-cadherin deficient SCC cells was associated with decreased levels of EGFR in membrane lipid raft domains. Upregulated expression of T-cadherin resulted in accumulation of EGFR in lipid raft domains. Thereby, we suggest that T-cadherin suppresses EGFR activity by sequestering the receptor into lipid raft domains.

4) *Signaling molecules contributing toward T-cadherin-dependent effects on squamous cell carcinoma cell invasive behavior.*
We found that underlying molecular mechanisms of T-cadherin effects on cell retraction and motility involve β1 integrin activation status and the Rho family of small GTPases. T-cadherin-deficient SCC cells exhibited lower levels of the active conformation of β1 integrin with further

reduced activity levels in response to EGF stimulation. T-cadherin over-expressing cells exhibited increased levels of active β1 integrin. Rac1 and Cdc42 activity were reduced and RhoA activity was enhanced in T-cadherin deficient cells.

5) T-cadherin regulates EGFR and IGF-1R activity and cross-talk in prostate cancer cells.

Alterations of T-cadherin expression in DU145 prostate cancer cells exert profound effects on cell morphology. Gain of T-cadherin is associated with a poorly polarized, disseminated morphology whereas loss of T-cadherin promotes highly polarized and compact colony characteristics. The effects of T-cadherin on morphology are related to effects of T-cadherin on IGF-1R and EGFR pathway activities.

6. PERSPECTIVES

The following analyses could be undertaken to elucidate molecular mechanisms underlying T-cadherin-dependent modulatory effects on cell behavior and RTK activity in prostate cancer:

(1) ***In vitro analysis***: Effects of T-cadherin over-expression and silencing on tumor cell behavior *in vitro*, such as cell proliferation, invasion, migration, apoptosis and differentiation. Experiments on cell transductants could be performed either under basal growth conditions to estimate the impact of T-cadherin over-expression *per se*, or after stimulation with IGF-1 or EGF to determine RTK ligand dependence. Function blocking antibodies against IGF-1R (mAb 24-60), EGFR (cetuximab), EGFR tyrosine kinase inhibitors gefitinib/lapatinib or IGF-1R tyrosine kinase inhibitor NVP-AEW541 could be variously included in the assays to study the impact of the IGF-1R and EGFR pathway involvement in T-cadherin effects on tumor cell behavior.

(2) ***Human tissue microarray analysis***: Evaluation of T-cadherin expression and distribution in human prostate carcinoma tissues using tissue microarray technology would help to determine whether and how alterations in T-cadherin expression are associated with clinico-pathological features of the tumors.

(3) ***In vivo analysis***: I*n vivo* application of T-cadherin-overexpressing and T-cadherin-silenced cell transductants to murine tumor models such as the xenograft (implantation) and/or experimental metastasis (tail vein injection) models to validate modulatory effects of T-cadherin in tumor cell signaling pathways and behavior as established *in vitro*. In order to estimate the impact of IGF-1R or/and EGFR pathway in T-cadherin-dependent regulation of tumor growth and metastasis animals could be treated with specific receptor blocking antibodies or specific receptor tyrosine kinase inhibitors.

7. NOMENCLATURE

A431	epidermoid carcinoma of skin
BCC	basal cell carcinoma
DU145	human prostate carcinoma
EGF, EGFR	epidermal growth factor and its receptor, respectively
EC	extracellular domain
HaCaT	immortal human keratinocytes
HCC	hepatocellular carcinoma
ICC	Immunocytochemistry
IGF-1, IGF-1R	insulin like growth factor and its receptor, respectively
LOH	loss of heterozygosity
RTK	receptor tyrosine kinase
SCC	squamous cell carcinoma
WB	Western blot

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10. CURRICULUM VITAE

Personal information

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Experience

10.2008 – present **Research assistant**, Department of Biomedicine, Basel University Hospital, Switzerland.
Investigated involvement of T-cadherin protein in signaling pathways that promote cancer cell proliferation, migration and invasion

Tasks & Skills

- Designed and executed experiments
- Analyzed, interpreted and communicated scientific data
- Experienced in writing of protocols and scientific progress reports
- Presented project work at (inter-)national conferences
- Independent and team work
- General laboratory organization

Hands-on experience

- Culture of primary human cells, cancer and endothelial cell lines
- Transduction of human cell lines with adeno- and lentiviral vectors
- Analysis of cellular signal transduction systems (immunoblotting, coprecipitation of membrane receptors, inhibitor studies)
- Cell adhesion assays, cell proliferation assays, migration assays (Boyden chamber, transwell assays, wound healing assay), cell apoptosis and survival assays
- Immunocytochemistry, fluorescent microscopy, confocal microscopy, time-lapse video microscopy, 3D image analysis
- Lipid rafts isolation
- 3D models for tumor invasion in vitro (spheroid assays of tumor expansion in collagen gel and Matrigel, transmigration and invasion through Matrigel)
-

12.2007 – 06.2008 Laboratory assistant at the New Technologies Center at the Novosibirsk State Research Institute of Circulation Pathology, Federal Agency of High Technology Medical Care, Russia.

- Biochemical and molecular biology techniques (cell culture, PCR, RT-PCR, gel electrophoresis)

Education

10.2008 – 06.2013 **Doctoral studies** at the Department of Biomedicine, University Hospital of Basel (Supervision: Prof. Dr. Thérèse Resink)

"Modulatory effects of T-cadherin on cell behavior and growth factor receptor activity in carcinoma cells"

09.2001 – 07.2007 **Master degree in Clinical Medicine and Biology,** Department of Medical biology, Faculty of Natural Sciences, Novosibirsk State University

Education in clinical medicine including extensive contact with patients

- Positions: hospital attendant, nurse, ambulance assistant, physician's assistant, operating room assistant

Volunteering

01.2011 – 01.2013 Volunteer at Healthcare Businesswomen's Association (non-profit organization)

- Launched and supervised a group-mentoring program at the HBA Europe chapter
- Event organization and liaison

Additional courses and Certificates

06.2012 – 06.2013 Participant in **Women Into Industry program,** a mentoring program of Novartis and the University of Basel, that introduced me into the world of pharmaceutical industry and helped me to determine my professional preferences through meeting of people from different divisions of the company

04.2013 Good clinical practice basic course, Swiss Tropical and Public Health Institute, Basel

Other Skills and Interests

Software Working knowledge of various computing packages including Microsoft Office (Word, PowerPoint, Excel), Cell^P, GraphPad, Aida, Scion, Photoshop, Lightroom

Languages Russian (mother tongue), English (fluent), German (good conversational)

Hobbies Photography

Publications

1. Kyriakakis E, **Maslova K**, Frachet A, Ferri N, Contini A, Pfaff D, Erne P, Resink TJ, Philippova M. Cross-talk between EGFR and T-cadherin: EGFR activation promotes T-cadherin localization to intercellular contacts. *The Journal of Cellular signaling,* 2013 Feb. doi: 10.1016/j.cellsig.2013.02.001.
2. Emmanouil Kyriakakis[1], **Kseniya Maslova[1]**, Maria Philippova, Dennis Pfaff, Manjunath B. Joshi, Stanislaw A. Buechner, Paul Erne and Thérèse J. Resink. T-cadherin is an auxiliary negative regulator of EGFR pathway activity in cutaneous squamous cell carcinoma: impact on cell motility. *The Journal of Investigative Dermatology,* 2012 Mai. doi: 10.1038/jid.2012.131.
   1 the authors equally contributed to the work.
3. Philippova M, Joshi MB, Pfaff D, Kyriakakis E, **Maslova K**, Erne P, Resink TJ. T-cadherin attenuates insulin-dependent signalling, eNOS activation, and angiogenesis in vascular endothelial cells. *The Journal of Cardiovascular research,* 2012 Mar. doi: 10.1093/cvr/cvs004.
4. Pfaff, D., Phillipova, M., Kyriakakis, E., **Maslova, K.,** Rupp, K., Buechner, S.A., Iezzi, G., Spagnoli, G.C., Erne, P., Resink, T.J. Paradoxical effects of T-cadherin on squamous cell carcinoma: up- and downregulation increase xenograft growth by distinct mechanisms. *The Journal of Pathology,* 2011 Mar 25. doi: 10.1002/path.2900.
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Conferences

Oral Presentations

1. **Kseniya Maslova**, Manjunath B. Joshi, Maria Philippova, Emmanouil Kyriakakis, Paul Erne, Thérèse Resink. T-cadherin attenuates insulin signaling in vascular endothelial cells.. ***17th Cardiovascular Biology and Clinical Implications Meeting***. *Muntelier, Switzerland, October 6-7, 2011*

Poster Presentations

1. **K. Maslova**, M. Philippova, E. Kyriakakis, D. Pfaff, P. Erne and T. J. Resink.The role of T-cadherin silencing in invasion of squamous cell carcinoma. ***36th FEBS Congress****, Turin, Italy, June 25-30, 2011.*
2. **Kseniya Maslova**, Maria Philippova, Manjunath B. Joshi, Emmanouil Kyriakakis, Dennis Pfaff, Paul Erne and Thérèse J. Resink. The role for lipid rafts in T-cadherin interaction with hormone receptors in endothelial cells. ***Annual Meeting of the Swiss Society of Cardiology,*** *Basel, June 8-10, 2011.*
3. **K. Maslova**, M. Philippova, M. B. Joshi, E. Kyriakakis, D. Pfaff, P. Erne, TJ. Resink.Novel mechanisms of T-cadherin dependent modulation of endothelial cell function: the role for lipid rafts in T-cadherin interaction with hormone receptors. ***16th Cardiovascular Biology and Clinical Implications Meeting,*** *Muntelier, Switzerland, 7-8 October 2010.*
4. **K. Maslova**, M. Philippova, M. B. Joshi, E. Kyriakakis, D. Pfaff, P. Erne, TJ. Resink. *In vivo* characterization of T-cadherin in angiogenesis and tumor angiogenesis ***15th Cardiovascular Biology and Clinical Implications Meeting***. *Muntelier, Switzerland, October 1-2, 2009*