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A Role for CD148 in the Control of Cell Growth and Cell-cell Interactions

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ABSTRACT

A variety of signal transduction events are controlled by the opposing, balanced activities of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). Until recently scientists have focused on PTKs, but recent findings have recognized that PTPs play specific and active roles in the regulation of many physiological processes.

CD148 is a receptor tyrosine phosphatase (RPTP) with a large extracellular domain and a single catalytic domain. It is expressed on a wide variety of cell types including fibroblasts, endothelial cells, and hematopoietic cells. A number of studies have addressed the role of CD148 in the regulation of cell growth and differentiation using fibroblasts or tumor cell lines. In cultured cells with epithelioid morphology, including endothelial cells and newborn smooth muscle cells, CD148 transcript levels are dramatically upregulated as culture density increases. Although CD148 is expressed on the surface of hemopoietic cells, only a few studies have been published examining the expression of CD148 in human lymphocytes, especially T cells. These reports showed that CD148 plays a role in T cell activation where it participates in the regulation of the T-cell receptor.

In this study, we have analyzed the possible involvement of CD148 in epithelial growth and cell-cell interactions. Using polarized epithelial cells as a model, we have applied a transcriptional silencing approach based on lentiviral-mediated RNA interference. We show that CD148 expression is required for epithelial cell growth control and proper assembly of epithelial sheets.

INTRODUCTION

Protein Tyrosine Phosphatases

Reversible tyrosine phosphorylation, which is governed by the balanced action of protein tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPs), regulates important signaling pathways that are involved in the control of cell proliferation, adhesion and migration.

The key regulatory role of protein kinases is clearly established; however, it has recently become apparent that protein phosphatases can no longer be viewed as passive housekeeping enzymes, but they partner with kinases in the regulation of signaling responses. The distinct but complementary function of these enzymes is emphasized by recent studies, in which kinases have been implicated in controlling the amplitude of signaling responses, whereas phosphatases are thought to have an important role in controlling the rate and duration of the response (R. Heinrich *et al.*, 2002; J.J. Hornberg *et al.*, 2005).

The first PTP was purified in 1988, approximately 10 years after the discovery of tyrosine kinases (N.K. Tonks *et al.*, 1988). It is now known that PTPs constitute a large, structurally diverse family of tightly regulated, highly specific enzymes with important regulatory roles (A. Alonso *et al.*, 2004; J.N. Andersen *et al.*, 2001; A. Ostman and F.D. Bohmer, 2001).

It is also clear that PTPs have both inhibitory and stimulatory effects on cancer-associated signaling processes, and that deregulation of PTP function is associated with

tumorigenesis in different types of human cancer. Recent reports have revealed the strategic importance of PTPs in the regulation of physiological processes, where they coordinate the interactions between neighboring cells, participating in tissue homeostasis, immune response, and embryogenesis (for a review, see T. Mustelin *et al.*, 2002).

The PTP-superfamily includes 109 genes, compared to 90 human PTK genes, suggesting similar levels of complexity between the two families. However, the number of genes only illustrates the minimal level of complexity in the family, as additional diversity is introduced through the use of alternative promoters, alternative mRNA splicing and post-translational modifications. This structural diversity is indicative of the functional importance of the PTPs in the control of cell signaling. It is now apparent that the PTPs have the capacity to function both positively and negatively in the regulation of signal transduction. Furthermore, PTPs have the potential to display exquisite substrate specificity *in vivo*. Substrate specificity is both dependent on interactions between the substrates and areas flanking the PTP active site, and on the subcellular compartmentalization that restricts substrate access (F.G. Haj *et al.*, 2002; A. Salmeen *et al.*, 2000).

The PTP Gene Superfamily

The members of the PTP superfamily can be classified in classical PTPs and dual-specificity phosphatases (*DSPs*). Classical PTPs can only dephosphorylate phospho-tyrosine residues, since this is the only phospho-aminoacid that can be accommodated in the depth of their active site cleft. DSPs, on the contrary, can also dephosphorylate serine and threonine phospho-residues and even phosphoinositol phospholipids.

Classical PTPs are broadly divided into receptor-like forms and non-receptor forms (J.N. Andersen, *et al.*, 2001). The receptor-like PTPs (*RPTP*) have a single transmembrane domain and variable extracellular domains. Non-receptor PTPs have striking structural diversity and often contain sequences that target them to specific subcellular locations or enable their binding to specific proteins (L.J. Mauro and J.E. Dixon, 1994; A. Ostman *et al.*, 2006).

The intracellular parts of most receptor-like PTPs contain two tandem PTP domains, the carboxy-terminal one having little or no catalytic activity but possibly functioning as a site of interaction with regulatory/targeting proteins (E.H. Fischer, 1999). The catalytic PTP domain spans approximately 280 amino-acids and contains a highly conserved active site with a cysteine residue that is required for catalytic activity. Dephosphorylation of substrates occurs through a two-step mechanism consisting of the formation of a covalent PTP–phosphate intermediate that is subsequently hydrolyzed (A. Ostman, *et al.*, 2006).

Dephosphorylation by PTPs occurs with a high degree of specificity, as demonstrated by the >1000-fold difference in the K_m for different phosphopeptides and by the selective substrate-binding of substrate-trapping PTP variants (A.J. Flint *et al.*, 1997; Z.Y. Zhang *et al.*, 1993). Some studies have also shown that PTPs preferentially dephosphorylate certain subsets of phosphotyrosines on proteins that have multiple phosphorylation sites (M. Kovalenko *et al.*, 2000; C. Persson *et al.*, 2004). Substrate specificity has at least two determinants; first, significant side-chain interactions occur between the substrates and areas that flank the PTP active site; second, subcellular compartmentalization restricts substrate access (F.G. Haj, *et al.*, 2002).

Receptor-like PTPs can be further subdivided into five types based upon common features found in the extracellular domain (R.J. Mourey and J.E. Dixon, 1994). Type I receptor-like molecules are represented by the hematopoietic cell-restricted CD45

family, which have multiple isoforms arising from differential splicing of sequences at the amino terminus. Type II molecules are the leukocyte common antigen-related (LAR)-like PTPs (e.g. DLAR in drosophila, mammalian LAR, PTPσ and PTPδ). This class of PTPs contains tandem repeats of immunoglobulin-like and fibronectin type III-like domains resembling neural cell adhesion molecules. With the exception of LAR, most other LAR-like PTPs are expressed preferentially in neurons, and play fundamental roles in neuronal development. Type III molecules have multiple fibronectin type III-like repeats (e.g. mammalian OST-PTP, drosophila DPTP10, and DPTP99A). PTPα and PTPε represent type IV isoforms, which generally have small extracellular domains. Type V molecules include PTPξ and PTPγ, which have amino terminal carbonic anhydrase-like domains. The extracellular domain (*ECD*) of RPTPs is important for extracellular interaction. For example, the fibronectin (Y. Fujita *et al.*, 2002) type III domain, which is found in different kind of proteins (including cell surface receptors and cell adhesion molecules), could mediate adhesion among PTPs (P.C. Tsiotra *et al.*, 1996). In fact, it was shown that PTPμs molecules interact homophilically mediating cell-cell aggregation through their FNIII domains (S.M. Brady-Kalnay *et al.*, 1993).

It is thought that FNIII domains present in the extracellular portion of CD148 might promote adhesion. In fact, some repeats in its coding sequence include the XGD sequence, similar to the cell attachment-promoting sequence RGD, which in fibronectin interacts with members of the integrin superfamily (**Fig. 1**).

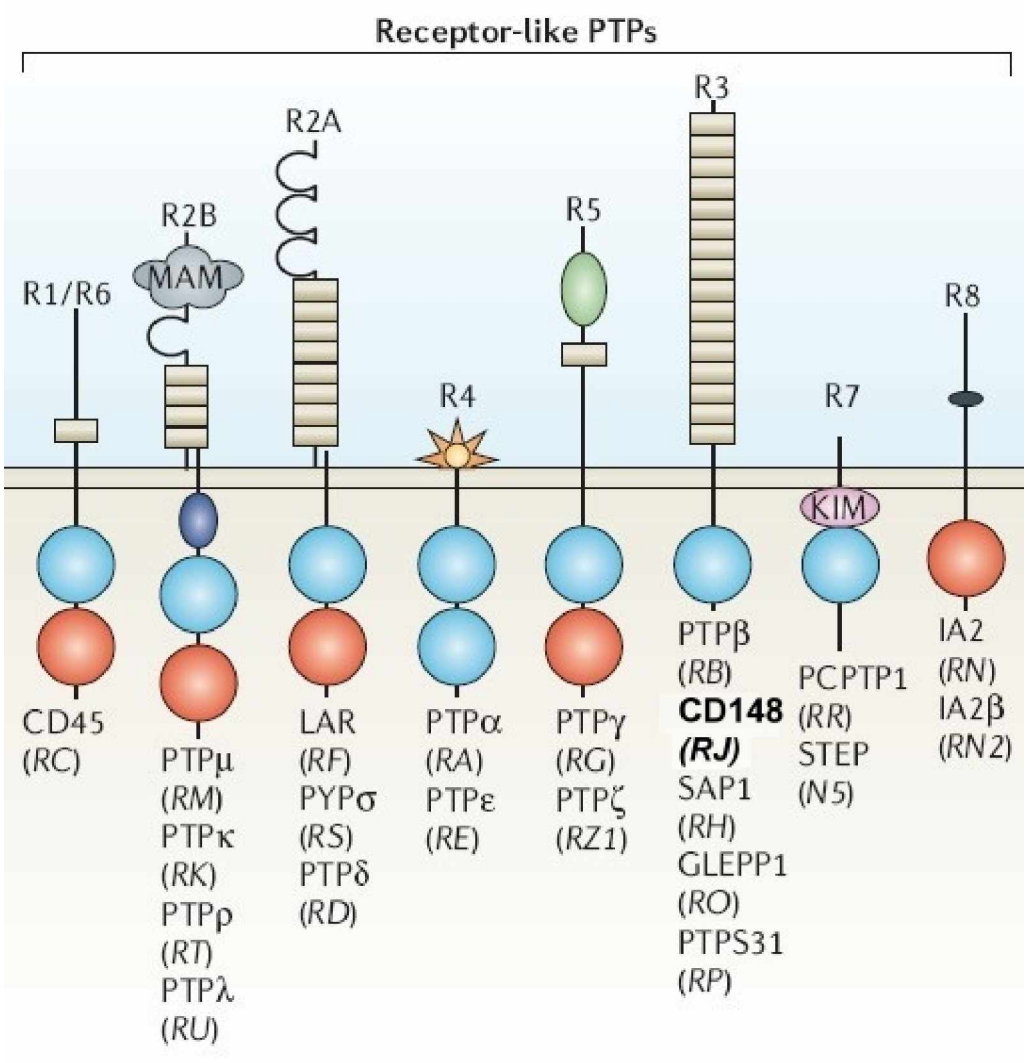


Fig. 1: Schematic view of the domain composition of all RPTP family members. The intracellular part consists of one catalytically active PTP domain (blue) and, in some subfamilies, a PTP domain with little or no catalytic activity (red), which is likely to have a regulatory function.

PTP Activity Is Tightly Regulated

As might be anticipated for a family of enzymes that play critical roles in the regulation of cell signaling, the activity of PTPs is tightly controlled *in vivo* by a variety of mechanisms.

Some PTPs are themselves regulated by phosphorylation, which can either increase or decrease their activity (reviewed in A. Ostman and F.D. Bohmer, 2001). This property has been extensively characterized in the case of CD45 (Y. Wang *et al.*, 1999) and PTP-PEST (A.J. Garton and N.K. Tonks, 1994). Moreover, both non-receptor and receptor-like PTPs can be regulated by proteolytic cleavage, which is normally associated with degradation or translocation of the target protein. Recently, reversible oxidation of a cysteine residue in the active site has also emerged as an important inhibitory control mechanism for PTP regulation. The production of reactive oxygen species (*ROS*) such as hydrogen peroxide and the resulting post-translational modification of proteins by reversible oxidation of the active site cysteine have been implicated in the regulation of tyrosine phosphorylation-dependent signaling pathways, that are initiated by a wide variety of stimuli including growth factors, hormones, cytokines and cellular stresses (T. Finkel, 2003)**.**

Interestingly, it was proposed that in a dimeric state the catalytic activity of RPTPs might be attenuated by reciprocal occlusion of the active sites (A. Weiss and J. Schlessinger, 1998). This is based on functional analyses of chimaeras of the epidermal growth factor receptor (EGFR) and the transmembrane PTP CD45**,** and on structural and functional studies of PTPα.

Several RPTPs Ligands Have Been Identified

Structural variability of the extracellular domains of RPTPs suggests that they undergo selective ligand interactions, potentially affecting RPTP activity.

The best characterized example of acute regulation of a RPTP by ligand binding is the inhibition of RPTPζ activity following binding of pleiotrophin (PTN) (K. Meng *et al.*, 2000). Potential downstream substrates of RPTPζ, which show increased phosphorylation in response to PTN, include β-catenin, which is a component of the cadherin–catenin cell–adhesion complexes, and β-adducin (H. Pariser *et al.*, 2005), which is a cytoskeletal protein that regulates actin filaments.

Moreover, the binding of heterophilic ligands including tenascin, contactin and N-CAM has been demonstrated for PTP-β/ζ (E. Peles *et al.*, 1998). Contactin also binds to PTP-α, and nidogen binding to LAR has been proven (P. O'Grady *et al.*, 1998; L. Zeng *et al.*, 1999). The extracellular domains of some receptor-like PTPs, such as PTPμ, PTPκ and PTPδ have been shown to mediate homophilic interactions (S.M. Brady-Kalnay *et al.*, 1995; J. Sap *et al.*, 1994).

In an intriguing symmetry with the regulation of RPTKs, heparin sulphate proteoglycans (HSPGs) have now been recognized as playing an important role in the regulation of RPTP functions. For example, the HSPGs agrin and collagen XVIII were identified as ligands for RPTPσ in neurons (A.R. Aricescu *et al.*, 2002). Recently, Sdc, which is a transmembrane surface protein on muscle cells, has been demonstrated to interact with LAR and to positively regulate the function of this RPTP in motor-neuron growth cones, thereby mediating signaling events that are associated with the axon-guidance decisions that lead to muscle innervation (A.N. Fox and K. Zinn, 2005).

Many PTP ligands are still unknown, such as the extracellular ligand of CD148. However, it was demonstrated that Matrigel, a preparation of extracellular matrix protein, activates the PTP activity of CD148 (M. Sorby *et al.*, 2001). The CD148 ligand should be searched among Matrigel components.

PTPs Play Crucial Physiological Roles in Mammalian Tissues

Many PTPs are believed to be involved in the complex role of orchestrating metabolism, but their specific activity at the moment is unclear. For example PTP1B, which directly dephosphorylates the insulin receptor, is considered a negative regulator of insulin and leptin signal transduction pathways (G. Liu, 2003). Many PTPs are believed to coordinate immune responses. The most representative case is CD45, a RPTP who acts as a positive regulator of T and B cell receptors by dephosphorylating the C-terminal negative-regulatory tyrosine residue of SRC-family PTKs (i.e., LCK, FYN and YES in T cells, and LYN, FYN and BLK in B cells. See T. Mustelin and K. Tasken, 2003).

It is believed that PTPs also work as regulators of development, but the studies carried out so far have achieved ambiguous results. Only in the case of neuronal development, RPTPs were proven to have a central role in modulating neuronal migration, axon guidance, and synapse formation (S.E. Ensslen-Craig and S.M. Brady-Kalnay, 2004).

PTPs Partecipate in Cell-cell Junctions

Cell-cell adhesion is critical to the development and maintenance of multicellular organisms. The stability of many adhesions is regulated by protein tyrosine phosphorylation of cell adhesion molecules and their associated components. Tight junctions (TJs) are responsible for the barrier function of many epithelia, whereas adherens junctions (AJs) and desmosomes mediate strong intercellular adhesion. The

assembly and function of TJs is typically dependent on the state of AJs, so that modulating AJ function often affects TJ barrier properties. The major adhesion molecules in both AJs and desmosomes belong to the cadherin family; however, cadherins can also contribute to adhesions between cells where distinct junctions do not develop and where adhesion is more dynamic. Whereas the extracellular domain of cadherins participates in calcium-dependent homophilic adhesion, the cytoplasmic domain binds p120*ctn* and β-catenin (Z. Kam *et al.*, 1995; R. Kemler, 1993; M.G. Lampugnani *et al.*, 1995). The former regulates the stability of cadherins on the cell surface (A.P. Kowalczyk and A.B. Reynolds, 2004), and β-catenin provides a link to α-catenin and the actin cytoskeleton, although the details of the bridge remain controversial (S. Yamada *et al.*, 2005). Tyrosine phosphorylation of cadherins and their associated proteins has major effects on the stability of adherens junctions (J.M. Daniel and A.B. Reynolds, 1997; M.G. Lampugnani *et al.*, 1997). In early work, it was shown that inhibiting PTPs with pervanadate elevated tyrosine phosphorylation in adherens junctions and promoted the disassembly of these structures (T. Volberg *et al.*, 1992). However, the same group later observed that in some situations elevation of tyrosine phosphorylation first transiently stimulated AJ assembly before resulting in the eventual disassembly of the same structures. Activation of PTKs or inhibition of PTPs can lead to increased tyrosine phosphorylation of members of the cadherin-catenin complex, dissociation of the AJ from the cytoskeleton, and disruption of cell-cell adhesion (J. Behrens *et al.*, 1993; N. Matsuyoshi *et al.*, 1992; B.A. Young *et al.*, 2003).

For example, phosphorylation of tyrosine residues 755 and 756 on E-cadherin leads to its ubiquitination and subsequent endocytosis, resulting in loss of junctional integrity (Y. Fujita, *et al.*, 2002). Similarly, phosphorylation of Tyr658 and Tyr731 in the cytoplasmic domain of VE-cadherin prevents binding of p120*ctn* and β-catenin, respectively, and causes a decrease in barrier function. Therefore, maintenance of

junctional integrity is regulated in part by reversible tyrosine phosphorylation that results from a competing balance of PTK and PTP activity.

Receptor-PTPs, such as PTPμ, VE-PTP, and also CD148, as well as the cytosolic PTPs, PTP1B, and Shp-2, have been shown to bind to members of the cadherin- catenin complex and to regulate cell-cell adhesion by regulating phosphorylation of the cadherin-catenin complex. (J. Balsamo *et al.*, 1996; L.J. Holsinger *et al.*, 2002; R. Nawroth *et al.*, 2002; J.A. Ukropec *et al.*, 2000; G.C. Zondag *et al.*, 2000). A number of PTPs interact with and dephosphorylate proteins that are involved in the formation and disassembling of focal adhesions, and are believed to control cell migration (M. Larsen *et al.*, 2003).

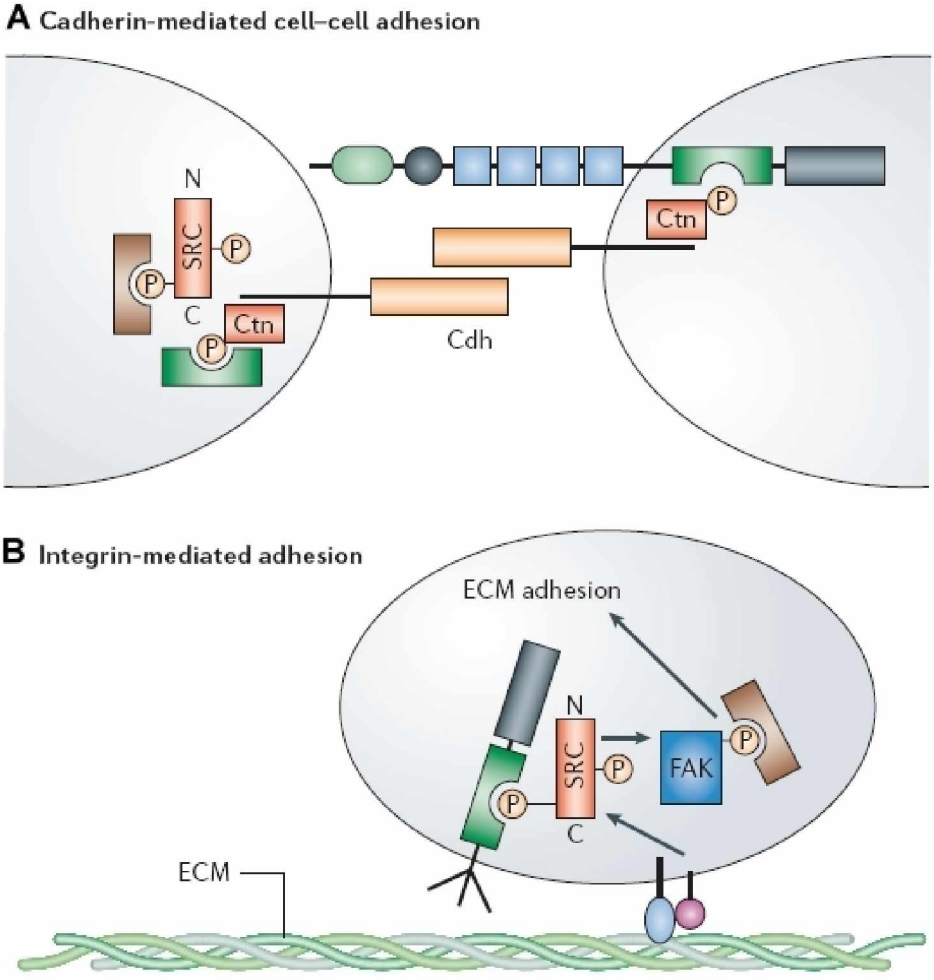


Fig. 2: Schematic illustration of the dual aspects of protein-tyrosine phosphatases in controlling cell–cell (**A**) and cell–matrix (**B**) adhesion. Signaling molecules are schematically drawn to illustrate the principles discussed. PTPs that antagonize mitogenic and adhesion signals are in brown, whereas PTPs that promote these signals are in green.

Tight junctions, or *zonulae occludentes*, are the most apical component of the intercellular junctional complex, which also includes adherens junctions, desmosomes, and gap junctions. Tight junctions visualized by electron microscopy are regions where the outer leaflets of plasma membranes from adjacent cells appear to fuse together and obliterate the intercellular space and may extend in depth from 0.2 to 0.5 μm (M.G. Farquhar and G.E. Palade, 1963). The tight junctions form a barrier to diffusion of molecules from the lumen to the tissue parenchyma (barrier function), which is not absolute but rather semi permeable, allowing the selective passage of certain solutes but

not others (J.M. Anderson *et al.*, 2004; M. Cereijido *et al.*, 2004). Tight junctions also help maintain cell polarity by forming an intramembrane diffusion fence that restricts diffusion of lipids in the exoplasmic leaflet of the plasma membrane (M.S. Balda and K. Matter, 1998; P.R. Dragsten *et al.*, 1981; G. van Meer and K. Simons, 1986).

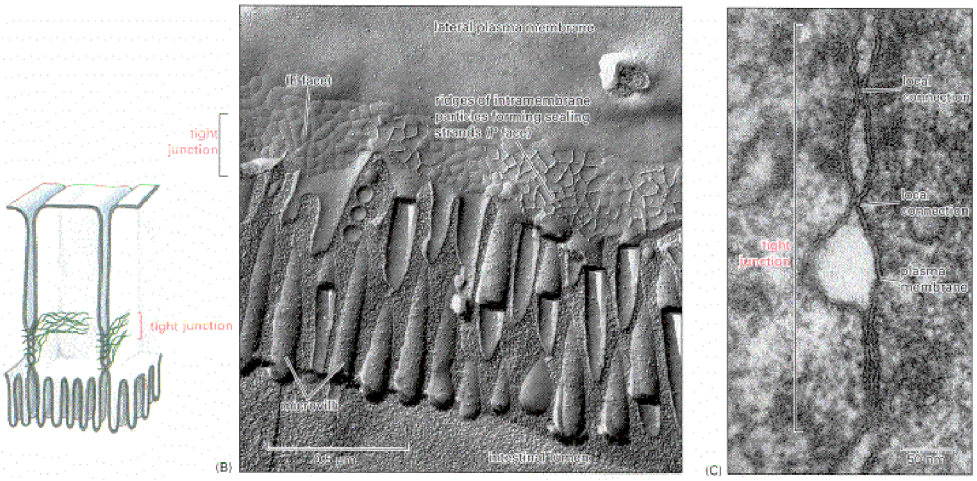


Fig. 3: The structure of a tight junction. The junctions are shown (A) schematically, (B) in a freeze- fracture electron micrograph, and (C) in a conventional electron micrograph (B. Alberts, 1994).

A set of different transmembrane proteins such as occludin, claudins, and junctional adhesion molecule (A.S. Fanning *et al.*) extend into the paracellular space and thus are candidates to create the seal, whereas the cytoplasmic plaque consists of a network of adaptor proteins that anchor the junction to the cytoskeleton and serve as a scaffold for the recruitment of signaling proteins.

The membrane-associated guanylate kinases (MAGUK), called ZO-1, ZO-2, and ZO-3, are thought to act as scaffolds for the transmembrane proteins, creating a link to the peri-junctional actin cytoskeleton, and as signal transducers controlling the

paracellular barrier (A.S. Fanning *et al.*, 1999). MAGUK proteins are composed of the following domains:

- Three PDZ (PSD95/Dlg/ZO-1) domains,
- an SH3 domain,
- a GK domain,
- an acidic domain, and
- an actin binding region (L. Gonzalez-Mariscal *et al.*, 2000).

The PDZ1 domain binds to claudins, while the GK domain is the binding site for occluding (K. Matter and M.S. Balda, 2003). SH3-GK domains are responsible for the binding to α-catenin and afadin (Y. Imamura *et al.*, 1999).

The best-studied protein within the cytoplasmic domain of TJs is the 220-kDa phosphoprotein ZO-1, which is likely to constitute the major backbone of the TJ plaque (S. Tsukita *et al.*, 1999). It has been reported that a fraction of ZO-1 accumulates in the nucleus in growing epithelial cells (C.J. Gottardi *et al.*, 1996).

ZO-1 is down-regulated in breast cancer tissues and during corneal wound repair, suggesting that expression levels of ZO-1 inversely correlate with the proliferation state of epithelial cells (Z. Cao *et al.*, 2002; K.B. Hoover *et al.*, 1998). This is also supported by the observations that ZO-1 becomes stabilized in confluent MDCK cells and that its expression levels increase with cell density (M.S. Balda and K. Matter, 2000; B. Gumbiner *et al.*, 1991). These results suggest that ZO-1 may serve multiple purposes within the cell, such as cell differentiation and regulation of transcription (K.J. Atkinson and R.K. Rao, 2001; C.J. Gottardi, *et al.*, 1996). Recent findings favor the notion that ZO-1 is directly involved in the establishment and sorting out of two distinct junctional domains, belt-like AJs and TJs, during epithelial polarization (J. Ikenouchi *et al.*, 2007).

Tight junction permeability in cell culture is generally measured using either of two techniques: transepithelial/transendothelial electrical resistance (TER) or

paracellular tracer flux. TER measurements are commonly used to assess the integrity of tight junctions. Electrical resistance across a monolayer represents the sum of the paracellular resistance, which consists of the resistance of the junction and the intercellular space, and the transcellular resistance, which consists of the resistances of the apical and basolateral cell membranes (P. Claude, 1978). Paracellular tracer flux may also be used to measure tight junction integrity. In this method, permeability to hydrophilic, uncharged paracellular tracers is measured using radioactive or fluorescently conjugated tracers such as mannitol or dextran.

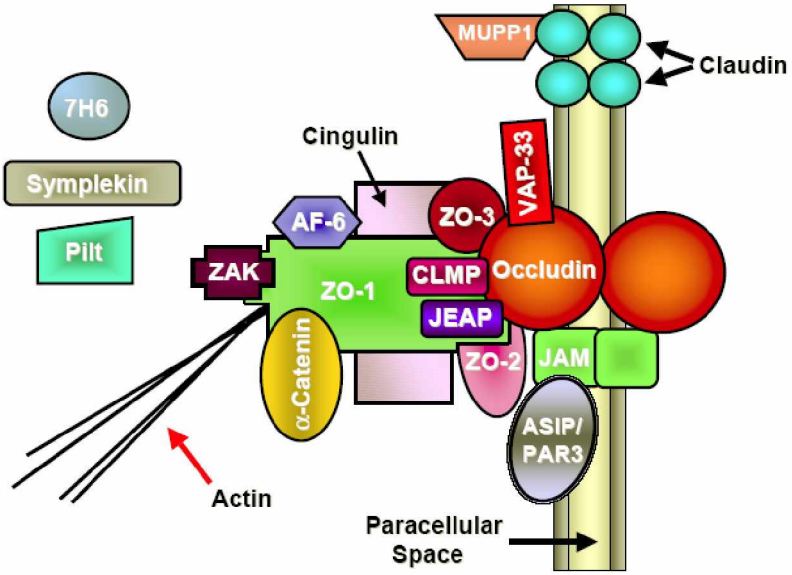


Fig. 4: Complexity of protein-protein interactions at the tight junction. This figure illustrates some of the many interactions that occur at the tight junction. Some of these interactions, such as those between claudins and ZO proteins, are necessary for the structural integrity of the tight junction. Others regulate signaling pathways that emanate from the junction.

Many PTPs Act as Tumor Suppressors

In cancer, oncogenic activation of tyrosine kinases is a common feature, and novel anticancer drugs have been introduced that target these enzymes. Although predictable on the basis of PTK/PTP antagonism, evidence showing that PTPs can function as tumor suppressors has become available only recently. The first example was PTEN, whose tumor suppressor function is associated with the dephosphorylation of the 3-position in the sugar head group of inositol phospholipids, thereby regulating phosphatidylinositol 3-kinase-dependent signaling pathways that are associated with cell survival (M. Cully *et al.*, 2006).

Another well-known tumor suppressor PTPs is SHP-1, a member of the non-TM-PTPs expressed predominantly in cells of the hematopoietic lineage. SHP-1 is an important negative regulator involved in signaling through receptors for cytokine/growth factors. Association of SHP-1 with the majority of these receptors is mediated by SRC-homology 2 (*SH2*) domains. SHP-1 acts by dephosphorylating the receptor and receptor-associated tyrosine kinases. Defective expression of SHP-1, as seen in the natural SHP-1 gene knock-out motheaten mice, results in hyperplasia of the erythroid and lymphoid lineages (Q. Zhang *et al.*, 2000).

A more complex picture is now emerging in relation to the roles of PTPs in cancer. In fact, overexpression of PTPα has been shown to cause persistent activation of the PTK Src, with concomitant cell transformation, suggesting a positive role for this PTP in tumorigenesis (X.M. Zheng *et al.*, 1992). More recently, several potential tumor suppressors have been identified among the classical PTPs, including receptor PTPs such as CD148 (C.A. Ruivenkamp *et al.*, 2002) and PTPκ (encoded by *PTPRK*). An analysis of mutations in *PTP* genes in colorectal cancers also identified several candidate tumor suppressors the most frequently mutated was the receptor PTPρ.

In addition to inactivating mutations in the intracellular PTP domains, a large number of missense mutations were detected in the extracellular segment of this receptor-like PTP, which highlights the importance of this domain for the function of the enzyme, and its potential role in ligand binding. Similar observations were made for two other RPTPs, LAR and PTPγ. The spectrum of potential tumor suppressor PTPs has now broadened with the observation of epigenetic regulation by hypermethylation of CpG sites in the promoters of several members of the PTP family (S.T. Jacob and T. Motiwala, 2005).

These include the receptor PTPRO (also known as GLEPP1) in hepatocellular carcinoma and lung cancer, Src-homology-2-domain-containing protein tyrosine phosphatase-1 (SHP1) in leukaemia and lymphoma, FAP1 in hepatocellular carcinoma as well as the dual specificity phosphatases PTEN in breast cancer and mitogen-activated-protein-kinase phosphatase-3 (MKP3) in pancreatic cancer. In principle, such gene-silencing events might be a target for anti-cancer therapies.

CD148

State of the Art

CD148 is a type-III RPTP with a broad pattern of tissue expression. It is expressed on fibroblasts and endothelium, as well as epithelial and hematopoietic cells (L.G. Borges *et al.*, 1996; H. Honda *et al.*, 1994; J.M. Osborne *et al.*, 1998). The CD148 gene, also known as *Ptprj*, is located on chromosome 11p11.12 in humans, and consists of 25 exons distributed over 150 Kb of genomic DNA (H. Honda, *et al.*, 1994). The

protein contains a bulky extracellular domain of 970 aminoacids organized in eight fibronectin type III repeats, a 25-aminoacid long transmembrane domain and a 342 aminoacid cytoplasmic portion with a single catalytic domain. Its mass varies from 200 to 250 KD depending on different N- or O-glycosylations in the extracellular domain.

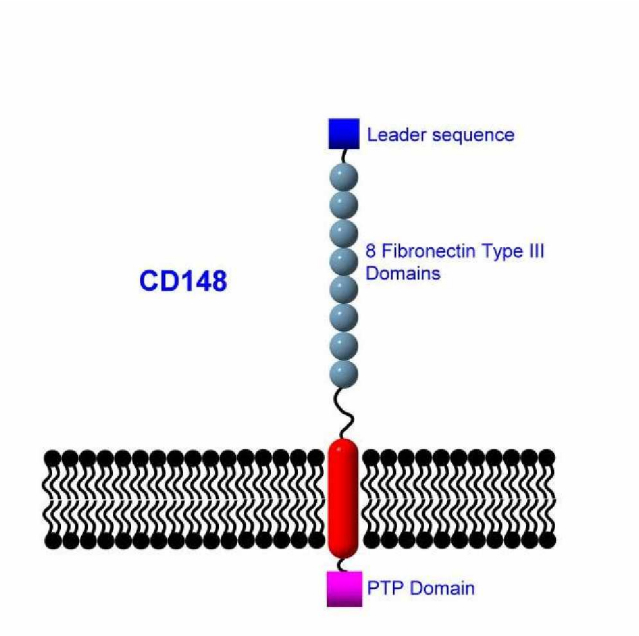


Fig. 5: Schematic view of the CD148 molecule.

Biochemical Activity of CD148

As mentioned above, the activity of many PTPs may be regulated by phosphorylation, and this seems to occur in the case of CD148. Jallal *et al.* reported that CD148 is tyrosine-phosphorylated after stimulation with epithelial growth factor (EGF) (B. Jallal *et al.*, 1997), although no change in phosphatase activity was documented.

Even if it does not affect CD148 phosphatase activity, this phosphorylation event might have an important role in promoting interactions with SH2-containing proteins involved in the metabolic networks in which CD148 signal transduction pathways participate.

Although protein dimerization is a key mechanism that simulates PTK activity, PTPs are generally not regulated in this way. Some lines of evidence, however, suggest that CD148 could indeed dimerize. In fact, the transmembrane domain contains the same dimerization consensus sequence (C.N. Chin *et al.*, 2005) found in RPTPα, whose dimerization inhibits its phosphatase activity (G. Jiang *et al.*, 1999). It is not clear if dimerization inhibits or induces CD148 phosphatase activity, since experimental data lead to conflicting results. For example, in lymphocytes CD148 dimerization (induced by cross-linking) was shown to induce proliferation (S.G. Tangye *et al.*, 1998); on the contrary, in endothelial cells dimerization induced by a bivalent antibody seems to act in an opposite way, promoting CD148 activation (T. Takahashi *et al.*, 2006).

CD148 Is Involved in Cell Contact Inhibition

CD148 is a PTP whose activity influences many aspects of cellular metabolism, but it is especially known for its role in promoting cell contact inhibition. The first paper on CD148 reported that its expression gradually increased in fibroblasts that became confluent. For this reason CD148 is also known as DEP-1 (i.e. Density Enhanced Phosphatase-1: A. Ostman *et al.*, 1994). The molecular mechanisms accounting for this phenomenon are unknown, but they might be induced by a homophilic interaction between the FNIII motifs of CD148 located in adjacent cells, as

similar observation have been already reported for PTPμ and PTPκ (M.F. Gebbink *et al.*, 1993; J. Sap, *et al.*, 1994).

Expression of CD148 is increased more than 10-fold in many cell types as they approach confluence, suggesting it contributes to cell-cell adhesion and growth contact inhibition (A. Ostman, *et al.*, 1994). CD148 is present at the apical surface of endothelial cells, but also co-localizes with VE- cadherin at intercellular junctions (T. Takahashi *et al.*, 1999; T. Takahashi *et al.*, 2003), and it indirectly associates with VE-cadherin by binding to p120*ctn*, γ-catenin (plakoglobin), and β-catenin. CD148 regulates their phosphorylation state, preserving their interactions with the cadherins and promoting cell-cell adhesion (L.J. Holsinger, *et al.*, 2002).

The role of CD148 in organizing cell-cell junctions is supported by the observation that in cells lacking strong cell-cell adhesion, such as fibroblasts, overexpression of CD148 results in a change in cadherins localization from discrete areas of cell-cell contact to large areas reminiscent of the continuous AJ found in epithelial cells (L.J. Holsinger, *et al.*, 2002; S. Kellie *et al.*, 2004).

CD148 Substrates

A number of studies in cell culture have indicated an antagonistic role of CD148 in growth factor signaling by direct dephosphorylation. Targets identified so far include the platelet-derived growth factor receptor (PDGFR), the vascular endothelial growth factor receptor, (VEGFR) and the hepatocyte growth factor/scatter factor receptor (HGFR/SF). Platelet-derived growth factors and their cognate receptors are important regulators of cell proliferation, cell migration, cell differentiation, and cell-matrix

interactions. CD148-mediated specific dephosphorylation of PDGFR (M. Kovalenko, *et al.*, 2000) was shown to influence PDGF transduction pathway, inducing a strong reduction of the phospholipase Cγ (PLCγ) activation and the extracellular signal-regulated kinase phosphorylation(M. Tijsterman *et al.*, 2002). Since these molecules are involved in cell proliferation, it is believed that CD148 could control cell proliferation through PDGFR. On the other hand, the phosphoinositide 3-kinase (PI3K) activation was only slightly affected, suggesting a less important role of CD148 in cell survival.

Kappert *et al.* identified CD148 as the dominating factor determining PDGF-phosphorylation and VSMCs proliferation in injury-induced neointima formation. In support of these findings, cultured $CD148^{-/-}$ cells displayed increased PDGF-dependent cell signaling. Hyperactivation of PDGF-induced signaling was also observed after siRNA-down-regulation of CD148 in VSMCs (K. Kappert *et al.*, 2007).

Moreover, CD148 indirectly increases cell-matrix adhesion by activating Src via its interaction with PDGFR (E. Jandt *et al.*, 2003). CD148-Src relationships were accurately investigated by Pera *et al*. Not only did they show a specific interaction between CD148 and Src, but they also identified the phosphotyrosine residue dephosphorylated by CD148, namely PY529, the one involved in Src activation. Interestingly, other proteins involved in the adhesion process, like the focal adhesion kinase (FAK) and paxillin, were shown to be phosphorylated and therefore activated after Src CD148-dependent dephosphorylation (I.L. Pera *et al.*, 2005).

Another growth factor receptor dephosphorylated by CD148 is Met, the receptor for HGF/SF, that has an expression pattern overlapping with CD148. Met induces mitogenic, motogenic, and morphogenic responses after ligand activation by recruiting a number of signaling and docking molecules, and has been implicated in the phosphorylation of cell junction proteins. Disruption of normal signaling through Met has been implicated in certain cancers. Met presents various tyrosine residues which

could potentially be dephosphorylated, but CD148 preferentially dephosphorylates PY1349 and PY1365. The first one promotes the recruitment of signaling and adapter molecules including Gab1, while the second one is essential in mediating morphogenic signals. CD148 does not dephosphorylate the phosphotyrosine of the activation loop, so it does not inhibit the kinase activity of Met. However, it may modulate Met downstream transduction pathway controlling the specificity of its activity (H.L. Palka *et al.*, 2003).

Although one of the first identified functions of CD148 was its role in contact inhibition (A. Ostman, *et al.*, 1994), the biochemical pathway that controls this mechanism is still poorly understood. Lampugnani *et al.* approached the problem by studying CD148 behavior in confluent endothelial cells, where CD148 was found to be enriched at cell junctions. Confluent endothelial cells stimulated with VEGF do not proliferate and show a reduced phosphorylation of VEGFR2. This study showed that CD148 specifically dephosphorylates VEGFR-2 only if it co-localizes with cadherin and VEGFR-2, a cluster occurring exclusively in confluent cells (M. Grazia Lampugnani *et al.*, 2003). Altogether, these data suggest an important role for CD148 in cell-cell contacts and adherens junctions turn-over.

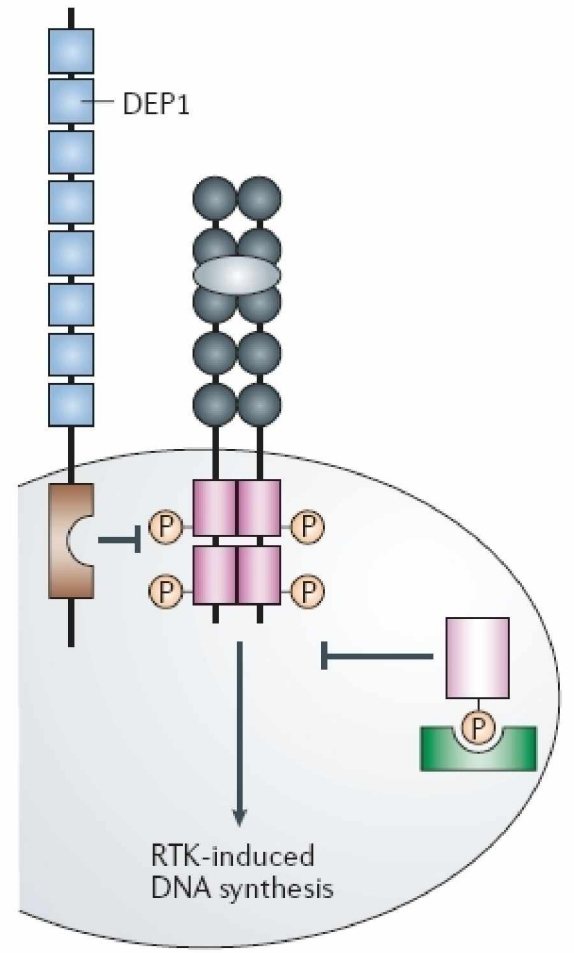


Fig. 6: Protein-tyrosine phosphatases (PTPs) can antagonize receptor-tyrosine kinase (RTK) signaling through direct receptor dephosphorylation, as described for DEP1-mediated dephosphorylation of platelet-derived growth factor (PDGF) receptors.

CD148 Acts as a Tumor Suppressor

Like many phosphatases, CD148 plays a role in cancer, its down-regulation being documented in different kinds of tumors. Additionally, in cultured breast cancer cells CD148 re-expression led to a five- to ten-fold reduction in cell growth (M.M. Keane *et al.*, 1996). Similar findings were later reported in pancreatic, thyroid, and colon cancer cells (C.A. Ruivenkamp, *et al.*, 2002; F. Trapasso *et al.*, 2000; F. Trapasso *et al.*, 2004).

CD148 re-expression was also described to induce differentiation in breast and thyroid undifferentiated tumor cells (R. Iuliano *et al.*, 2003; M.M. Keane, *et al.*, 1996). To understand the role of CD148 in cancer, many studies were carried out to analyze *Ptprj* gene status in human tumors. The most thorough analysis identified *Ptprj* as the functional gene at the mouse colon-cancer susceptibility locus, Scc1 (C.A. Ruivenkamp, *et al.*, 2002). This locus was originally defined based on its segregation with colon cancer susceptibility after crossing cancer-resistant and cancer-susceptible mouse strains (C.J. Moen *et al.*, 1996). Sequence differences in *Ptprj* between the cancer-susceptible and cancer-resistant strains were also identified, supporting the theory that tumor-susceptibility is conferred by certain *Ptprj* variants.

Ptprj status has also been analyzed in human tumors (R. Iuliano *et al.*, 2004; C.A. Ruivenkamp, *et al.*, 2002). Loss of heterozygosity, occurring in the absence of acquired mutation in the remaining allele, has been found in breast, colon, lung and thyroid cancers, implicating *Ptprj* haploinsufficiency as a transforming mechanism in humans. In association with these studies, different allelic variants of human *Ptprj* were identified; these variations result in CD148 proteins that have differences in their extracellular-domain residues (C. Ruivenkamp *et al.*, 2003).

CD148 Regulates Cell Growth and Cell Cycle

The tumor-suppressive role of CD148 was clearly displayed in experiments where highly malignant thyroid cells, after transduction by a retrovirus carrying CD148, appeared to acquire a normal phenotype and showed increased adhesion, restoration of differentiation, reduced proliferation and decreased tumorigenicity (F. Trapasso, *et al.*,

2000). The same report established that CD148 induced a G1 growth arrest by suppressing the degradation of the cyclin-dependent kinase inhibitor p27kip1 protein.

The antiproliferative role of CD148 has also been associated with somatostatin, an endogenous negative regulator of proliferation whose activity is mediated by a family of five G protein-coupled receptors (named SSTR1–5). Interestingly, somatostatin antiproliferative effect is reported only in CD148-coexpressing cells. The inhibition of cell proliferation caused by somatostatin treatment is due to an increased activity of CD148, which directly dephosphorylates ERK1/2 (A. Massa *et al.*, 2004).

CD148 role in controlling endothelial cell proliferation is essential for the development of a well structured vascular system, which is at the basis of a correct embryo formation. Homologous recombinant mice were generated expressing a mutant CD148 (CD148ΔCyGFP), from which cytoplasmic phosphatase sequences had been removed and substituted in frame by sequences encoding enhanced green fluorescent protein (EGFP) to create a CD148/GFP fusion protein. In the first days of embryo development, a normal endothelial differentiation occurred, but after E8.5, when CD148 expression appears evident, endothelial progenitor cells began to proliferate in an uncontrolled way. Developmental consequences were severe and knock-in embryos died at 10.5 days of gestation, with failed developmental vascularization and growth retardation (T. Takahashi, *et al.*, 2003).

It must be noted however, that recently Trapasso *et al.* showed that mice deficient for CD148 were viable, fertile, and showed no gross anatomical alterations (F. Trapasso *et al.*, 2006). This result could be accounted for by redundant pathways operating in early development.

CD148 Is Expressed in the Hematopoietic Tissue

CD148 is ubiquitously distributed throughout the hematopoietic tissue, but its expression level is quite variable among cell lineages. The highest levels are found in macrophages, then in B cells, neutrophils and erythroid cells. T cells show the lowest level of CD148, but its expression markedly increases upon T-cell receptor (TCR) activation (J. Lin *et al.*, 2004).

Despite its abundant distribution, CD148 role in hematopoietic cells is far from being elucidated; only few studies have investigated the increased expression of CD148 in T cells. In these cells, CD148 is thought to act as a critical regulator of the signal transduction cascade induced by TCR activation, contrasting T cell proliferation. The identified CD148 molecular targets are LAT and PLCγ1, whose dephosphorylation inhibits fundamental transduction pathways (J.E. Baker *et al.*, 2001). The inhibitory role of CD148 only occurs in the last phases of T-cell activation, due the formation of the immunologic synapse at the interface between T-cell and the antigen presenting cell (APC). The immunologic synapse is formed by proteins organized in an ordered structure, from which proteins not involved in T-cell activation are excluded. Also due to its large extracellular domain, CD148 is excluded from the immunologic synapse and normally does not inhibit TCR activation. Only at the end of T-cell activation, with the dissociation of the immunologic synapse, does CD148 interact with its substrates and down-regulates TCR activity. Exclusion from the immunologic synapse is therefore a stratagem to prevent the premature termination of T-cell immune response (J. Lin and A. Weiss, 2003).

Macrophages are the hematopoietic cells where CD148 is more abundantly expressed, suggesting its important role in their function. As previously discussed, CD148 controls proliferation and differentiation in many cell types. Consistent with this

finding, in macrophages a down-regulation of CD148 expression after colony-stimulating factor-1 (CSF-1) stimulation and an up-regulation after lipopolysaccharide (LPS) was documented (J.M. Osborne, *et al.*, 1998). Little is known about CD148 role in eosinophils; the only available data suggest that it might control degranulation and induce superoxide anion generation (V. del Pozo *et al.*, 2000).

The work described herein addresses the role of CD148 in epithelial tissues with a somatic knock-down approach.

Choice of the Experimental System

RNA Interference

Gene silencing mediated by double-stranded RNA (dsRNA) is a sequence specific, highly conserved mechanism in eukaryotes. Originally recognized in plants and lower organism (C. Napoli *et al.*, 1990; F. Ruiz *et al.*, 1998) the phenomenon was recently extended to almost all eukaryotic organism, including mammals (S.M. Elbashir *et al.*, 2001a). RNA interference (RNAi) is believed to have originated in order to protect the genome against invasion by mobile genetic elements such as viruses and transposons (M. Tijsterman, *et al.*, 2002; P.M. Waterhouse *et al.*, 2001), and to orchestrate developmental programs. The elements responsible for RNAi are short interfering double stranded RNA (siRNA) molecules originated in the cell cytoplasm by the activity of an RNase III, called Dicer, which cleaves long doubled-stranded RNA. siRNAs are 21–23 nucleotide long and present symmetric 2-3 nt 3' overhangs and 5'-phosphate and 3'-hydroxyl groups (S.M. Elbashir *et al.*, 2001b). siRNAs are then

incorporated into an RNA inducing silencing complex (L. Gonzalez-Mariscal, *et al.*, 2000), which unwind the siRNA duplex and guide the antisense strand to its homologous target mRNA for the endonucleolytic cleavage (A.M. Denli and G.J. Hannon, 2003). RNAi mediated by the introduction of long dsRNA has been used as a method to investigate gene function in various organisms; however, the applicability of this approach is limited in mammals because the introduction of dsRNA longer than 30 nt induces a sequence non-specific interferon responses. This impasse was broken with the introduction of synthetic siRNAs which can efficiently and specifically silence gene expression (S.M. Elbashir, *et al.*, 2001b).

The design of this molecules should be accurately studied considering not only the sequence of the fragment but also the target mRNA whose secondary structure can sometimes affect the efficiency of silencing. Another mechanism that is lacking in mammalians cells is the siRNA amplification mechanisms that confer RNAi potency and longevity in lower organisms (Y.L. Chiu and T.M. Rana, 2002). Prolonged gene silencing has become possible even in mammalian cells with the development of expression vectors allowing for the production of shRNAs that can be converted by Dicer into functional siRNAs. There are different choices of vector-driven systems but most are based on the production of a single RNA from a pol III-driven plasmid that forms a stem-loop structure in which the sense and antisense strands form the stem of the hairpin. Termination of transcription at a stretch of thymidine bases results in the generation of a 2–4 bp uridinenucleotide overhang at the 3' end, identical to the overhang that is normally produced by the Dicer enzyme (J.Y. Yu *et al.*, 2002). Recently, another even more powerful strategy was developed, retroviral-mediated delivery of siRNA, which allows the integration of the siRNA into the target cells in order to almost permanently silence the expression of a gene (G. Tiscornia *et al.*, 2003).

Lentiviral Vectors

Lentiviral vectors are versatile tools for gene transfer given their ability to stably integrate into target cells irrespective of proliferative status. They are replication-incompetent derivatives of Lentiviruses, therefore belonging to the large Retrovirus family of viruses (which also includes Oncoroviruses and Spumaviruses).

Lentiviruses are lipid-enveloped viruses containing a homodimer of linear, positive-sense, single stranded RNA genomes of 7-11 kilobases. Following entry into the target cells, the RNA genome is retro-transcribed into linear double stranded DNA (provirus) and integrated into the host genome and serves as a template for the production of progeny virions. All retroviral genomes have two long terminal repeat (LTR) sequences at their end. LTRs and neighboring sequences act in cis during viral gene expression and packaging, retrotranscription and integration of the genome. The LTR sequences frame the tandem gag, pol and env genes encoding the structural proteins, nucleic-acid polymerases/integrases and surface glycoprotein, respectively (S.P. Goff, 2001). Lentiviruses have a more complex genome; in addition to the gag, pol, and env genes, they encode two regulatory genes, tat and rev, essential for expression of the genome, and a variable set of accessory genes (R. Desrosiers, 2001).

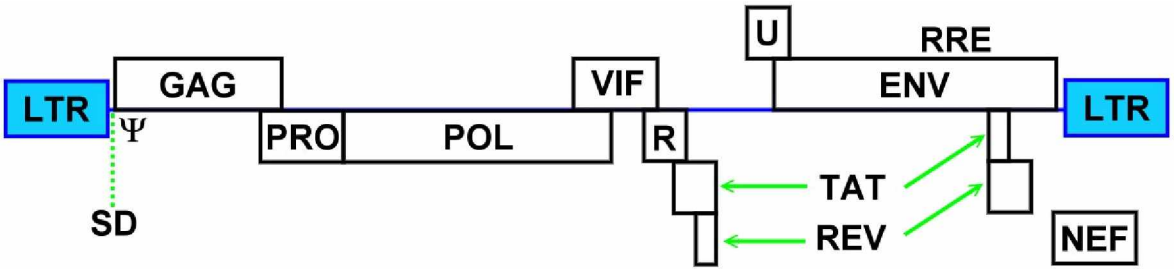


Fig. 7: Schematic drawing of the HIV provirus.

Integration into the host genome is a fundamental feature of retroviruses. An important difference between prototypic retroviruses and lentiviruses are the basic requirements for integration: the former are dependent on cell cycling, and viral gene transfer can only occur in host cells that are actively replicating at the time of infection (D.G. Miller *et al.*, 1990). In contrast, lentiviruses can transduce non-dividing cells (L. Naldini *et al.*, 1996), because the lentiviral genome is actively transported into the nucleus (A. Follenzi *et al.*, 2000; V. Zennou *et al.*, 2000). Lentiviral vectors are replication-incompetent derivatives of lentiviruses (HIV-1 in our system) whose genome has been segregated into distinct units to improve the biosafety properties of the system. A number of non essential viral genes have been removed, and *cis*-acting sequences have been split from genes required in *trans*. The first generation of HIV-based vectors comprised all HIV-1 protein except Env and was constituted by 3 plasmids: (1) the packaging construct, which contained all the viral genes driven by the CMV promoter; (2) the transfer vector, which contained HIV-1 *cis*-acting sequences and the expression cassette for the trangene; (3) the envelope-coding plasmid, which encoded a heterologous envelope protein for pseudotyping the vector particles (L. Naldini, *et al.*, 1996).

A major step towards improved biosafety was the deletion of four additional genes that encode virulence factors (Vpr, Vif, Vpu and Nef) whose removal did not affect the transducing vector effectiveness (R. Zufferey *et al.*, 1997). The more recent third-generation lentiviral vectors (see **Fig. 8**) retain only three of the nine genes present in the genome of the parental virus (gag, pol and rev), minimizing the risk for the development of replication-competent particles (RCR). In fact, the tat gene was deleted and the transfer vector transcription was supported by replacing the 5' U3 region with the RSV promoter. The Rev gene was also removed from the packaging construct and inserted in a fourth plasmid with an upstream RSV promoter (T. Dull *et al.*, 1998). In

addition, these vectors carry a 400 bp deletion in the U3 region of the 3' LTR of the provirus, which is copied at the 5' during reverse transcription thus generating transcription-dead LTR sequences. Vectors with this design are known as "self-inactivating" (SIN), and strongly reduce the likelihood of insertional mutagenesis (R. Zufferey *et al.*, 1998).

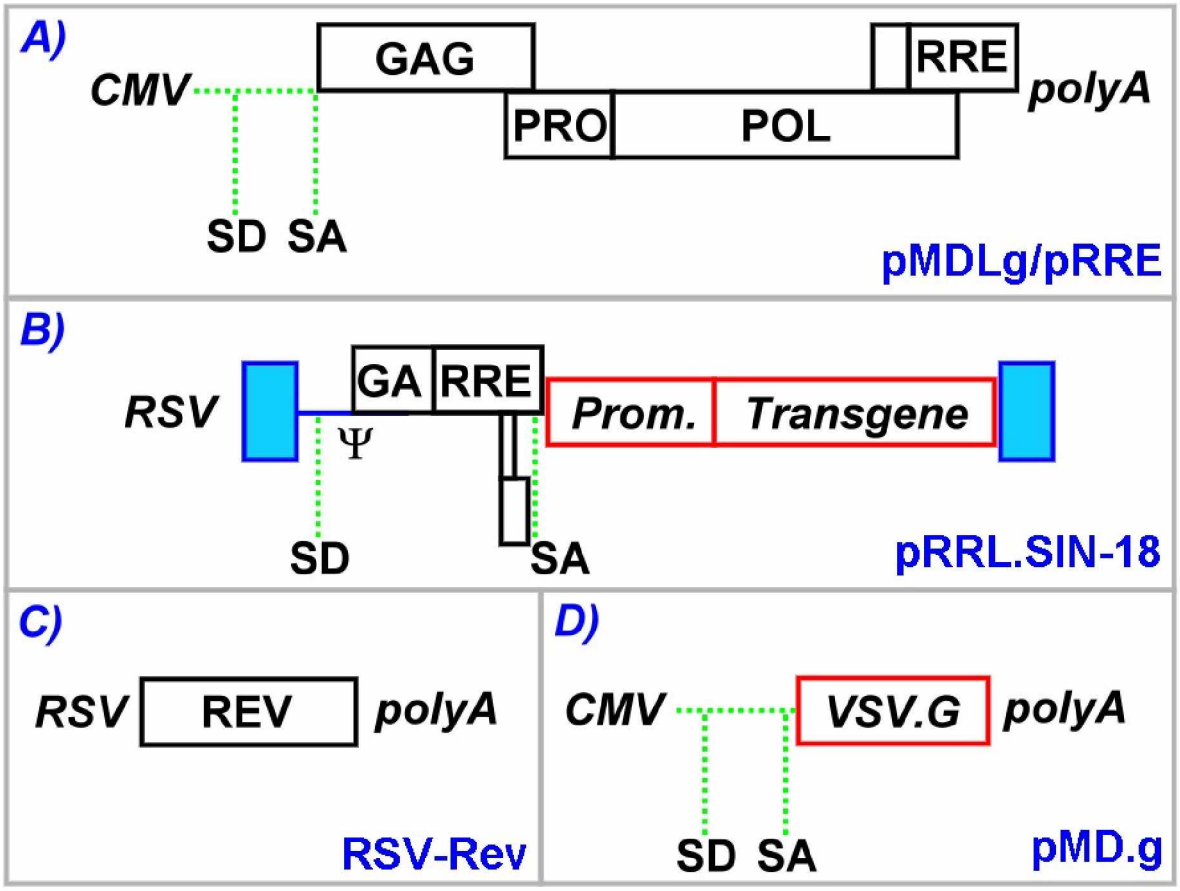


Fig. 8: Basic structure of the 4-plasmid lentiviral system used (T. Dull, *et al.*, 1998). The construct in B is the viral genome containing viral genetic components required in cis and the expression cassette for the transgene; A contains structural viral genes required in trans; C drives expression of REV (required for efficient nuclear export of viral transcripts), and D drives the expression of the envelope (VSV-G).

Further improvements were made to enhance viral transduction efficiency, such as the insertion of the WPRE (Woodchuck hepatitis virus post-transcriptional regulatory element) in the 3' untranslated region of the vector (R. Zufferey *et al.*, 1999), or the incorporation of the polypurine tract (cPPT) of the pol gene upstream of the expression cassette (A. Follenzi, *et al.*, 2000). The procedure for lentiviral vector production consists in the co-trasfection of the four plasmids into 293T cells, a particularly efficient packaging cell line. Vector preparations are usually concentrated by ultracentrifugation (see Materials and Methods) to purify particles and increase titers.

MATERIALS AND METHODS

Cell Lines

A431 (human epidermoid carcinoma), Caco-2 (human colon adenocarcinoma), and 293T (human renal epithelium) cells were cultured at 5% CO2 in Dulbecco's modified Eagle's medium containing 10 % fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 2mM L-glutamine. D-MEM/F-12 (1:1) medium and RPMI 1640, containing 10 % fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 2mM L-glutamine were used for T84 (human colon carcinoma) and THP-1 (human acute monocytic leukemia) cells, respectively, and they were cultured in the same conditions.

ShRNA Design

shRNAs were constructed according to previous identified siRNAs (Galimi unpublished). Each hairpin contained a double-stranded stem of 19–29 bp, identical in sequence to the target mRNA; the 2 strands of the stem are connected by a loop of 6–9 bases, which is removed by Dicer to generate effective siRNAs. The constitutive expression of the shRNAs is driven by an RNA polymerase III (pol III) promoter.

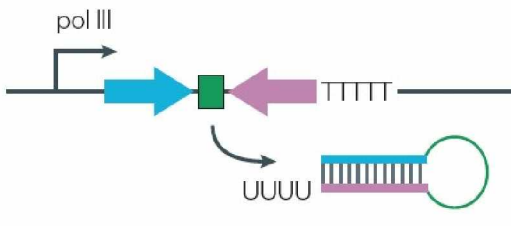


Fig. 9: Schematic drawing of a shRNA (D.M. Dykxhoorn *et al.*, 2003).

We used a human U6 snRNA promoter to obtain stable and efficient suppression of CD148 gene. Transcription terminates at a stretch of five thymidine nucleotides, and the nascent RNA is processed in such a way that the 3'-terminus will contain two uridine nucleotides, resembling the ends of natural siRNAs. Three different shRNA (# 1525, # 785 and # 884) were produced against the nucleotide sequence of the gene for the extracellular domain of human CD148 from validated target sequences (Galimi unpublished).

As appropriate controls we used lentiviral vectors carrying a silencing cassette against human cyclophilin gene (PPIB), or lacking a silencing cassette, carrier of the GFP cassette only.

siRNA sequences (sense strand) for human CD148 and human cyclophilin B gene are (**Tab. 1**):

CD148-1525 siRNA	5' GAG TCG TCA TCT AAC TAT A –dTdT 3'
CD148-745 siRNA	5' CCA ACC AAT GTG ATC TTA A –dTdT 3'
CD148-884 siRNA	5' CAG CGA CTT CAT ATG TAT T –dTdT 3'
HuCyclophilin siRNA	5' TGG TGT TTG GCA TTG TTC –dTdT 3'

Tab: 1.

Lentiviral Vector Constructs

In the old version, the shRNA cassette was cloned between the GA and the REE sequence of the pLV616 transfer vector plasmid. The pLV616 vector contains a gene marker cassette between the cPTT and the WPRE sequence. The cassette is composed of a hEF1a promoter followed by the GFP and neo genes, which are connected by an IRES (internal ribosome entry site) sequence.

In the new version, a *NheI* site was inserted within the 3' U3 region of the p156RRL-sinPPTCMVGFPPRE LTR. The hU6shRNA#1525Pol III Terminator template was cloned from the old constructs by using the primers GGCATTGAGCTAGCAGCA-GGCTTAGATCTGAA LV (forward) and GGCATTGAGCTAGCATCTCCTCCTCC-AGGTCT (reverse). The design of the primers incorporated a *NheI* site which allowed the insertion of the 2 shRNAs into the *NheI* site of the p156RRLsinPPTCMVGFPPRE plasmid.

The resulting genome construct was therefore characterized by two shRNA cassettes inserted into the 2 LTR and by a shorter gene marking cassettes composed by the CMV-GFP gene.

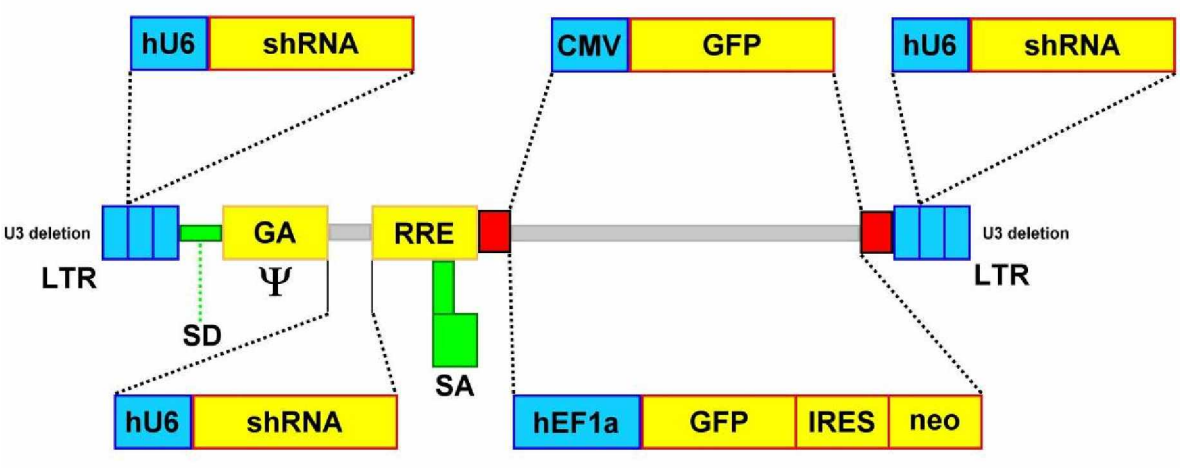


Fig. 10: Alternative genome structures of nLV-shRNA (P. Carmeliet *et al.*, 1999) and LV-shRNA (down) lentivectors. The structure shown at the top allows for high titer and higher level of gene silencing, due to the shortest overall genome size and double copy of the siRNA cassette.

ShRNA Hairpin Cassette Design for hCD148#745 and hCD148#884

The generation of shRNA-hCD148#745 and shRNA-hCD148#884 initially required designing PCR primers with siRNA coding sequence. Briefly, two separate PCR reactions have been performed for each siRNA: the first one produced the U6 region and the sense strand of the hairpin, using primers forward *NheI*-pLVshRNA and reverse specific for each siRNA (Rw-U6-745, Rw-U6-884).

The second PCR reaction amplified the loop region with the antisense strand of the hairpin, followed by the pol III terminator by using primers reverse *NheI*-pLVshRNA and forward specific for each siRNA (Fw-U6-745, Fw-U6-884) , (see **Tab. 2**, below). Then, PCR fusion has been set up. The PCR products were subcloned into the new p156RRLsinPPTCMVGFPPRE plasmid after restriction enzyme digestion with *NheI*.

It was restriction mapped and also the sequencing reaction to confirm the integrity of the plasmid.

PCR1	FwNheI-pLVshRNA	5' GGC ATT GAG CTA GCA GCA GGC TTA GAT CTG AA 3'
	Rw-U6-primer-745	5' TTA ATC TCA TGA ATT AAG ATC ACA TTG GTT GGC GGT GTT TCG TCC 3'
PCR2	Fw-U6-primer-745	5' TTA ATT CAT GAG ATT AAG ATC ACA TTG GTT GGC TTT TTA CCC AGC 3'
	RwNheI-pLVshRNA	5' GGC ATT GAG CTA GCA TCT CCT CCT CCA GGT CT 3'

PCR1	FwNheI-pLVshRNA	5' GGC ATT GAG CTA GCA GCA GGC TTA GAT CTG AA 3'
	Rw-U6-primer-884	5' TAT TTC TCA TGA AAA TAC ATA TGA AGT CGC TGC GGT GTT TCG TCC 3'
PCR2	Fw-U6-primer-884	5' TAT TTT CAT GAG AAA TAC ATA TGA AGT CGC TGC TTT TTA CCC AGC 3'
	RwNheI-pLVshRNA	5' GGC ATT GAG CTA GCA TCT CCT CCT CCA GGT CT 3'

Tab. 2: Up: shRNA 745 PCR primers. **Down:** shRNA 884 PCR primers.

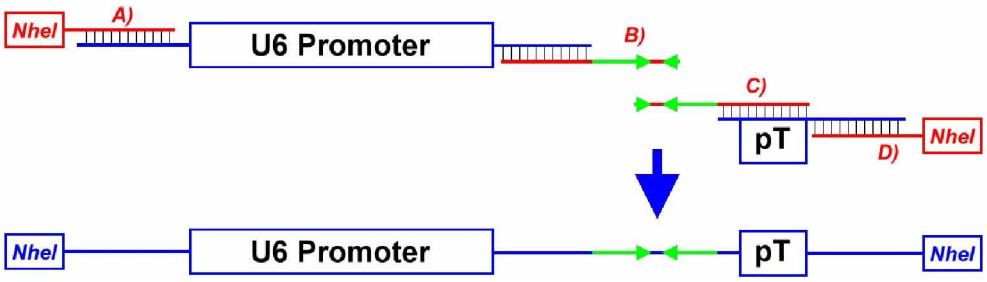


Fig. 11: Construction of shRNA hairpin cassette by PCR fusion.

Lentiviral Vector Production

Recombinant lentiviruses were produced by transient co-transfection in 293T cells using the calcium-phosphate protocol. About $3X10^6$ cells were plated the day before in a poly-L-lysine coated 15 cm plates. A total of 50.6 μg of endotoxin-free plasmid mix was used for the transfection of one dish: 22.5 μg of the transfer vector plasmid, 14.6 μg of the packaging plasmid pMDLg/p RRE, 5.6 μg of pRSV.Rev, 7.9 μg of the envelope plasmid pMD.G. The precipitate was formed by adding plasmids to a final volume of 1.125 μl of CaCl2 0.25 M, mixing well, then adding 1.125 μl of 2X BBS while vortexing. After an incubation of 15' the mix was added dropwise onto 293T cells and the plate was incubated at 3% CO2 for 16 hours, when medium was changed and cells were reincubated at 5% CO_2.

Infectious lentiviruses were harvested at 48 and 72 h post-transfection and filtered through 0.22-μm-pore cellulose acetate filters as described (T. Dull, *et al.*, 1998; L. Naldini, *et al.*, 1996). Recombinant lentiviruses were concentrated by ultracentrifugation (2 h at 50.000 g) and subsequently purified on a sucrose 20% gradient (2 h at 46.000 g). The resulting pellet was resuspended in 200 μl of Hank's buffer. Vector concentrations were analyzed by using an immunocapture p24-gag ELISA (Alliance, DuPont/NEN).

GFP Lentiviral Vector Titration

293T cells were plated in a 24-well-plate and transduced in 200 µl medium 36 hour later with 3-fold dilution of the virus prep (0.1 µl to 1.46 10^4 µl from the concentrate). Medium was changed after over-night incubation (0.5 ml/well).

Cells were counted in one well at the time of infection, they should be 10-15 10^4 per well. After 4-5 days the last effective dilution was checked in the following way. 293T cells were detached with 0.5 ml of EDTA 0.5 mM, resuspended and the percentage of green-fluorescent cells were analyzed by flow cytometry.

The specific activity (T.U./ml) on the samples was calculated in the linear range, according to the formula (P x N) / (100 x V). Where P is the number of GFP-positive cells in a given well (in %), N is the number of cells per well, V is the volume of virus used for infection of that well (in ml).

Safety Check

$5X10^4$ of HIV-permissive cells (C8166, 293G, or HeLa-CD4) were plated in 24-well plate and transduced with 1 µl of concentrated vector. Confluent cells were split 1:10 for 7 passages and 200 µl medium were checked for p24. The undiluted medium should have no detectable p24.

Lenti-shRNA Validation

A431, Caco-2 and THP-1 cells were seeded on a 24-well dish and transduced with 1 μl of nLV-sh-hCD148, or with 1 μl of nLV-GFP at a confluence of 70%. CD148 expression was detected by flow cytometry 7 days, 2 weeks and 5 weeks post transduction as described. Cells were harvested, counted, and resuspended in ice-cold staining buffer (PBS + 3% FBS). Subsequently, primary antibodies were added. A431, Caco-2 and THP-1 cells were stained with a mouse anti-human CD148 antibody 143-41 (Biosource) at a final concentration of 10 μg/ml. The cells were then incubated at 4°C for 30 minutes, and they were washed twice in staining buffer. The secondary antibody used in these experiments was Alexa Fluor® 647 goat anti-mouse IgG1 at a final concentration of 10 μg/ml. Cells were then incubated at 4°C for 25 minutes in the dark, washed twice, and then resuspended in 500 μl of staining buffer. Samples were finally analyzed on FACScan flow cytometer (Becton Dickinson) using Cellquest software (Becton Dickinson). CD148 expression was analyzed 10 days post-transduction.

Transcript Analysis

T84 wild-type cells were plated in a 24-well plate, at different cell densities (160.000, 80.000, 40.000, 20.000, 10.000 and 5.000 cells/well). 4 days later, medium was removed and CD148 mRNA levels were measured by bDNA assay (QuantiGene® Reagent System, Bayer).

A431 and Caco-2 Growth Curves

A431 and Caco-2 wild-type or CD148 KD were plated in a 96-well plate at a density of 1000 cells and 2000 cells per well for A431 and Caco-2 cells, respectively. Cell proliferation was measured every 24 hours for 9-14 days by an ATP quantitation assay (CellTiter-Glo). The percentage of died cells was calculated by PI incorporation. 200.000 cells were resuspended in PBS and 2 μl of PI (BD Bioscience) was added immediately before flow cytometry analysis.

Immunocytochemistry

To examine the subcellular distribution of CD148 in Caco-2 cells, 12 X 10^4 cells per well were planted onto glass coverslips (155411 Nalge Nunc International, Rochester, NY), and transduced the following day with 1 μl of nLV-sh-hCD148 or with CMV-GFP vector.

After 8 days, the cells were washed twice in PBS and fixed in 4% w/v ice-cold paraformaldehyde.

Following fixation, cells were washed twice in PBS, permeabilized in 0,1% Triton X-100, washed 3X in PBS, and then blocked in PBS containing 1% w/v BSA for 60 min.

Subsequently, primary antibodies were added. Caco-2 cells were stained with rabbit polyclonal anti ZO-1 (Zymed, San Francisco, USA), rabbit polyclonal anti occluding (Zymed) and mouse monoclonal anti CD148 (143-41).

The cells were then incubated at 4°C over night, and then washed twice in PBS buffer.

As secondary antibody, Alexa Fluor® 647 goat antirabbit IgG and Alexa Fluor® 488 goat antimouse IgG were used. Stained cells were then added to DAPI to label cell nuclei and kept in the dark until evaluated with confocal microscopy.

Confocal Microscopy

A confocal laser scan microscope (LSM 510) was used to analyze the intracellular localization of ZO-1, occluding and CD148. All images were imported into Adobe Photo Impact 8 for preparation of figures. No digital manipulations have been performed.

Transepithelial Electrical Resistance (TER) Measurements

TER was measured using an Endohm for 24 mm & Costar Snapwell™ cup (World Precision Instruments, Sarasota, Fla.) and calculated as Ω cm^2 by multiplying it with the surface area of the monolayer. The resistance of the supporting membrane in Snapwell filters is subtracted from all readings before calculations.

Caco-2 cell monolayers were grown as confluent monolayers on a Snapwell insert permeable support containing a 12 mm diameter 0.4µm pore polycarbonate membrane, until they were polarized. Cells were seeded in 500µl at a density of $2X10^5$ cells/well and, at confluence, transduced with 1 µl of LV-sh-hCD148#1525, or with 1 µl of LV-GFP.

Cells were fed apically and basolaterally with 1ml and 1,5ml fresh medium, respectively every other day. Cells were incubated at 37^{o}C, 5% CO_2 for 28 days. CD148 knock-down cells were compared to untransduced and to LV-GFP-transduced. TER was measured at 0 day before adding lentiviral vector, and then every other day.

Three independent experiments were done to ensure consistent results. Mean values ± standard deviation for TER were calculated.

RESULTS

Expression of CD148 is Cell Density-Dependent in Epithelial Cells

In order to study the density-dependent behavior of CD148 expression, T84 epithelial cells were plated at different densities and after four days in culture CD148 transcript levels were measured by bDNA analysis. As shown in **fig. 12**, CD148 mRNA levels increased as the culture became confluent.

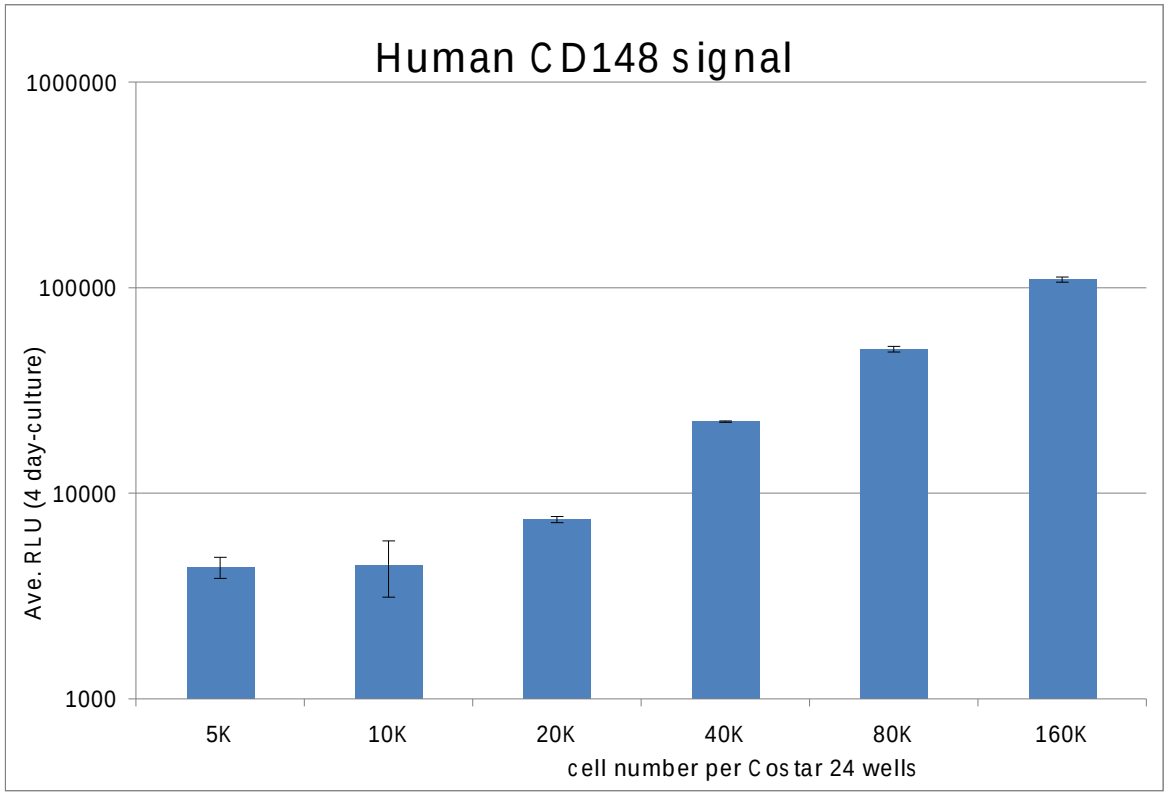


Fig. 12: Analysis of CD148 transcripts at various cell densities.

Production of LV-shRNAs Specific for Human CD148

To investigate the role of CD148 in cultured cells, we chose a gene silencing approach mediated by short interfering RNAs (siRNAs). A previous investigation had identified a number of siRNA triggers specific for human CD148 that efficiently down-regulated its protein expression (Galimi, unpublished results).

To achieve high-efficiency and long term CD148 silencing, we used a lentiviral gene delivery system. Lentiviral vectors allow for the integration of expression cassettes for short-hairpin RNAs (shRNAs) into the genome of the target cell, resulting in down-regulation of the specific gene. Moreover, lentiviral vectors can transduce a wide variety of cell lines and primary cells, including non-dividing cells.

For the initial experiments, three different lentiviral vectors were generated: one carried shRNA specific for the human CD148 (LV-sh-hCD148), a second carried an unrelated shRNA sequence with no detectable silencing effect used as a negative control (LV-sh-scrambled), and an "empty" vector carried the GFP expression cassette only. All three vectors had a common expression cassette including a hEF1α-driven bicistronic cassette for the GFP and neo genes (see **Fig. 10 down**). Transduced cells could be easily identified by fluorescence microscopy or flow cytometry, or could be selected by adding neomycin to the culture medium, depending on the experimental needs. Lentiviral vector preparations were produced as described in the Materials and Methods section. Typically, biological titers were in the range of 10^8 TU/ml for lenti-shRNA, and 10^{10} TU/ml for GFP-only vectors (**Fig. 13**)

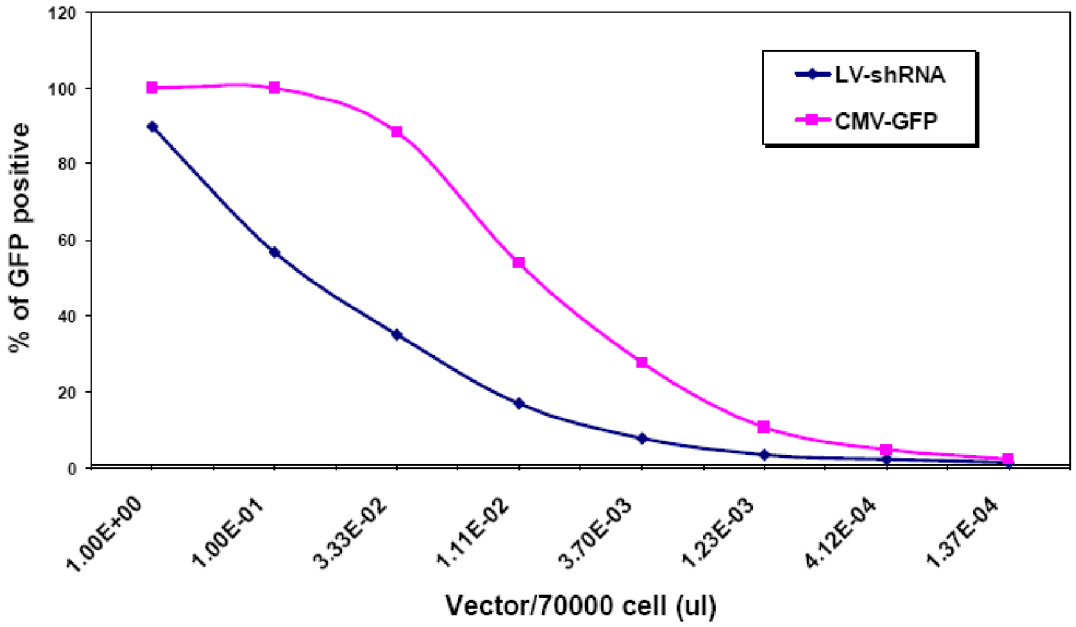


Fig. 13 Comparison between a CMV-GFP and LV-siRNA lentiviral titers.

Human shRNA characterization: the efficiency of human CD148 silencing was tested in A431 cells, an epidermoid carcinoma-derived cell line. Cells were transduced as described in Materials and Methods; after 2 weeks of selection in G418, CD148 levels were measured by flow cytometry with a monoclonal antibody specific for human CD148. The plots in **fig 14** show a nearly complete disappearance of CD148 in cells transduced with LV-sh-hCD148 compared with control cells transduced with LV-sh-scrambled.

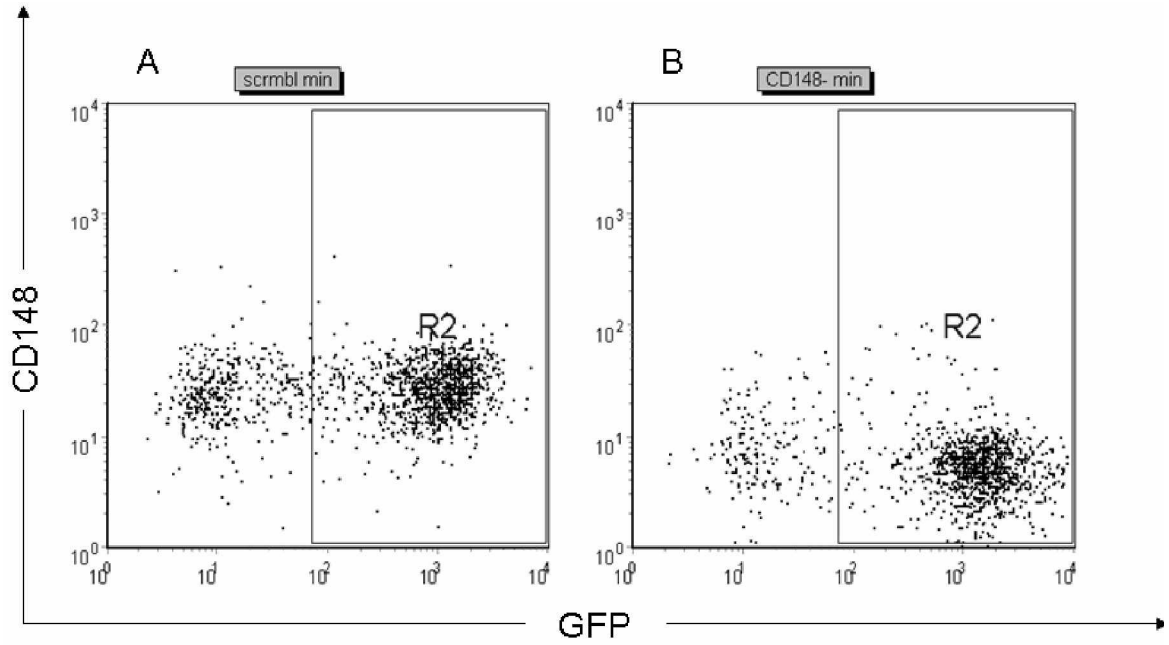


Fig. 14: CD148 silencing measured by flow cytometry on A431 cells following transduction with the LV-sh-scrambled (A) or with the LV-sh-hCD148 (B).

A New LV-shRNA Design Increased the Transduction Efficiency

Considering that the size and complexity of the vector genome affects the titer of the vector preparations and the efficiency of gene expression (F. Galimi *et al.*, 2005; M. Kumar *et al.*, 2001), we optimized the design of the LV-shRNA vectors to improve the silencing efficiency of the construct.

In order to simplify the vector design and reduce its genome size, the IRES and the neo gene were removed, and the bicistronic EF1a-GFP-IRES-NEO cassette was replaced by a CMV-GFP cassette. In addition, the shRNA cassette was removed from its original position and inserted within the 3' U3 region of the LTR. Reverse transcription of the transfer vector by reverse transcriptase duplicates the 3' end of the lentiviral genome so that the 3' U3 is copied at the 5'. The resulting provirus therefore carries two copies of the shRNA cassette in its genome, doubling the silencing power of

the Lenti-shRNA (see **Fig. 10**). The titer of the improved vectors (nLV-shRNA) was much higher compared to the previous vector design as measured on 293T cells, yielding 10^{10} TU/ml. Furthermore, the green fluorescence intensity was also much brighter. Vectors were validated as described above on Caco-2, A431 and THP-1. The new vector design allowed for very high efficiencies of transduction and full CD148 knock-down without G418 selection.

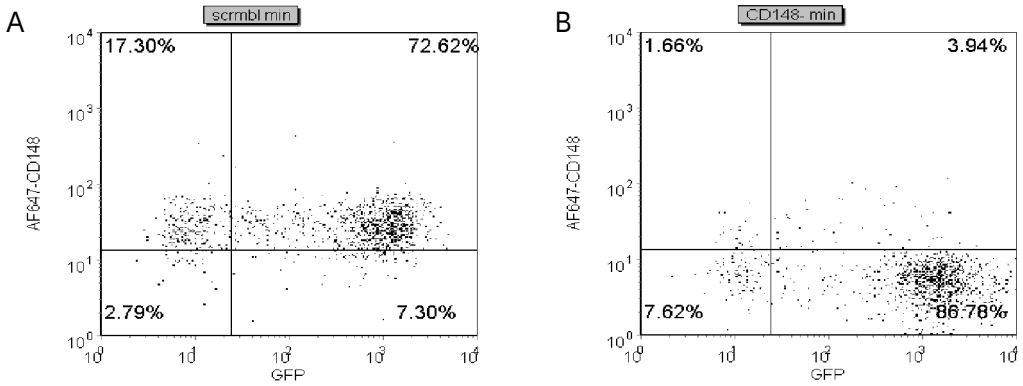


Fig. 15: Flow cytometry analysis of CD148 expression in A431 cells infected with LV-sh-scrambled (A) or with nLV-sh-hCD148 (B) carrying a shRNA#1525 against human CD148.

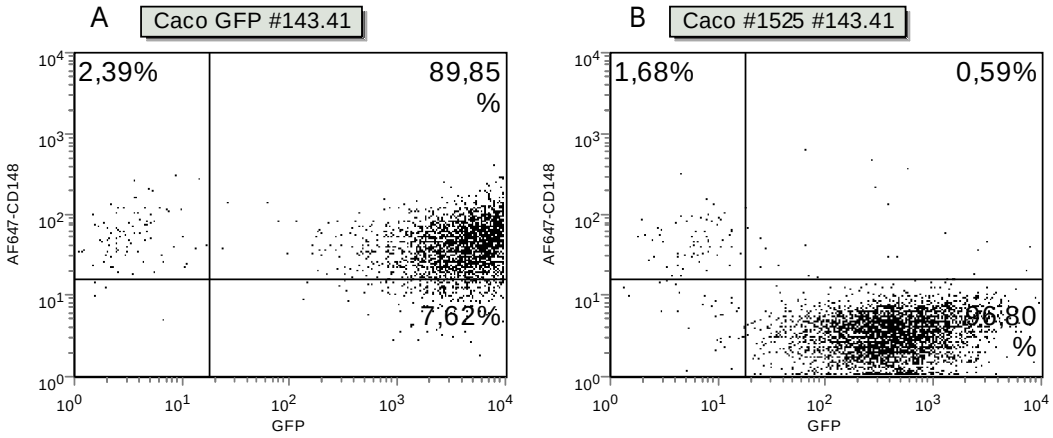


Fig. 16: Flow cytometry plots of Caco-2 cells transduced with the LV-CMV-GFP (A) or with nLV-sh-hCD148 (B)

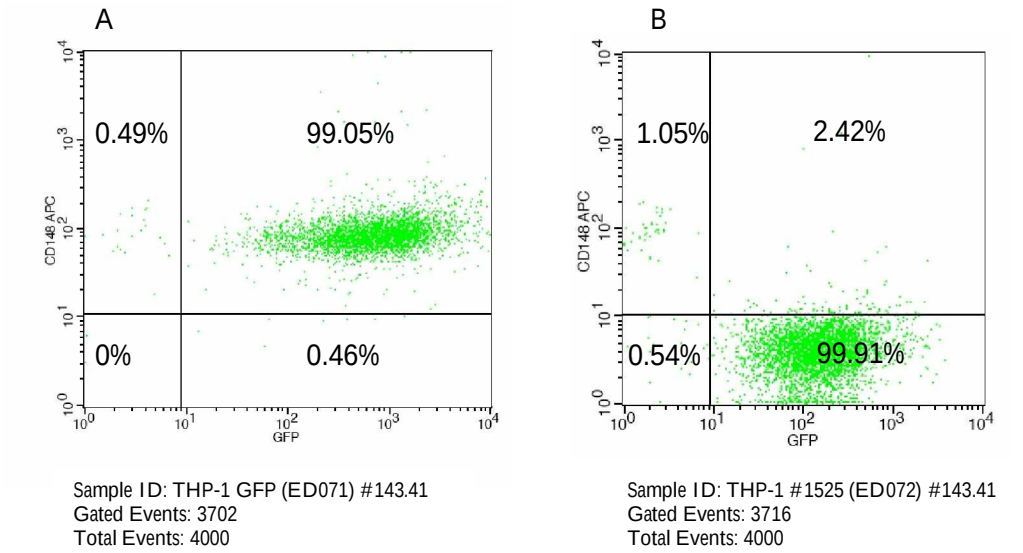


Fig. 17: Flow cytometry plots of THP-1 cells transduced with the LV-CMV-GFP (A) or with nLV-sh-hCD148 (B).

CD148 Knock-down Alters the Growth of Several Cellular Types

Overexpression of CD148 in tumor cell lines is known to reduce cell proliferation (S. Kellie, *et al.*, 2004; F. Trapasso, *et al.*, 2004). We therefore tested whether down-regulation of CD148 increases cell proliferation in epithelial cells.

A431 cells, previously transduced with nLV-sh-hCD148, nLV-sh-scrambled, or untransduced were plated at the same density and grown for 10 days. Proliferation then was measured by ATP quantification (see Materials and Methods) and values were reported in a growth curve as shown in **Figure 18**.

Surprisingly, our results showed that during the initial six days of the culture, CD148 knock-down (KD) cells grew faster than controls; once CD148KD cells reached confluence, they started to detach and die. A likely explanation for the accelerated growth pattern is an increased activity of kinases mediating growth signals. Their dephosphorylation by CD148 in normal conditions is relieved after CD148 silencing,

with a resulting increase in proliferation. However, we have no good explanation for the second finding. As discussed above, CD148 expression is increased in confluent cultures, and is known to interact and dephosphorylate proteins involved in cell-cell contact (L.J. Holsinger, *et al.*, 2002). It is therefore conceivable that CD148 is required for proper formation and stabilization of epithelial monolayers, and that its absence its absence might interfere with cell-cell interactions. The same growth pattern described for A431 cells lacking CD148 was observed in RAW264.7 cells (a mouse monocyte cell line) under similar experimental conditions (Ninniri, unpublished results and Doctorate Thesis, XIX ciclo).

Caco-2 cells, a human colon adenocarcinoma cell line, were not as sensitive to CD148 knockdown, allowing us to study epithelial cell populations in culture. We compared three populations of Caco-2 cells, transduced with anti-CD148 lenti-shRNA, or with a GFP-only control vector, or untransduced (see **Fig. 19**). The cells were plated at the same density and grown for 14 days. As shown in **figure 19**, CD148 KD cells grew very slowly, apparently never reaching confluence. This different response in the growth of the Caco-2 CD148 KD cells was confirmed by fluorescence microscopy of the same cells near the end of the growth curve (day 12). **Fig. 20** shows a high number of CD148KD cell population lacking the ability to form monolayers and unable to form cell-cell contacts compared to CMV-GFP treated cells. Interestingly, cell shape changes resulted in a more irregular appearance of CD148 KD cells.

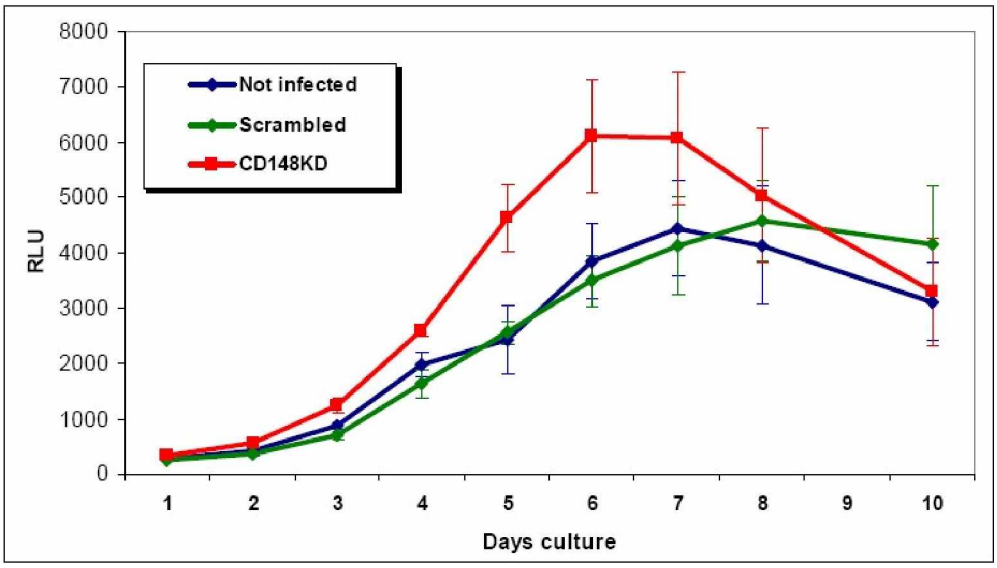


Fig. 18: Growth curve of A431 cells expressing or not expressing CD148.

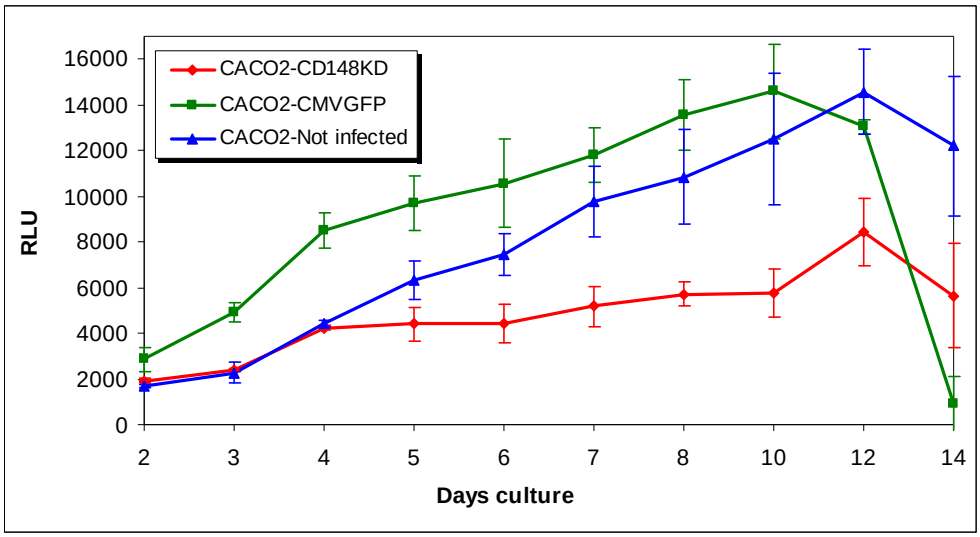


Fig. 19: Growth curve of CaCo-2 cells expressing or not expressing CD148.

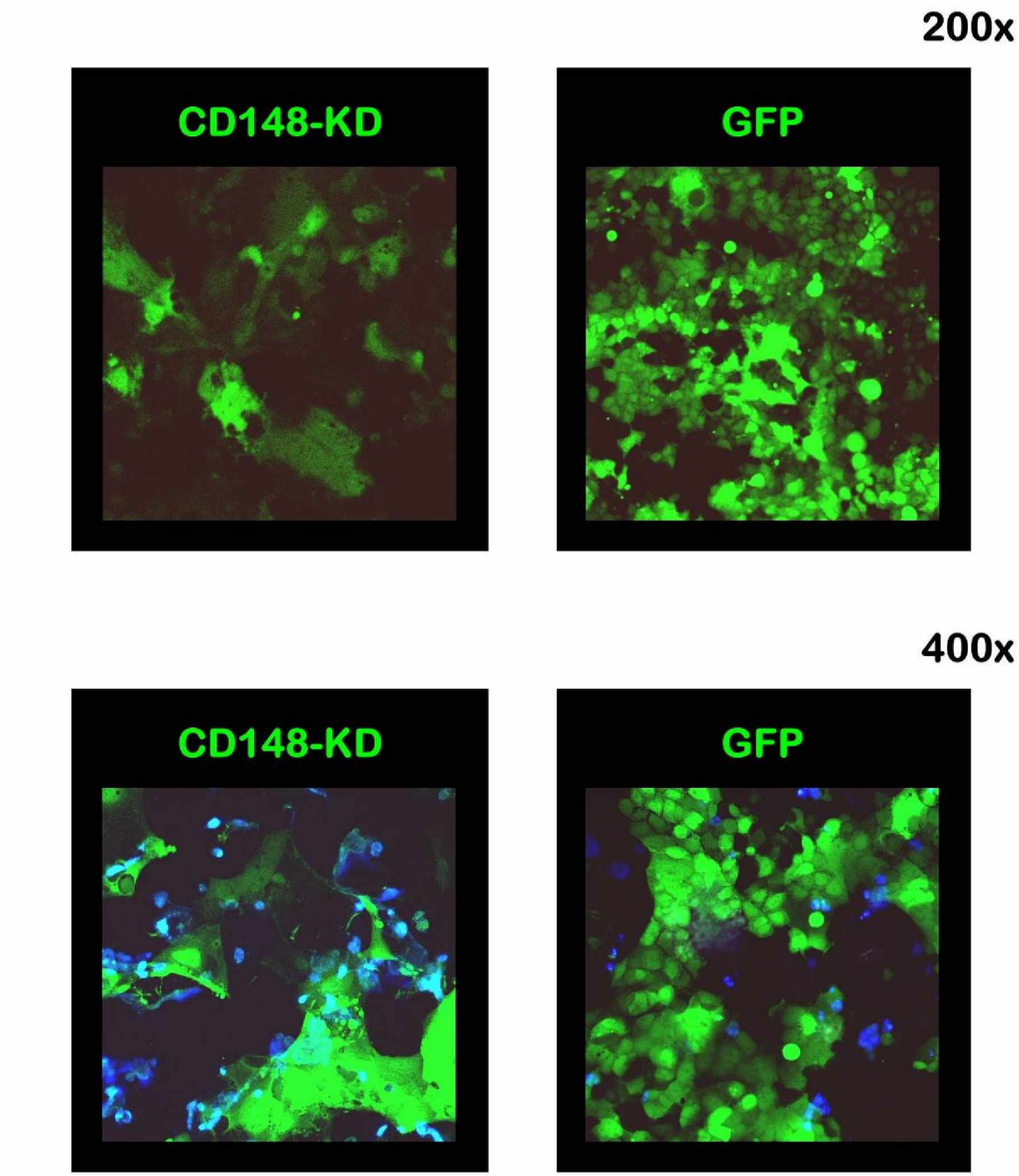


Fig. 20: Confocal microscopy of CD148 KD- and GFP- transduced Caco-2 cells (left and right, respectively), plated onto 96-well dish at 2000 cells/ well, at 12-day of their growth in culture. Magnification: 200X and 400X.

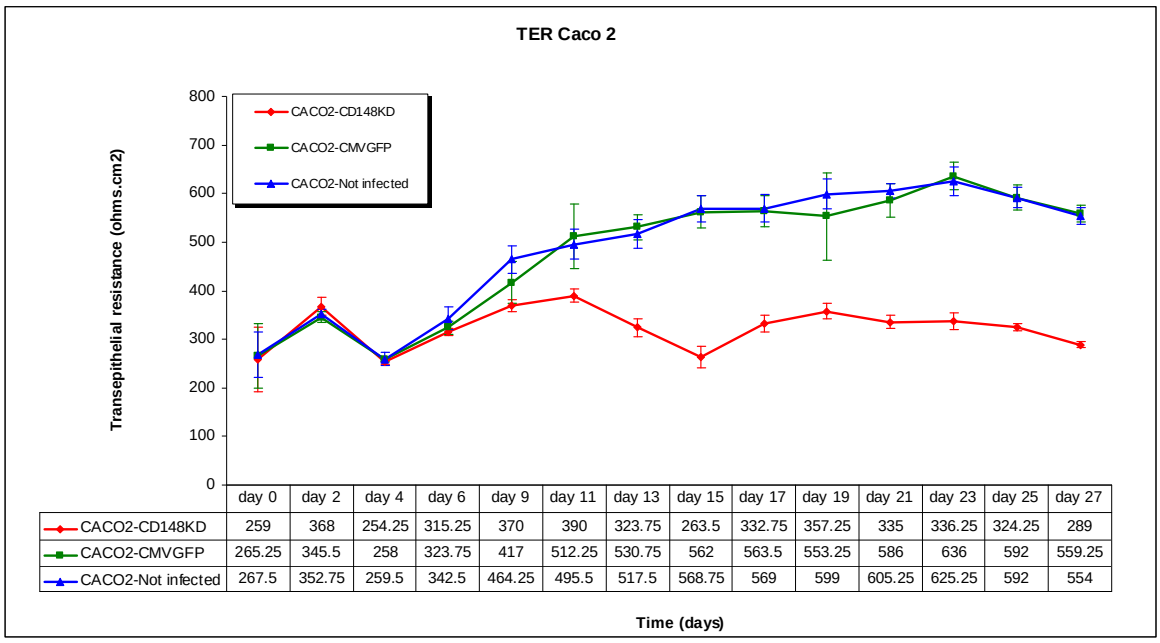
Measurements of TER in CD148 KD Caco-2 Cells

Based on these observations, we decided to test the hypothesis that the absence of CD148 alters a specific tyrosine phosphorylation equilibrium in intracellular targets involved in the formation of cellular contacts. We therefore examined the effect of CD148 knock-down on the integrity of tight junctions (TJs). To determine the ion permeability of TJs and to assess their integrity, we performed measurements of transepithelial electrical resistance (TER) of Caco-2 monolayers transduced with the lentiviral vector carrying the anti-CD148 shRNA compared to non-transduced controls.

To investigate the potential role of CD148 in cell-cell junction formation, we chose to study the effects of its down-regulation in Caco-2 cells, which represents a broadly used in vitro cellular model for polarized epithelia. The effect of endogenous CD148 in the regulation of the assembly of TJ was evaluated in these cells by measurements of transepithelial resistance. For this purpose, equal numbers of Caco-2 cells, plated on polycarbonate 12 mm Snapwell filter (Costar) and allowed to form a confluent monolayer, were transduced with 1 µl of LV-sh-hCD148#1525 or with 1 µl of LV-GFP, as described in Materials and Methods. Resistance was measured just before virus addition and at various times thereafter up to 28 days.

In monolayers exposed to LV-sh-hCD148#1525, a significant drop in resistance was first observed at day 9 and continued to decline over, while no significant difference has been revealed between control and GFP infected monolayers. Domes are formed by Caco-2 monolayers grown on nonporous surfaces. These structures are thought to represent areas of net fluid absorption. Domes are present in untreated and GFP control monolayers (not shown), but are diminished after LV-sh-hCD148 infection. Although our current experimental design does not allow to formally rule out non-specific effects of transduction or off-target gene silencing, this result is suggestive

of a CD148 involvement in the establishment of the epithelial barrier. Experiments with alternative triggers and inducible vector systems are ongoing.



	day 0	day 2	day 4	day 6	day 9	day 11	day 13	day 15	day 17	day 19	day 21	day 23	day 25	day 27
CACO2-CD148KD	259	368	254.25	315.25	370	390	323.75	263.5	332.75	357.25	335	336.25	324.25	289
CACO2-CMVGFP	265.25	345.5	258	323.75	417	512.25	530.75	562	563.5	553.25	586	636	592	559.25
CACO2-Not infected	267.5	352.75	259.5	342.5	464.25	495.5	517.5	568.75	569	599	605.25	625.25	592	554

Fig. 21: Time course for the decline in TER observed after infection of Caco-2 cells with nLV-sh-hCD148, compared with CMV-GFP vector infected cells and uninfected. The values represent the average of three independent experiments (each performed in quadruplicate; Mean ± SD).

Localization of CD148 by Confocal Microscopy

Because an alteration of the transepithelial resistance may be accompanied by alterations in the subcellular distribution of tight junction proteins, we used immunohistochemistry to detect changes in the localization of two tight junction specific proteins, occludin, and ZO-1 in Caco-2 cells. To positively identify the subcellular localization of the proteins being examined, we analysed the samples by confocal microscopy.

Caco-2 cells were transduced with LV-CMV-GFP or nLV-sh-hCD148 lentiviral vectors carrying a shRNA against CD148. After 10 days in culture, they were stained with an antibody against h-CD148 (143-41). CD148 was efficiently down-regulated, as shown in **fig. 16**.

Next, we determined the subcellular localization of CD148 by immunofluorescence in the same cell line. Epithelial monolayers of Caco-2 cells were grown on glass coverslips (Nalge Nunc International, Rochester, NY) until they were polarized, as described in Materials and Methods. The cells were stained with mouse monoclonal anti CD148 (143-41), and a confocal laser scan microscope (LSM 510) was used to analyze the intracellular localization of CD148.

In a serial confocal *xy*-sections of Caco-2 labeled with the anti-CD148 antibody, CD148 was detected at cell-cell contacts (**fig. 22**, B1 and B2). In order to identify the intracellular targets/partners of CD148, the cells were stained with rabbit polyclonal anti ZO-1 (Zymed, San Francisco, USA), rabbit polyclonal anti occludin (Zymed) and mouse monoclonal anti-CD148 (143-41). Co-labeling with an anti-ZO-1 and with an anti-occluding antibody revealed that CD148 co-localizes with ZO-1 and with occludin (**fig. 22**, C1 and C2). This was supported further by *xz*-section (**fig. 23**), showing that ZO-1 and CD148 co-localizes at the apical end of the lateral membrane. Thus, endogenous CD148 is expressed in the plasmatic membrane and is associated with intercellular junctions.

Moreover, untransduced cells were compared to CD148 knock-down cells (transduced the day after seeding into the glass coverslips with nLV-sh-hCD148, as described in Materials and Methods). Although no significant quantitative difference in labeling intensity was discernible, we observed a striking qualitative change in labeling pattern: the ZO-1 staining at cell-cell contacts was redistributed in a zigzag pattern, which differed considerably from the straight labeling pattern observed at cellular

junctions in control monolayers (**fig. 24, 25**). Our preliminary results revealed that the down regulation of CD148 in Caco-2 cells interfered with cell-cell junction formation, as detected by abnormal ZO-1 localization, and also that the knock-down of CD148 in Caco-2 cells resulted in loss of linearity of ZO-1 expression.

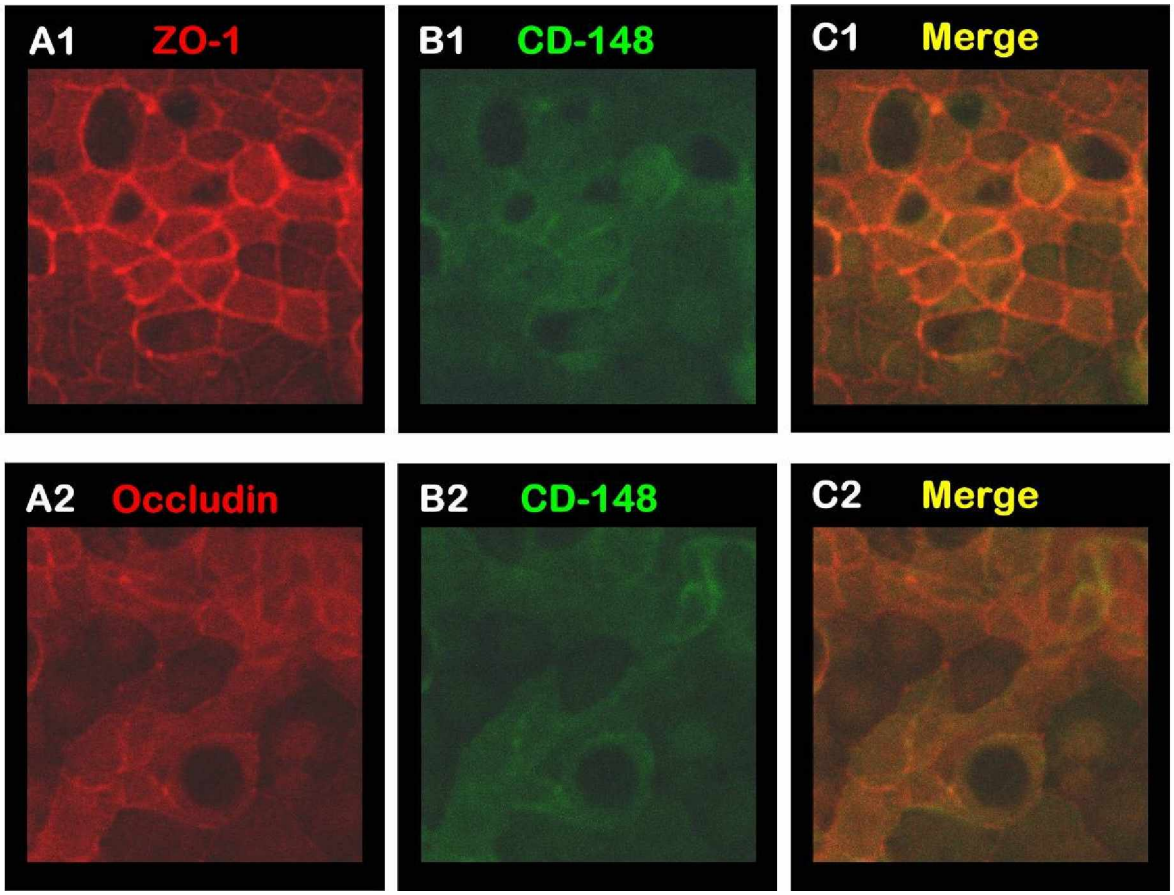


Fig.22: Localization of ZO-1 (A1), CD148 (B1 and B2) and occludin (A2) to the apical tight junction domain in confluent polarized Caco-2 cells. The distributions of CD148, ZO-1 and occludin (red) were visualized by double immunofluorescence; shown is a confocal *xy* section. Merged images show overlapping of CD148 and ZO-1 in yellow (C1), CD148 and occludin in yellow (C2). Magnification 200X.

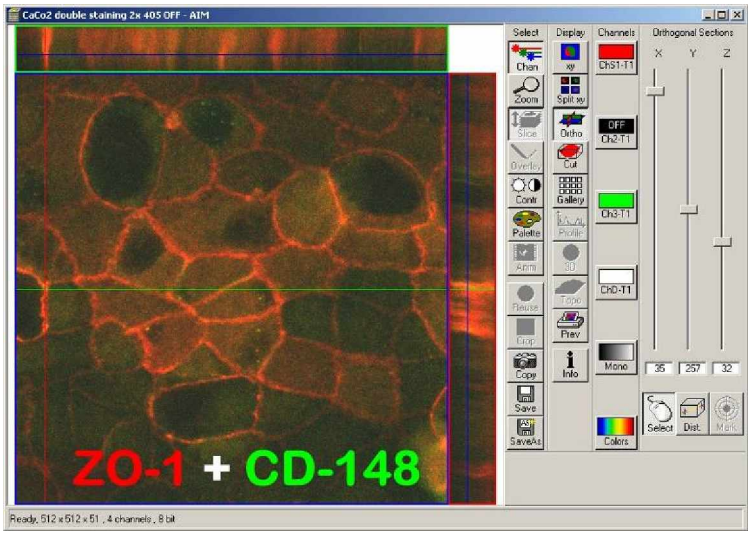


Fig. 23: Confocal image of Caco-2 cells. ZO-1 (red) and CD148 (green) are localized to the apical tight junction domain. Merged image shows both proteins appearing together in the same confocal planes (*xz* section, on the top). Magnification 200X, scaling: 0,29 µm X 0,29 µm X 0,30 µm.

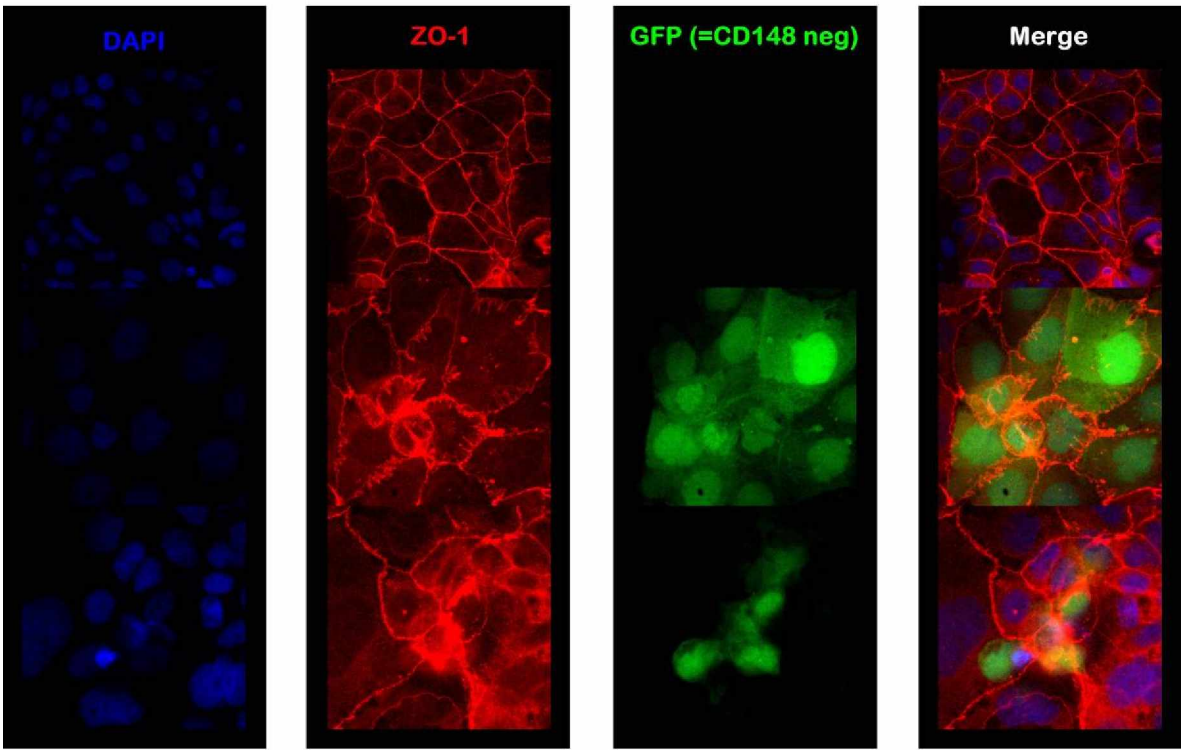


Fig. 24: Effect of the down regulation of CD148 on the junctional localization of ZO-1 in Caco-2 cells**.** Confluent polarized cells, untransduced (1st row) or transduced (2nd and last row) with a lentiviral vector carrying a shRNA against CD148, were fixed, permeabilized and immunostained for ZO-1. Although ZO-1 labeling was seen to be localized at cell-cell contacts in the KD cells, the distribution assumed a zigzag pattern in all samples examined, in contrast to the untransduced cells. Magnification 200X, scaling: 0,22 µm X 0,22µm.

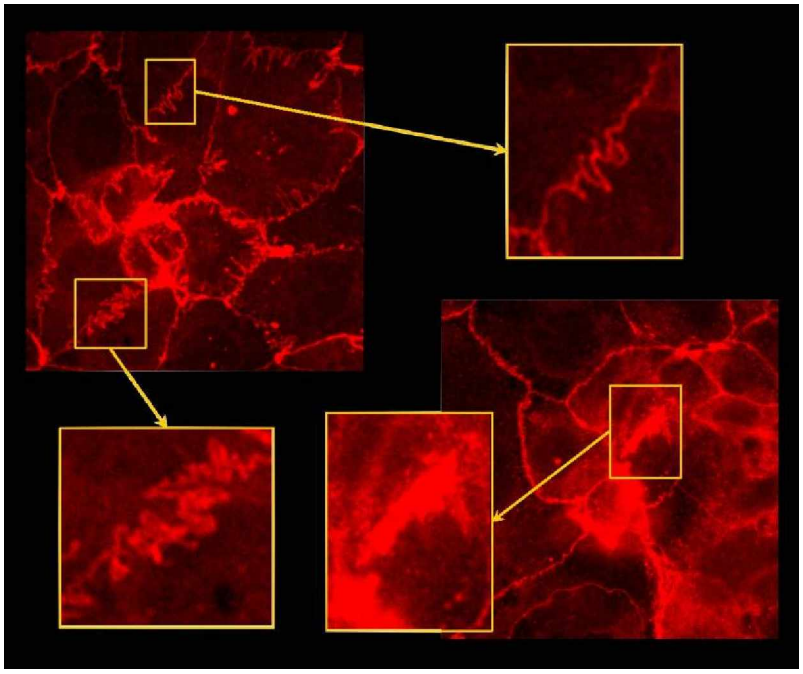


Fig. 25: Abnormal distribution of tight junction protein ZO-1 (red) expression in Caco-2 cells expressing undetectable levels of CD148. Magnification 200X.

Discussion

Protein phosphorylations on tyrosine residues are essential events involved in cellular processes, such as proliferation, differentiation and migration. Triggering of cellular surface receptors by specific ligands leads to the transient activation of protein tyrosine kinases (PTKs) which subsequently phosphorylate and thereby modify the function of intracellular molecules. These early steps of the signaling cascade are also influenced by protein tyrosine phosphatases (PTPs), an increasing number of which have been described in recent years. CD148 is a RPTP that exhibits a wide tissue distribution, being expressed in hematopoietic and endothelial cells, as well as in fibroblasts, epithelial and several other cells. The widespread expression of CD148 suggests its involvement in diverse signaling pathways, but so far little is known about its physiological functions. A number of published reports suggest a role for CD148 in contact inhibition and cell proliferation (M. Grazia Lampugnani, *et al.*, 2003; A. Ostman, *et al.*, 1994). Like many RPTPs, CD148 has a bulky extracellular domain whose role in the physiological activity of the protein is unknown. It has been proposed that CD148 extracellular domain may mediate homophilic or heterophilic interactions with protein expressed on adjacent cells, playing a role in cell contact inhibition. Additionally, soluble ligands may also modulate the activity of CD148, as shown in experiments with Matrigel, a mixture of several extracellular matrix compounds, which can potentiate the catalytic activity of CD148 (M. Sorby, *et al.*, 2001).

In order to study the biology of CD148, we used the technology of RNAi and lentiviral vector gene delivery to achieve somatic knock-down of the molecule on a

broad range of proliferating cell types. This method allowed us to achieve long-term silencing of the target mRNA and profoundly reduced CD148 expression. Distinct vector designs were used to achieve CD148 knock-down on cell lines and primary tissues, the latter remarkably more difficult to transduce and handle in culture. With the appropriate vector design, we were able to obtain a complete knock-down of the CD148 molecule in all the cell types tested.

The most apparent effect of CD148 knock-down on a number of cell lines was their increased growth rate compared to cells expressing normal level of the phosphatase. This phenomenon occurred epithelial and monocytic cell lines of human and mouse origin (Ninniri, unpublished results and Doctorate Thesis, XIX ciclo). The regulatory role of CD148 in cell proliferation has been investigated, in the last few years, by many researchers. Iuliano reported that the over expression of CD148 in thyroid carcinoma cells could suppress their proliferation (R. Iuliano, *et al.*, 2003). A similar study was recently conducted by Balavenkatraman in colon carcinoma cells (K.K. Balavenkatraman *et al.*, 2006). What appears in these studies is that CD148 could control cell cycle, even though the molecular details are currently unknown. Cell expressing CD148, in fact, show higher level of p27kip1 protein, a cyclin-dependent kinase inhibitor involved in the regulation of G1/S transition. It has been assumed (F. Trapasso, *et al.*, 2000) that CD148, by preventing the degradation of p27kip1, promotes the inhibition of MAP kinases activity, so that cell proliferation is suppressed. Therefore, the behavior of CD148KD cells could be explained assuming that CD148 is a cell regulator that maintains cells in a controlled proliferation state. When CD148 is over-expressed, cells show a reduction in cell proliferation (S. Kellie, *et al.*, 2004), while when there is a deficiency in CD148 cells proliferate uncontrollably.

Interestingly, CD148 knock-down resulted in accelerated death rate once the culture reached confluence. Although we do not have a definitive explanation for this

finding, it suggests that CD148 expression is required at cell confluence, therefore implying its role in contact inhibition. CD148 role in contact inhibition is known since Ostman (A. Ostman, *et al.*, 1994) in 1994 described that the expression of this phosphatase increased with increasing cell density. Various studies have tried to elucidate the molecular mechanisms of this phenomenon, but with limited results.(M.M. Mareel *et al.*, 1991)

Recently, an (F. Galimi, *et al.*, 2005) interesting work focused on contact inhibition in endothelial cell and pointed out the role of CD148 in cell proliferation (M. Grazia Lampugnani, *et al.*, 2003). The Authors show that the interaction between cadherins involved in intercellular contacts is responsible for the inhibition of proliferation. Cadherins work in the arrangement of protein clusters that co-localize VEGFR with CD148. Because the formation of these clusters occurs only in confluent cells, CD148 is able to dephosphorylate VEGFR and therefore to inhibit its proliferative activity only once cells reach confluence.

ZO-1 directly binds to the cytoplasmic tail of occludin (M. Furuse *et al.*, 1994) and forms a heteromeric complex with two others MAGUKfamily proteins, called ZO-2 (B. Gumbiner, *et al.*, 1991; L.A. Jesaitis and D.A. Goodenough, 1994) and ZO-3 (J. Haskins *et al.*, 1998). ZO-1 and occludin also establish a link with the actin cytoskeleton by directly interacting with actin filaments (A.S. Fanning, *et al.*, 1998; E.S. Wittchen *et al.*, 1999). Previous studies have shown that a number of perturbations induce alterations in paracellular permeability associated with protein redistribution of the junctional complex (M.A. Jepson *et al.*, 1995; C.X. Li and M.J. Poznansky, 1990). One of these perturbations involves phosphorylation on residues of intercellular junction proteins. Among the various mechanisms (D. Hecht and Y. Zick, 1992) reported to induce this effect we can mention: (a) overexpression of oncogenic protein tyrosine kinase (PTK) (R. Brackenbury *et al.*, 1984; M. Hamaguchi *et al.*, 1993; T.

Volberg *et al.*, 1991); (b) inhibition of several phosphotyrosine phosphatases (PTP) by vanadate in conjunction with H_2O_2 (C.B. Collares-Buzato *et al.*, 1994; D. Hecht and Y. Zick, 1992; J.M. Staddon *et al.*, 1995; T. Volberg, *et al.*, 1992) and (c) activation of protein kinase C (PKC) by phorbol esters (J.M. Mullin and M.T. McGinn, 1988; J.M. Mullin and T.G. O'Brien, 1986; G.K. Ojakian, 1981; W.F. Stenson *et al.*, 1993). In addition, the agents mentioned have been reported not only to affect the TJs but also AJs (C.B. Collares-Buzato *et al.*, 1998; J.M. Staddon, *et al.*, 1995; T. Volberg, *et al.*, 1992). Thus it has been generally assumed that both types of junctions are commonly regulated and that permeability of TJs depends on the integrity of AJs.

Moreover, it was previously reported that ZO-1 protein is directly involved in the establishment of two distinct junctional domains, belt-like adhesion junctions (AJs) and tight junctions (TJs), during epithelial polarization (J. Ikenouchi, *et al.*, 2007). It was also demonstrated that CD148 is associated with a number of proteins involved in cell-cell contacts including p120ctn and other members of the catenin gene family. The interaction of these proteins with CD148 has been revealed dependent on their tyrosine phosphorylation.

As shown by confocal microscopy (**Fig. 24**), we noted significant changes in ZO-1 distribution in CD148 KD Caco-2 cells, at day 8 after seeding into glass coverslips, a time when TER was beginning to decline. In control cultures, ZO-1 immunostaining was present as a strong, continuous and distinct pericellular band, as shown in figure 24. A notable change in ZO-1 immunostaining was observed with nLV-shRNAhCD148 infection: intercellular gaps in ZO-1 staining were frequently observed (**Fig. 24 and 25**) in all the experiments, perhaps as a consequence of altered cell shape.

In this study, we showed that the downregulation of CD148 in Caco-2 monolayers decreases transepithelial macromolecular paracellular permeability. We also showed that the infection with the shRNA anti human CD148 alters the distribution of ZO-1 that

serves to link occludin and the actin cytoskeleton. These structural alterations in the tight junctions occur contemporaneously with changes in TER.

Our experimental design does not allow to exclude non-specific effects of transduction or off-target gene silencing. “Off-target effects of siRNA” are concentration dependent, responsible for up to threefold suppression of dozens of genes, and mediated by either the sense or antisense strand of the siRNA.

If not adequately addressed, these effects can lead to false positives in broad RNAi-based phenotypic screens, thus clouding data interpretation. The transduction of the cell lines with several triggers anti human CD148 will also help us exclude the unlikely possibility of citotoxicity of the lentiviral preparations used. For that purpose, we are also constructing tetracycline-inducible vectors.

Our data offer a novel perspective to the role of CD148 in the establishment and maintenance of epithelial sheets. If confirmed in other experimental systems in vitro as well as in vivo, our approach will identify CD148 as a central player in cell-cell interactions, possibly opening the way to the identification of its extracellular ligand(s), which until now have proven elusive.

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