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Role of Rac1 protein in the acquisition and maintenance of epithelial cell polarity

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Introduction

1. Introduction

1.1 Cell polarity

The polarity is a characteristic of some types of cells that can be defined in two different ways depending of the point of view from which we analyze the phenomenon: structural polarity and functional polarity. In the first case, cell polarity is defined as the asymmetric distribution of membrane proteins and lipids, the oriented distribution of organelles and cytoskeleton elements. Functional polarity refers to the property to perform tasks in a oriented manner, as the transport of ions, the transfer of proteins and migration. Cell polaritation, which is fundamental to many aspects of cell and developmental biology, is involved in the processes of differentiation, proliferation and morphogenesis in both unicellular and multicellular organisms. In a wide range of elementary cellular processes, many constituents of the cell, such as plasma membrane proteins, organelles, and cytoskeletal components are organized asymmetrically within the cell. This asymmetrical pattern of organization is enhanced by cell differentiation processes resulting in dynamic cell compartments specialized in complex vectorial functions. Cell polarity is essential for processes such as the growth of budding yeast, cell division, the development of a fertilized egg into an organism, the transmission of nerve impulses, the transport of molecules across an epithelial cell layer, cell crawling and lymphocyte homing, etc. Many cell types, if not all, express, or are capable of expressing, the polarized phenotype. Epithelial cells and nerve cells are the most common examples. Other cells, such as blood cells, do not manifest an explicit polarity, This property may become apparent when they interact with other cells or when they migrate. The epithelial cells express both types of polarity and the polarized phenotype is morphologically visible: 1) The functional specializations of the apical surface, like invaginations (eg, microvilli, or cilia), 2) the position of the Golgi in the apical zone and 3) accumulation of the products secreted into the apical cytoplasm, 4) in the specializations of the lateral surface (i.e the presence of numerous cell junctions) . The establishment of the polarized phenotype occurs in several steps and generates a reorganization of both the the cytoskeleton ant the cell surface. Isolated, dispersed cells are non-polarized, but when such cells are seeded in a culture dish, the initial events observed are attachment of the cells to the substratum and establishment of cell-cell contact (Rodriguez-Boulan and Nelson 1989). The attachment of non-polarized single cells of epithelial origin to the substrate generates a signal to form an immature

surface containing specific apical surface markers (Vega-Salas, Salas et al. 1987); basolateral membrane proteins are, at this stage, randomly distributed at the entire cell surface. The molecular mechanism of this process involve redistribution of membrane proteins via cytoskeleton rearrangements. Cell-cell contact in epithelia is mediated by a Ca2 + dependent cell adhesion molecule, E-Cadherin (Takeichi 1990). After cell-cell contacts are established, an intracellular vacuolar apical compartment, lined by a membrane wich form microvilli and contains apical surface markers, fuses with the plasma membrane, thus contributing to the completion of the apical cell surface (in the thyroid, the vacuolar apical compartment probably corresponds to intracellular lumina, wich are frequent in isolated thyrocytes in suspension). Formation of a distinct basolateral membrane requires foregoing establishment of cell-cell contact; specific basolateral membrane proteins, such as Na+/K+ ATPase then gradually accumulate (Nelson and Veshnock 1987) .

Once acquired, the polarity is maintained through several mechanisms. The first is the formation of tight junctions, which, besides constituting a sealing element in the barrier function of epithelia, acts as a fence to prevent intermixing of membrane proteins and lipids in the apical and basolateral membranes (Gumbiner 1987). Another mechanism by which these components are maintained membrane-anchored is restriction of their mobility. This can accomplished by anchorage to components of the submembranous cytoskeleton (Nelson and Veshnock 1987), by interaction with extracellular matrix components (Parry, Cullen et al. 1987), or CAMs of neighbouring cells (McNeill, Ozawa et al. 1990) and by association with immobile glycolipids domains. In fact, many membrane proteins occur in microdomains both in apical and the basolateral plasma membrane. The different composition of two domains is continuously supported by intracellular sorting mechanism witch regulate the insertion of new proteins and degradation of old proteins in specific plasma membrane domains by, respectively, exocytosis and endocytosis of membrane. Ca2 +- dependent cell adhesion is important also for the preservation of cell polarity. Cell-extracellular matrix contacts, mediated by the family of transmembrane receptors, and integrins also contribute to the formation of the polarity axis.

1.2 The polarity in epithelial cells

The epithelial cells are contiguous, they work closely together and are connected together by junctional complexes that enable the creation of barriers with selective permeability. They thus form the epithelia which “cover” the free surfaces and cavities of an organism and constitute real barriers in relation to its content. The cells in an epithelium are highly polarized. Indeed, the plasma membrane of epithelial cells can be divided into two domains: an apical domain and a basolateral domain (Simons and Fuller 1985; Simons 1993). The two domains have a different composition of lipids and proteins. The apical domain of epithelial cells is usually in contact with the external surface of an organism or with the body cavities, while the basolateral surface can be divided into two regions: the basal surface lies on a basement membrane and it is in close proximity to blood vessels and capillaries, whereas the lateral surface is adjacent to other cells in the same epithelium, and contains specialized junctional domains that allow interactions among adjacent cells (either simple mechanical adhesion as in the case of tight junctions, adherent junctions and desmosomes, or metabolic cooperation via gap junction). The apical domain, is often provided with specialized structures like cilia or flanges brush and is rich in sphingolipids (glycosphingolipids and sphingomyelin). The basolateral domain is rich in glycerophosfolipids and phosphatidylcholine. Thus, the different roles that these two domains play is reflected in a different lipid and protein composition due to the presence of a ring of tight junctions (TJs) (**Figure 1**). To maintain this asymmetry molecular proteins and lipids should be directed to the proper domain of residence. This process requires both the presence of signals on protein sorting and the recognition of these signals by a cellular machinery of "sorting" able to decipher them. .Through a series of studies based on both biochemical and imaging approaches in living cells, it has been suggested that the sorting of proteins toward the two compartments of the plasma membrane occurs at the level of trans-Golgi network (Mostov, Su et al. 2003; Rodriguez-Boulan, Kreitzer et al. 2005). In epithelial cells, the apical domain is particularly rich in glycolipids, cholesterol, H/K ATPase and ionic channels. The basolateral domain contains proteins involved in communication with adjacent cells. The thyrocytes, thyroid epithelial cells, are an excellent example of polarized epithelial cells. Studies on isolated follicles have given important contribution to the general concept of how cell polarity is established and maintained in epithelia (Mauchamp, Margotat et al. 1979; Nitsch and Wollman 1980; Garbi,

Tacchetti et al. 1986). In vivo, the thyrocytes are organized into follicles, spherical structures bounded by a closed monolayer of cells resting on basement membrane. Follicles are the functional unit of the thyroid because they are able to perform the essential functions of the entire gland to produce hormones (Tacchetti, Zurzolo et al. 1986). In follicle lumen is secreted thyroglobulin and iodide, which is transported in the cells through a peroxidase, that is localized in apical plasma membrane; iodide binds the thyroglobulin and this generates the subsequent formation of thyroid hormones. Iodide is transported across both the basolateral and the apical plasma membrane. The basolateral membrane contains two components essential for the execution of thyroid-specific functions: the TSH receptor and the iodide pump (Westermark, Westermark et al. 1986). At this surface the thyroid hormones are also released after their liberation from intracellularly degradated thyroglobulin. Apical secretion of thyroglobulin, the direct transport from base to apex of iodide, the internalization of iodinated thyroglobulin by the apical domain and secretion from the basolateral membrane of thyroid hormones are all expressions of the phenotype of these polarized epithelial cells.

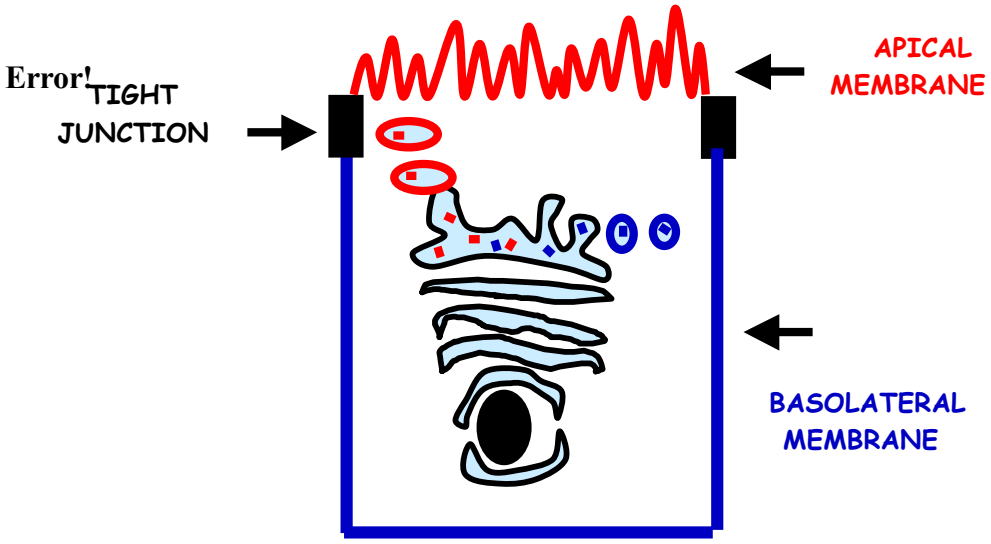

Fig. 1 Image of an epithelial cell. As a result of polarization and the presence of TJs the plasma membrane of epithelial cells can be divided into two domains: an apical domain and a basolateral domain. The apical domain of epithelial cells is usually in contact with the external surface of an organism or with the body cavities, while the basolateral surface can be divided into two regions: the basal surface lies on a basement membrane and it is in close proximity to blood vessels and capillaries, whereas the lateral surface is adjacent to other cells in the same epithelium.

1.3 Protein complexes involved in the acquisition of cellular polarity

The cell polarization is achieved through the combined action of three protein complexes: the complex Crumbs-PALS1-Patj, called the CRB complex, the complex Par3-Par6-Apkc complex, called PAR (Partitionig defective) and the complex Scribble -Disc large (DGL)-letal giant larvae (LGL) (Assemat, Bazellieres et al. 2008). These three complexes have a different localization in cells. The first two are located in the apical region of the membrane,the third is concentrated along the lower side of the membrane. In some processes of polarization they cooperate in the induction of polarity, whereas in other systems can also act as antagonists. (**Figure 2**).

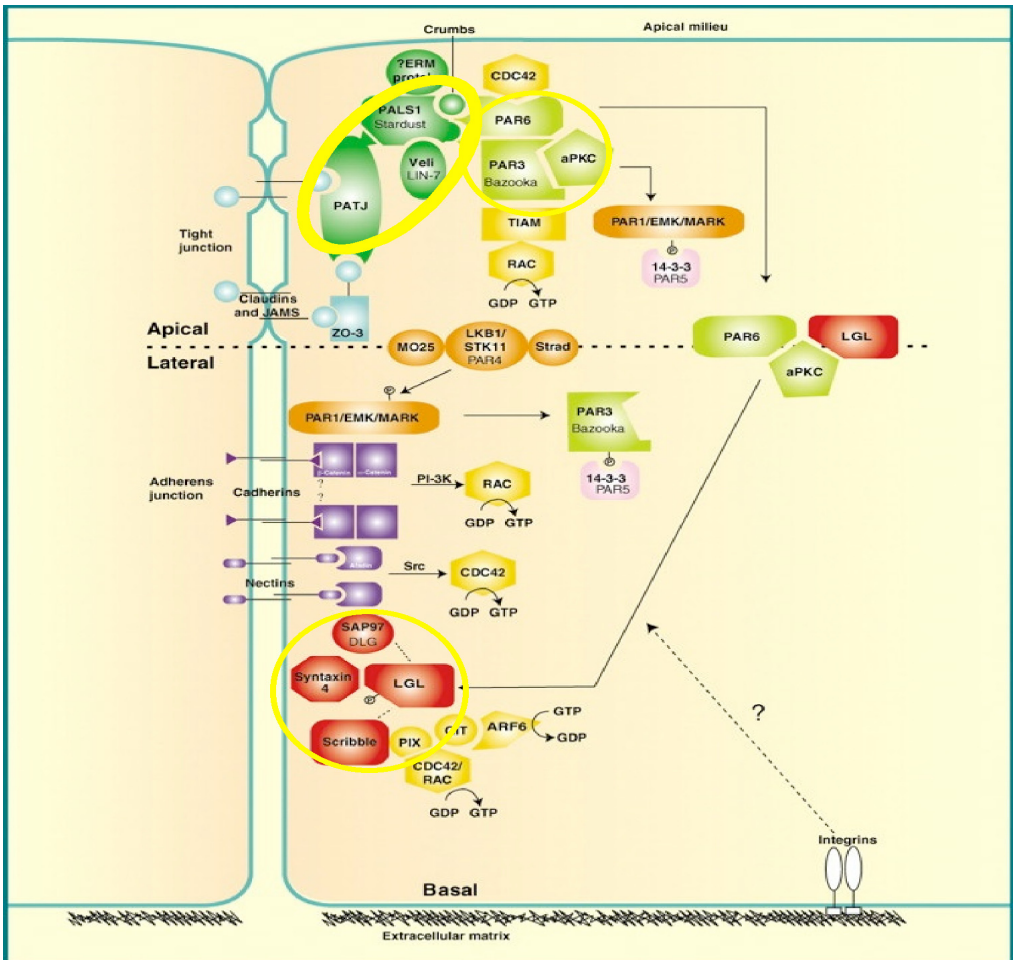

Fig. 2 Schematic view of apicobasal polarity complexes. The scheme indicates the major proteins thought to play a role in the initiation of apicobasal polarity.Three protein complexes are involved: the complex Crumbs-PALS1-Patj, called the CRB complex, the complex Par3-Par6-Apkc complex, called PAR and the complex Scribble DGL-LGL. The first two are localize near the apex, the third is concentrated along the lateral . At the molecular level, LGL and Scribble are connected to trafficking machinery. LGL associates with syntaxin 4, a component of the basolateral exocytotic machinery whereas Scribble binds to PIX and GIT, two regulators of the ARF6 and CDC42/RAC small GTPase. Protein After the interaction of Tiam1 with the Par complex, by direct binding to Par3, Rac1 binds PAR6 in the complex with PAR3/aPKC and this interaction is involved in the maturation of tight junctions faciliting the onset of cell polarity. (taken from Margolis and Borg 2005).

The PAR complex initially described in the nematode C. elegans and later in the fruit fly D. melanogaster and vertebrates is composed of two scaffold proteins, PAR6 and PAR3 and an atypical protein kinase C, aPKC. This tripartite complex named PAR6/PAR3/aPKC is conserved from worms to vertebrates. PAR6, PAR3 and aPKC were first described as essential proteins for asymmetric division of the C. elegans zygote (Kemphues 2000). PAR6 protein has a molecular weight of 37 kDa, functions as part of a protein complex and contains three conserved domains mediating his interactions with the other members of the complex. A Phox/Bem 1 (PB1) domain that binds to other PB1-domain-containing proteins such as aPKC is located at the N-terminal. The adjacent Cdc42/Rac interaction binding (CRIB) motif binds to the Cdc42 or Rac GTPases only in their activated GTP-bound state. Lastly, it has a PDZ domain that binds to other proteins such as PAR3 and CRB3 (Joberty, Petersen et al. 2000; Hung and Sheng 2002). The exact function of PAR6 has not yet been elucidated, but since Garrard et al. (Garrard, Capaldo et al. 2003) established that Cdc42-GTP can induce a conformational change in PAR6B, Gao and Macara (Gao and Macara 2004) a functional model for PAR6 can be proposed, in which the N-terminal folds back and interacts with the CRIB-PDZ domain. Cdc42-GTP binding to PAR6 results in the unfolding of PAR6, thus exposing the PALS1 binding site (PDZ domain inPAR6). The key function of PAR6 should be to allow the interaction between aPKC and its downstream effectors such as PAR3 and LGL. Phosphorylation of LGL will result in its detachment from the aPKC/PAR6 dimer, leading to the formation of another functional complex: PAR6/PAR3/aPKC (Yamanaka, Horikoshi et al. 2003). PAR3 protein contains three PDZ domains and the first PDZ of PAR3 interacts with PAR6. PAR3 has been extensively studied in epithelial cells. It seems likely that the starting point required for PAR3 to target the tight junctions is its ability to form self-associations and to bind to the junctional adhesion molecules (JAMs). PAR3 forms a homodimer via its N-terminal region; this association seems to be required for the correct association of PAR3 at the apical side of the cell–cell contact region during the process of polarization (Mizuno, Suzuki et al. 2003). PAR3 may then be stabilized upon binding directly to JAM via its first PDZ domain, and these two proteins may then be co-distributed to the sites of cell–cell contact (Ebnet, Suzuki et al. 2001). As JAMs are present at newly formed cell-cell contacts prior to PAR3, they can serve as anchors for the recruitment of PAR3 to the junctional complex at an early stage in the junction formation process (Itoh, Sasaki et al. 2001). Once PAR3 occupies this site, it can play the role of a

scaffold in the recruitment of proteins involved in the formation of the junctions, such as PAR6 or aPKC. Many studies have in fact shown that overexpression or depletion of PAR3 in epithelial cells leads to the disruption of tight junctions, along with the mislocalization of PAR6, aPKC and tight junction markers (Joberty, Petersen et al. 2000; Mizuno, Suzuki et al. 2003; Chen and Macara 2006). aPKC has a molecular weights of 75 kDa and functions as part of the polarity complex PAR. It has a PB1 domain in the N-terminal, which is known to interact with PAR6, and a catalytic domain present within the C-terminal region. This domain is known to phosphorylate several proteins such as PAR3 and LGL. In addition, Yamanaka et al. (Yamanaka, Horikoshi et al. 2003) have demonstrated that both LGL and PAR3 can form independent complexes with aPKC/PAR6 to regulate epithelial cell polarity. In MDCK epithelial cells, aPKCs localize with the other members of the PAR complex at tight junctions (Izumi, Hirose et al. 1998); and it is worth noting that aPKCs are the only members of the complex showing catalytic activity and this kinase activity is required for the formation of tight junction. As Cdc42-GTP binds to PAR6, it seems likely that Cdc42-GTP may form a complex with aPKC via the adaptor PAR6, the expression of Cdc42-GTP leads to the translocation of aPKC from the nucleus to the cytoplasm and cell periphery, where the complex will be involved in tight junction formation. Cdc42 is activated upon E cadherin mediated cell–cell adhesion, resulting in phosphorylation and thus activation of aPKC, and this chain of events is crucial to tight junction formation.

The Crumbs complex was identified in the epithelia of Drosophila and subsequently in vertebrates. Mammalian CRB are transmembrane proteins, whereas the other proteins present in this complex, PALS1 and PATJ, are cytoplasmic scaffolding proteins.

CRB (crumbs) protein has a molecular weight of 13 kDa and has a very short extracellular domain but no recognizable protein domain and includes O and N glycosylation sites. This protein has a transmembrane domain and a cytoplasmic domain very well conserved. In the cytoplasmic tails, CRB protein contains two motives: a FERM (band 4.1-ezrin-radixinmoesin) protein-binding domain consisting of 12 amino acids containing a GTY motif and a PDZ-binding domain consisting of ERLI residues. The FERM domain is a protein–protein interaction domain which exists in various proteins, many of which serve as adapters linking transmembrane proteins to the cortical actin cytoskeleton. In addition to playing a role in the formation of tight junctions, CRB is involved in the differentiation of the apical membrane (Fogg, Liu et

al. 2005). PALS1 (77 kDa) is an adaptor protein mediating indirect interactions between CRB and PATJ , then, it is a scaffold protein that has multiple protein–protein interaction domains and belongs to the MAGUK (membrane-associated guanylate kinase) family. PALS1 consists of two L27 domains, a PDZ domain, an SH3 domain, a hook domain and a GUK domain anh with his PDZ domain interacts with CRB. Knockdown of PALS1 in MDCK cells leads to tight junction and polarity defects (Straight, Shin et al. 2004) and to the mis-targeting of E-cadherin to the cell membrane (Wang, Chen et al. 2007). Furthermore, loss of PALS1 resulted in concomitant loss of PATJ expression and the presence of PALS1 in mammalian epithelia depends on interactions with PATJ, it seems possible that the stability of these two proteins may depend on interactions between them (Michel, Arsanto et al. 2005). The PATJ L27 domain present at the N-terminal is followed by up to ten PDZ domains. The 6th and 8th PDZ modules of PATJ interact directly with ZO3 and Claudin1, respectively, via the PDZ-binding domain present at the C-terminal ends of these proteins. The fact that overexpression or downregulation of PATJ in epithelial cells disrupts the tight junction-specific localization of ZO1, ZO3 and Occludin, suggests that PATJ might be involved in stabilizing tight junctions (Lemmers, Medina et al. 2002; Michel, Arsanto et al. 2005). **SCRIB, LGL and DLG** are localized in the basolateral domain of epithelial cells, the exact nature of the physical interactions between SCRIB, LGL and DLG has not yet been clearly defined. SCRIB (175 kDa) is a large cytoplasmic multidomain protein that plays many roles in flies and mammals. SCRIB is a member of the LAP protein family and has 16 LRR at its N-terminal, followed by 2 LAP-specific domains (LAPSD), a linker region, 4 PDZ domains and a C-terminal lacking any identifiable motives. SCRIB binds directly to the C-terminal motif of ZO2 via its PDZ domains 3 and 4, theSCRIB/ZO2 interaction probably takes place at the cell junctions before ZO2 is segregated in the tight junctions of the apical membrane (Metais, Navarro et al. 2005). Mammalian DLG shows the characteristic MAGUK structural domains, including three PDZ domains, a SH3 domain, a hook domain and a GUK domain, in addition to this basic structure, DLG has a L27 domain at the N-terminal. LGL is localized to the lateral membrane, the region below the adherens junctions and it contains repeated WD40 domains that are known to form β-propellers that act as protein interacting modules for SCRIB. Previously data indicated that LGL has to be phosphorylated to be able to adopt its restricted basolateral localization, as non-phosphorylatable LGL leaked into the apical domain of MDCK cells (Musch, Cohen et

al. 2002). This phosphorylation is mediated by aPKC during the epithelial polarity establishment phase, leading to the detachment of LGL from the PAR6/aPKC dimmer (Yamanaka, Horikoshi et al. 2003). The formation of a polarized epithelial cell layer with functional tight junctions requires spatio-temporal coordination of the activity of the polarity complexes that regulate the establishment and maintenance of the apical polarity in the cell. E-cadherin/E-cadherin interactions in the cell–cell adhesion region trigger Cdc42 GTP activation (Kim 2000) and the phosphorylation of aPKC, which in turn phosphorylates LGL. Phosphorylated LGL dissociates from PAR6/aPKC dimer and distributes to the lateral membrane, where it could interact with DLG and SCRIB (Plant, Fawcett et al. 2003), aPKC is then able to interact with and phosphorylate PAR3, allowing the formation of the active PAR complex at the apical junctions. A direct connection therefore exists between the activity of the basolateral complex containing LGL and the active apical PAR complex. aPKC is required for the stable localization of PAR3, and PAR3 phosphorylated at S827 residue accumulates at tight junctions (Nagai-Tamai, Mizuno et al. 2002; Suzuki, Ishiyama et al. 2002). Once it has been phosphorylated at the S827 residue, PAR3 therefore dissociates from aPKC and this protein is able to bind directly to the CRB cytoplasmic tail that contains two threonine residues (T6 and T9) in an evolutionarily conserved region, which are potential targets for aPKC phosphorylation. CRB binds to PAR6 directly or via PALS1, to promote the differentiation of the premature junctional structure into mature epithelial structures. SCRIB complex restricts the localization of CRB and PAR complexes to the apical region of epithelial cells, where they may act together to regulate tight junctions formation (Hurd, Gao et al. 2003; Lemmers, Michel et al. 2004).

1.4 Tight junctions and cell polarity

In epithelial cells, apical-basal polarity is manteined through the formation of several intercellular adhesion systems consisting of adherens junctions (AJs), desmosomes, and tight junctions (TJs). The tight junctions in addition to estabilish the adhesive contacts between cells, essential property for the function of many tissues, also act as barriers to the diffusion of lipids and proteins between the apical domain and other domains in the membrane. In addition to membrane proteins that mediate the direct contact between cells, these junctions contain a large number of cytoplasmic proteins associated with transmembrane proteins, which function as adaptors that link the integral membrane

proteins with the actin cytoskeleton, thus stabilizing the tight junctions structure (Shin, Fogg et al. 2006). The tight junctions are composed of a network of sealing filaments. Each sealing strand is composed of a long line of transmembrane adhesion molecules immersed in each of the two interacting membranes. The extracellular domains of these proteins are united directly with one another to occlude the intercellular space. The TJ consist of transmembrane proteins claudins, occludins and JAM proteins (junctional adhesion molecules), wich are organized in intramembranous strands and are linked to the F-actin cytoskeleton, either directly or indirectly through intracellular membrane protein called ZO-1 (zonula occludens), ZO-2, ZO-3 (**Figure 3**). The ZO family proteins belong to the family of guanylate kinases (MAGUKs) and are composed of three domains: a PDZ domain that allows ZO proteins binding to claudins, an SH3 domain responsible for binding to α-catenin and guanylate kinase (GUK) domain, which lacks catalytic activity, but interaction with other proteins, such as occludins (Gonzalez-Mariscal, Betanzos et al. 2000). ZO Proteins colocalize with E-cadherin in primordial junctions in not polarized cellsbut succesively they concentrate in tight junctions by interacting

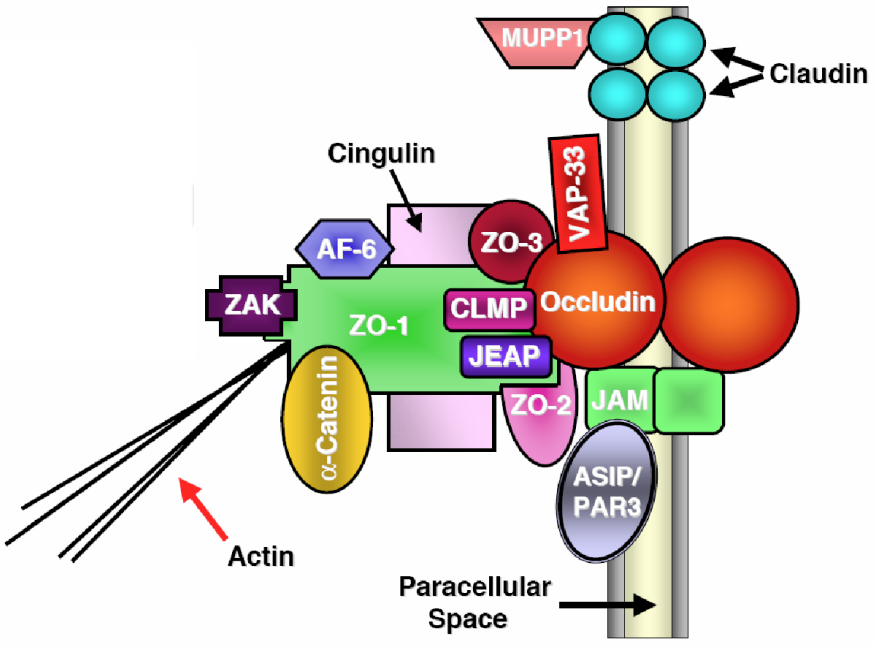

Fig. 3 Tight junctione structure. Tight junctions are multiproteic structures involved in cell-cell contacts and in other processes such as the regulation of cell polarity, proliferation and differentiation. They are platform from wich depart several cellular pathways. The TJs consist of transmembrane proteins claudine, occludine and proteins JAM, wich are organized in intramembranous strands and are linked to the F-actin cytoskeleton either directly or indirectly through intracellular membrane protein called ZO-1, ZO-2, ZO-3.

with occludins and claudins, thus allowing the maturation of this adhesions. The proposed mechanism for the formation of junctional structures is a gradual and sequential mechanism, consisting of various stages and achieved by the cooperation of the three complexes that regulate cell polarity: Crumbs-Patje-Pals, Scribble-Leg-Dgl and par3-Par6 aPKC-protein with the proteins involved in cell-cell contacts. Indeed, this process is accompanied by reorganization of the cortical actin cytoskeleton and establishment of cell polarity. The first event is given by the formation of the first cell-cell contacts, which occur following the formation of lamellipodia and filopodia. These structures allow two neighbor cells to move and come into contact with each other (Figure 4**A**). Following this step, the first proteins, that are locally recruited in regions of cell-cell contacts are the nectins, Nectins, along with a scffolding protein afadin form homophilic and heterophilic trans-dimers and play a key role in identifying cell partners in the primordial cell-cell adhesions (Figure 4**B**). These junctions are then stabilized by recruiting the E-cadherin, that expand the lateral surface and allow the concentration of all the proteins that will form the tight junctions, in this domain Finally, the JAM proteins assemble and allow the recruitment of claudins, occludins, thereby stabilizing cell-cell junctions (Figure 4**C** e 4**D**). It has been proposed that an activator of the small Rho-GTPase Rac1, Tiam1, is associated directly with the Par complex by binding directly Par3 and is involved in the maturation of tight junctions, after the formation of the primordial adhesion (PA) (Mertens, Pegtel et al. 2006). In keratinocytes following the clustering of cadherins, both the Par complex and the Rac1 activator, Tiam1 are recriuited in the sites of primordial adhesions (PA) (**figure 5**). The polarity complex, par3-Par6-aPKC, interacts with the plasma membrane through the binding of Par-3 with JAM-A protein, also recruited in response to the clustering of cadherins (Itoh, Sasaki et al. 2001). Par3 directly interacts with Tiam1, which allows the activation of Rac and thus allows the interaction between Rac and the Par complex. This results in a translocation of occludins, claudins and JAM in the apical membrane with the subsequent formation of tight junctions. In conclusion, the activation of Rac1 mediated by Tiam1 is required for the formation of tight junctions and the activation of these proteins occurs upstream from the activation of the complex polarity Par. This consideration emphasize the role of the small GTPase Rac1 in the acquisition of polarity. The polarity complexes function to determine the site of the tight junction in the process of cell polarization, indeed, CRB and PATJ play an important role in the correct localization of tight junction proteins. In MDCK cells, iRNA-mediated reduction

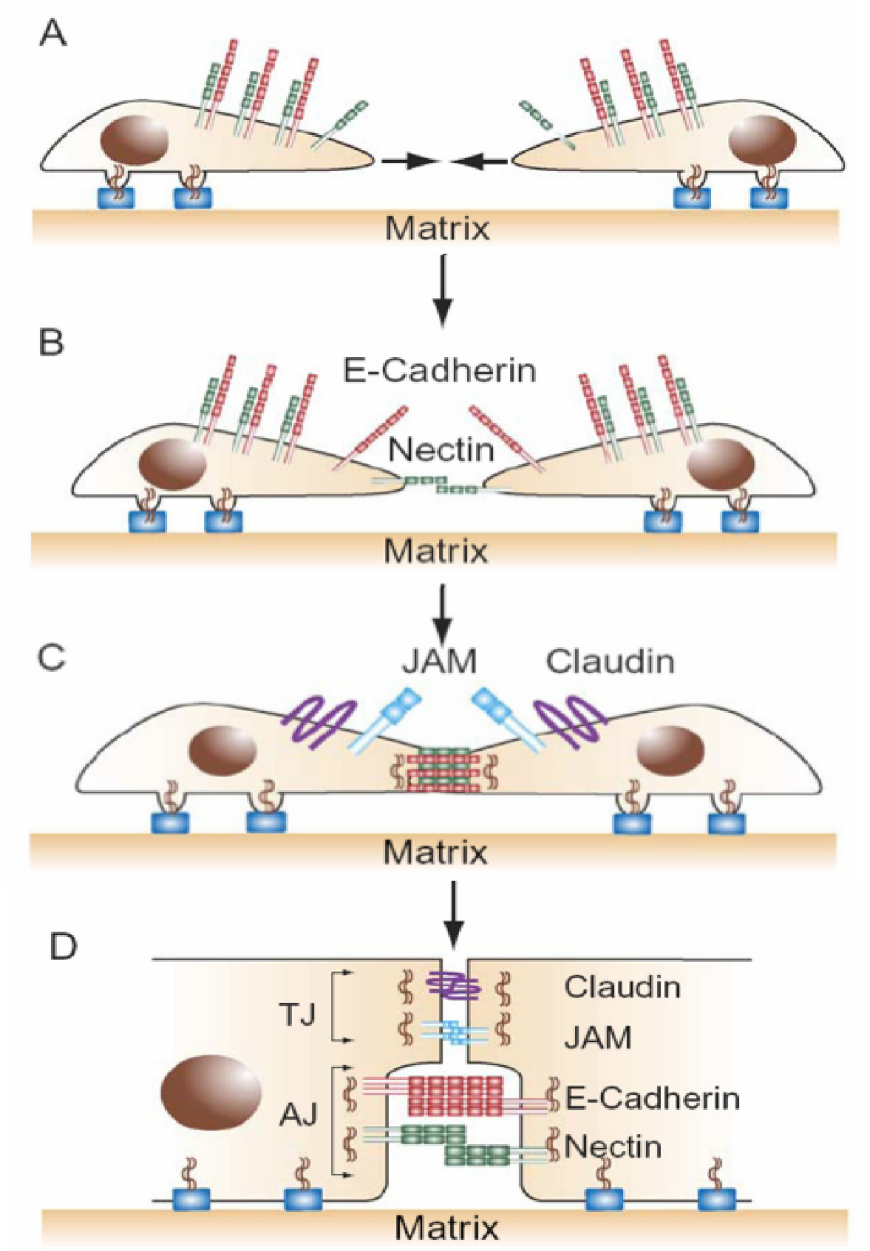

Fig. 4 Sequenzial phases during tight junction formation. The first step is the formation of the first cell-cell contacts (primordial junctions), which occur following the formation of lamellipodia and filopodia (**A)**, in the scond step nectins are recruited, leading to the formation of the first type of cell-cell junctions ((primordial junctions, **B**) These junctions are then stabilized by recruiting the E-cadherin, that expand the lateral surface and allow the concentration of all the proteins that will form the tight junctions in this domain **;** the JAM proteins assemble and allow the recruitment of claudins, occludins, thereby stabilizing cell-cell junctions **(**Fig **C e D**)This picture was taken from the review of Miyoshi and Takai in Advanced Drug Delivery Reviews 57,205, 815-855).

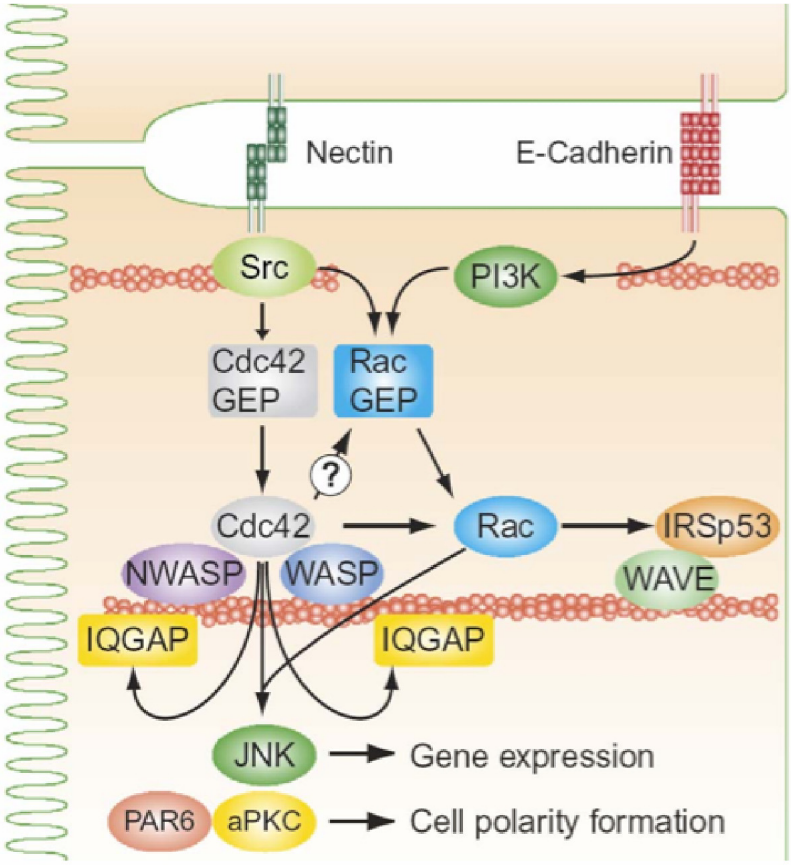

Fig. 5 Signaling pathways from the nectin-based and E-cadherin-based Ajs. These signaling lead to reorganization of the actin cytoskeleton, gene expression, and cell polarity formation. The nectin and E-cadherin–catenin systems induce activation of Cdc42 and Rac. Activated Cdc42 and Rac regulate reorganization of the actin cytoskeleton through their downstream effectors, IQGAP1, IRSp53/WAVE, NWASP, and WASP. Furthermore, they induce gene expression via JNK activation, and facilitate epithelial cell polarity. The picture illustrate salso the role of the Rac protein.
This picture was taken from the review of Miyoshi and Takai in Advanced Drug Delivery Reviews 57,205, 815-855).

of PATJ expression results not only in polarity defects but also in a severe delay of tight junction formation, including mislocalization of occludin and ZO-3 to the lateral membrane (Latorre, Roh et al. 2005; Shin, Straight et al. 2005). In a study in mammary epithelial cells it was shown that the cells expressing little endogenous CRB don't form tight junctions in vitro (Fogg, Liu et al. 2005). An emerging concept is that this complexes define the apico-basal polarity and create the landmark where the tight junction will form. This leads to a close interdependence between polarity complexes and tight junction structural components. Polarity complexes target proteins to the tight

junction, then serves to reinforce polarity by preventing mixing of apical and basolateral membrane proteins.

1.5 Small Rho GTPases

The Rho GTPases are monomeric proteins of 20-30 kDa and belong to the superfamily of GTP-binding protein Ras, which is composed of over 50 members divided into 6 families: Ras, Rho, Arf, Sar, Ran and Rab (Takaji, Sasaki et al 2001). They act as molecular switches in a wide range of signalling pathways upon stimulation of cell surface receptors (Ellenbroek and Collard 2007). These signalling cascades regulate gene transcription, vesicle trafficking and cytoskeleton reorganisation, junctonal complexes formation, processes which affect growth, differentiation, adhesion, and migration of cells (Bar-Sagi and Hall 2000; Mitin, Rossman et al. 2005).

20 different mammalian Rho GTPases, some of them having different isoforms: Rho (isoforms A, B, C), Rac (isoforms 1,2,3), Cdc42 (cdc42Hs isoforms, G 25 k), Rnd1, Rnd2 ; Rnd3, Rho D, Rho G, TC10, and TTF.

Among these the best characterized Rho GTPases are Rho, Rac and Cdc42. Small GTPases can be found either in an active conformation (bound to GTP) or in an inactive conformation (bound to GDP). Only in the GTP-bound state these proteins are able to bind effector proteins and transducer signals from a large variety of membrane receptors including adhesion receptors (such as integrins) and G-protein coupled receptors (Juliano 2002; Buchsbaum 2007). However they are also involved in processes of neoplastic transformation and metastasis. Two main classes of proteins regulate the activity of Rho GTPases, influencing the transition from the active to the inactive state: GEFs (guanine nucleotide exchange factors) and GAPS (GTPase-activating proteins) (Figure 6). The GEFs promote the exchange of GDP with GTP, thus stimulating the dissociation of GDP and the subsequent recruitment of GTP from the cytosol, leading to activation of Rho GTPases. more than 70 GEFs have been identified (Schmidt and Hall 2002). They can be divided into 2 large families. One group is defined by the presence of two characteristic domains, which account for the catalytic activity. These GEFs contain a catalytic Dbl homology (DH) domain, which is almost invariantly followed by a pleckstrin-homology (PH) domain. The PH domain interacts with phospholipids, which may activate the catalytic DH domain of GEFs and localise them to the plasma

membrane (Mertens, Roovers et al. 2003; Rossman, Der et al. 2005). The second group of GEFs for Rho GTPases consists of proteins related to Dock180 (dedicator of cytokynesis 180). These proteins contain a Dock-homology region-2 (DHR2 or CZH2) domain, which renders these proteins catalytically active (Brugnera, Haney et al. 2002; Meller, Merlot et al. 2005). Besides promoting the exchange of nucleotides, GEFs contain various additional domains and are able to influence and determine the signalling route downstream of Rho GTPases by direct binding to different effector molecules, or to serve as scaffold proteins that associate with components of downstream effector signalling pathways (Mertens, Roovers et al. 2003; Rossman, Der et al. 2005). The inactive state is achieved with the GTPase-activating proteins, GAPs, which enhance the intrinsic ability of small GTPases to hydrolyze bound GTP to GDP, which is intrinsically very low. Thus, GAPs promote inactivation and reverse effector binding, thereby shutting down the signalling pathway. About 60 GAPs have been identified (Moon and Zheng 2003; Tcherkezian and Lamarche-Vane 2007), all characterized by an arginine residue, called arginine finger, which interact with the active site of small GTPase. With a few exceptions, Rho family members have a N-terminal portion that interacts with trinucleotides GTP and GDP, followed by the switch I and switch II regions essential for binding to effector proteins and a C-terminal sequence that ends with a CAAX motif (Wherlock and Mellor 2002). Post-translational modifications of Rho GTPases at the C-terminus, such as prenylation (farnesylation or geranylgeranylation) or palmitoylation, determine their intracellular localisation. They act as a lipid anchor and allow GTPases to localise (and attach) to the plasma membrane where they can be activated by GEFs. The regulatory GDI proteins, of which three mammalian members have been identified (DerMardirossian and Bokoch 2005), are cytosolic proteins that form complexes with inactive, GDP-bound Rho GTPases. This binding occurs via an immunoglobulin-like domain present at the C-terminus of GDI protein containing a hydrophobic pocket that is able to accommodate the geranyl-geranyl anchor. Their N-terminal domain, they bind to the switch I and switch II regions of Rho GTPases. Herewith they prevent cycling of the GTPases between cytosol and the plasma membrane and therefore also activation of Rho GTPases by GEFs. Furthermore, GDIs are able to interact with active, GTP-bound GTPases, preventing hydrolysis and interaction with downstream effectors. Association with GDIs thus keeps Rho GTPases in the cytoplasm, inactive or unable to signal towards downstream effectors (Robbe, Otto-Bruc et al. 2003) (**Figure 6**).

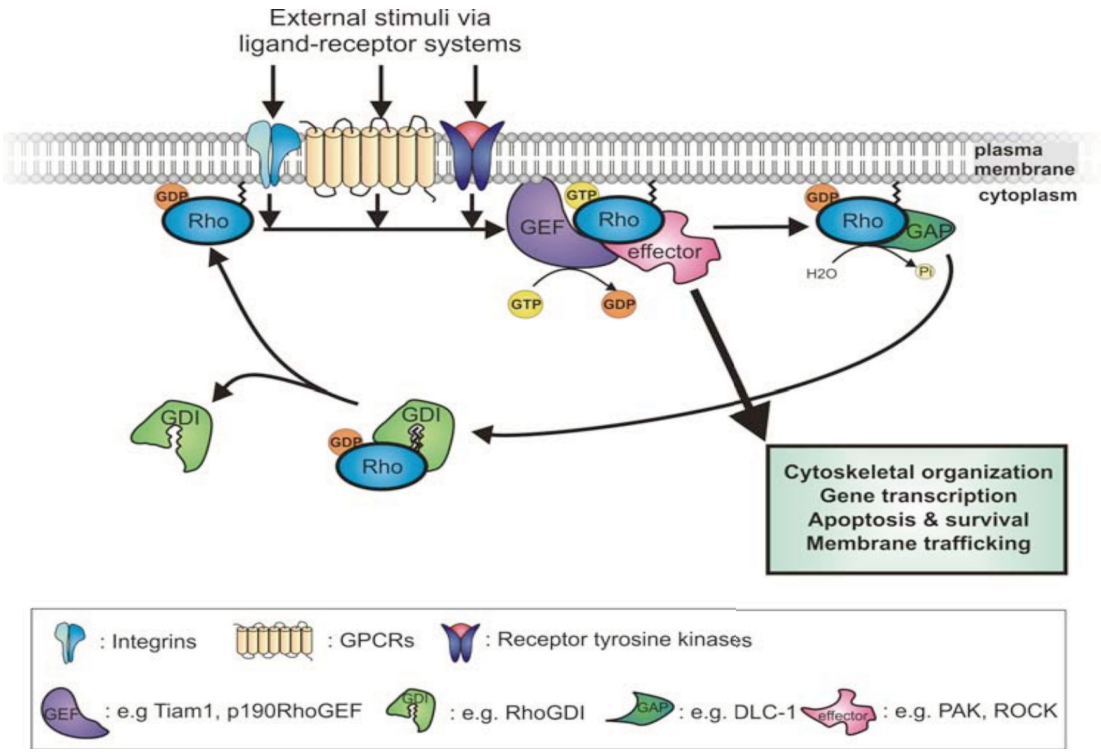

Fig. 6 Regulation of GTPase cycle: GDP-bound inactive GTPases are mainly cytoplasmic, maintained thereby GDIs masking the Cterminal tail required for plasma membrane localisation. Upon dissociation of the GDI, GTPases translocate to the plasma membrane, where they can be activated by GEFs upon external stimuli from surface ligand-receptor systems such as adhesion receptors, G-protein coupled receptors (GPCRs) and receptor tyrosine kinases. Upon activation by GEFs, Rho GTPases can bind different effector proteins, and induce downstream signalling pathways. GAPs inactivate the Rho GTPases and switch off the downstream signalling (this picture is taken by Saskia , Ellenbroek et al. 2007)

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Phosphorylation of Rho GDIs may lead to dissociation of the complex and allow Rho GTPases to translocate from the cytoplasm to the plasma membrane, where they can be activated by GEFs and bind effectors. Specific signals mediated by integrins or other proteins, promote the dissociation of GDI from the Rho GTPase protein. The activation state of all Rho GTPases is dependent on the balance of the activities of their regulators (GEFs, GAPs and GDIs). Thus, the local amount of GTP-bound protein and the time during which the protein is active determines the downstream signalling at specific sites in cells.

1.6 Functions of Rho GTPases

1.6.1. Regulation of the actin cytoskeleton

The Rho family of GTPases have been best characterised for their particular function in the regulation of the actin cytoskeleton in response to receptor signalling. All Rho proteins play an important role in organizing the actin filament system. RhoA regulates the formation of contractile actomyosin bundles (stress fibres) and focal adhesions, Rac1 regulates the formation of actin-rich protrusions (lamellipodia) a well as membrane ruffling and Cdc42 regulates the formation of filopodia (Hall 2005). These actin dynamics are regulated by coordinated activation of different signalling pathways downstream of the small GTPases. RhoA can interact with its effector protein ROCK, which can subsequently activate myosin light chain kinase, leading to activation of myosin (by phosphorylation), increased contractility and formation of stress fibres. Furthermore, RhoA can stimulate actin polymerisation via its effector proteins mDia1 and mDia2, which catalyze F-actin assembly in filopodia and lamellae (Hotulainen and Lappalainen 2006; Gupton, Eisenmann et al. 2007). Cdc42 can signal to the Arp2/3 complex via its effector N-WASP, which results in actin polymerisation and the formation of filopodia (Miki, Yamaguchi et al. 2000; Ten Klooster, Evers et al. 2006). Rac1 regulates actin organisation by activating WAVE or PAK, resulting in altered actin nucleation activity of the Arp2/3 complex. Furthermore, the Rac-activator Tiam1 can bind IRSp53 and p21Arc, one of the components of the Arp2/3 complex, providing a mechanism to directly regulate Tiam1/Rac mediated actin polymerisation processes by the Arp2/3 complex (**Figure 7**).

1.6.2. Regulation of the microtubule cytoskeleton

Besides regulating the actin cytoskeleton, small Rho GTPases are also important regulators of the microtubule cytoskeleton, via regulation of activity of several downstream effector proteins. Interestingly, mDia is a downstream effector protein of RhoA, which is involved in both the regulation of the actin cytoskeleton as well as the microtubule cytoskeleton. RhoA can promote the formation of stable and aligned microtubules via signalling through mDia (Palazzo, Cook et al. 2001; Yamana, Arakawa et al. 2006). Both Rac1 and Cdc42 can influence microtubule stability by mediating PAK signalling to stathmin, an important microtubule destabilizing protein (Daub, Gevaert et al. 2001). Furthermore, Rac1 and Cdc42 are able to promote microtubule capture at the cell cortex (leading edge of migrating cells) by stimulating

binding of their effector protein IQGAP to the capture protein CLIP-170 (Fukata, Watanabe et al. 2002). Microtubule capture is necessary for the stabilisation of microtubules, which is essential for polarisation of cells. Cdc42 plays another crucial role concerning microtubule organisation, during polarisation and directional cell migration.

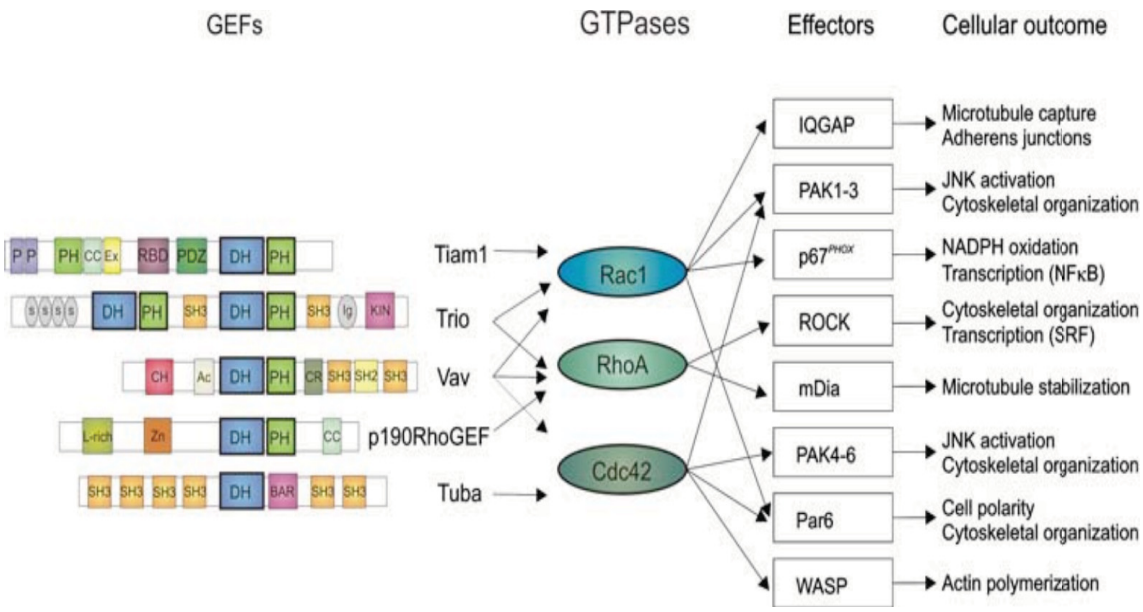

Fig. 7 Rho GTPases and interacting proteins: selected overview of GEFs that can activate RhoA, Rac1 and/or Cdc42 and allow interaction with various downstream effector proteins, resulting in diverse cellular responses. Domain structures of GEFs are schematically represented (this picture is taken by Saskia , Ellenbroek et al. 2007).

It binds to one of its effector proteins, Par6, which forms a polarity complex with atypical PKC and together transduce signals to downstream targets resulting for example in the reorientation of the Golgi apparatus and microtubule organizing centre (MTOC) (Etienne-Manneville 2004). The Rac-GEF Tiam1 also associates with proteins of the Par polarity complex (Par3, Par6 and atypical PKC) and promotes microtubule stability, thereby allowing cells to stably polarise and to migrate in a persistent fashion (Pegtel, Ellenbroek et al. 2007).

1.6.3. Rho GTPases and cytoskeleton-dependent processes

Because of their function in cytoskeletal organisation, small Rho GTPases regulate various cytoskeleton-dependent processes such as changes in cell shape, cell adhesion, cell spreading, cell migration and cell polarity. Adhesive structures such as tight junctions (TJs), adherens junctions (AJs) and desmosomes are linked to the cytoskeleton and determine epithelial morphology and functionality and therefore play an essential role in the maintenance of tissue architecture (Halbleib and Nelson 2006). Rho GTPases have been shown to regulate the formation and maintenance of these adhesive structures (AJs and TJs) (Mertens, Pegtel et al. 2006). Interestingly, Rho GTPase signalling can contribute not only to stabilisation but also to disassembly of AJs leading to EMT. RhoA signalling via mDia and subsequent actin polymerisation is required for formation and maintenance of AJs, whereas RhoA signalling through ROCK results in disruption of AJs caused by increased contractility (Sahai and Marshall 2002). In vitro studies have shown that Tiam1-mediated Rac activity in conjunction with the Par polarity complex is essential for the establishment of apical-basal polarity of epithelial cells and interference with either Tiam1 or the Par complex facilitates EMT and migration of cells (Mertens, Rygiel et al. 2005). Interestingly, the same Par-Tiam1 complex also regulates front-rear polarity and directional migration in dissociated migratory epithelial cells. Rho GTPases also function in polarisation processes in other cell types (T cells, neutrophils and neuronal cells). The outcome of signalling of the Par complex in conjunction with Rho GTPases is clearly context and cell type dependent. Rho GTPase signalling is required for the regulation of vesicular trafficking, including exocytosis, endocytosis and phagocytosis, processes which are dependent on actin and microtubule dynamics and essential for establishment of asymmetrical distribution of proteins in polarized cells (Symons and Rusk 2003).

1.6.4. Rho GTPases and regulation of transcription

Rho proteins have also been implicated in the control of gene transcription. RhoA controls the activation of the nuclear transcription factor serum response factor (SRF), by which it can regulate the transcription of many genes. Furthermore, RhoA, Rac1 and Cdc42 regulate cell cycle progression and growth as well as apoptosis, by regulating transcription of specific genes, including cyclin D1 (Olson, Ashworth et al. 1995; Welsh 2004). Cyclin D1 belongs to a family of proteins that regulate cell cycle progression by stimulating G1 to S phase transition. The transcription of cyclin D1 is

controlled by transcription factors like ETS, AP-1 and NFjB of which activity is regulated by Rho GTPases. These transcription factors may also control anti-apoptotic survival signalling. Rac promotes cell survival by activating the NFjB pathway or by the production of ROS that promotes ROS dependent Erk-mediated survival signalling. Rac1 also stimulates survival signalling through activation of the phosphatidylinositol 3-kinase (PI3K)-Akt signalling pathway (Murga, Zohar et al. 2002). Because of their regulatory function in various signalling cascades, it is not surprising that aberrant signalling by Rho GTPases has been found to be involved in disturbed cellular phenotypes in a wide range of diseases, including neurodegenerative disorders and cancer (Boettner and Van Aelst 2002; Sahai and Marshall 2002).

1.7 Rac1

Rac1, one of the most extensively studied members, was initially discovered as Ras-related C3 botulinum toxin substrate 1 in 1989.

There are 3 isoforms of Rac: Rac1 is ubiquitously expressed, RAC2 is specific for hematopoietic cells and Rac3 is espressed in neurons. Isoforms of Rac1 and Rac 2 have an amino acid sequence identical to 92%. They have an identical effector domain, critical for interactions with both the GEF and with effector proteins. Rac1 exists in two conformational states, an inactive GDP-bound form and an active GTP-bound form. In response to extracellular signals, the interconversion of these states occurs via guanine nucleotide exchange factors (GEFs) which convert Rac1 to its active form, and GTPase-activating proteins (GAPs), which inactivate (Van Aelst and D'Souza-Schorey 1997; Etienne-Manneville and Hall 2002). Among the first described Rac1 effector proteins was the family of p21 activating kinases (PAK). PAK1 binds Rac1 in aGTP-dependent manner, potently stimulating PAK kinase activity and leading to cytoskeletal dynamics, adhesion, and transcription. Rac1 signals through PAK to activate c-Jun Nterminal kinase (JNK), placing Rac1 between Ha-Ras and MEKK in a signaling cascade from growth factor receptors and v-Src to JNK activation. Rac1 has also been shown to influence nuclear signaling through its effectors MLK2/3, which have been shown to activate the JNK pathway (Teramoto, Coso et al. 1996). Rac1 signaling can be important for cellular transformation via modulation of anti-apoptotic and cell cycle

machineries. Rac1 positively regulates transcription at NFkB transcription factor-dependent promoters and facilitates phosphatidylinositol-3 kinase (PI3K)-dependent activation of AKT ser/thr kinase, thereby permitting the survival of transformed cells (Perona, Montaner et al. 1997). Rac1 can also influence transformation through regulation of cyclin D1, a cell cycle protein that is frequently overexpressed in cancer (Westwick, Lambert et al. 1997; Joyce, Bouzahzah et al. 1999) (**figure 8**). The first studies of Rac1 function were performed in the context of clonal cell lines and a team of work implicated Rac1 in reorganization of the actin cytoskeleton, specifically lamellipodia formation, which is thought to contribute to cell movement (Ridley, Paterson et al. 1992). Rac1 was shown to reside at the leading edge of migrating cells, and microtubule growth can activate Rac1 to promote lamellipodial protrusion. The actin-rich lamellae formed at the leading edge in fibroblasts are similar to the membrane dynamics at developing cell-cell contacts in epithelial cells, where actin is recruited to physically strengthen adherens junctions following E-cadherin activation of Rac1 (Vasioukhin, Bauer et al. 2000; Ehrlich, Hansen et al. 2002). Furthermore, because actin cytoskeletal dynamics are intimately linked to vesicular trafficking, it is not surprising that Rac1 has been implicated in this process. Rac1, also participates in reactive oxygen species (ROS) generation in primary cells via NADPH oxidase o via Nox, and it can regulate diverse functions, including transcription factor activation, proliferation, transformation, apoptosis, and cellular innate immune responses. In addition to its effects on the actin cytoskeleton, Rac1 signaling can affect cell growth through a variety of mechanisms. Rac1 can modulate gene transcription through the activation of NFkB, JNK, and p38 mitogen-activated protein kinase (MAPK), all of which induce activator protein-1(AP1) transcription factors that can upregulate the expression of proteins that control cell cycle progression, such as cyclin D1 and c-myc, to induce G1/S progression (Olson, Ashworth et al. 1995; Chiariello, Marinissen et al. 2001). Rac1 is a pleiotropic regulator of multiple cellular functions, some of which are unique in specific cellular contexts. Another role of Rac is in the process of cell-cell adhesion. In fact, it was demonstrated the presence of active Rac and its downstream effectors, in the early contact zone between cell-cell, suggesting that activation of Rac promotes the early stages of adhesion (Price, Leng et al 1998). Indeed, the active form of Rac is able to bind IQGAP preventing it from mediating the dissociation between the β-catenin dall'α-catenin and thus to promote the cell-cell adhesion mediated by cadherins (Kuroda, Fukata et al. 1999). A recently characterized function specific for epithelial cells is the

maintainance of apico-basolateral cell polarity. In a model that use human keratinocyte is shown that, the Rac-specific guanine nucleotide exchange factor Tiam1 (T-lymphoma invasion and metastasis) controls the cell polarity of epidermal keratinocytes. Similar to wild-type (WT) keratinocytes, Tiam1-deficient cells establish primordial E-cadherin-based adhesions, but subsequent junction maturation and membrane sealing are severely impaired (Mertens, Pegtel et al. 2006). Tiam1 and V12Rac1 (a constitutively activated form) can rescue the TJ maturation defect in Tiam1-deficient cells, indicating that this defect is the result of impaired Tiam1-Rac signaling. Tiam1 interacts with Par3 and aPKCzeta, which are two components of the conserved Par3-Par6-aPKC polarity complex, and triggers biogenesis of the TJ through the activation of Rac and aPKCzeta, which is independent of Cdc42. Rac is activated upon the formation of primordial adhesions (PAs) in WT but not in Tiam1-deficient cells. The data indicate that Tiam1-mediated activation of Rac in PAs controls TJ biogenesis and polarity in epithelial cells by association with and activation of the Par3-Par6-aPKC polarity complex.

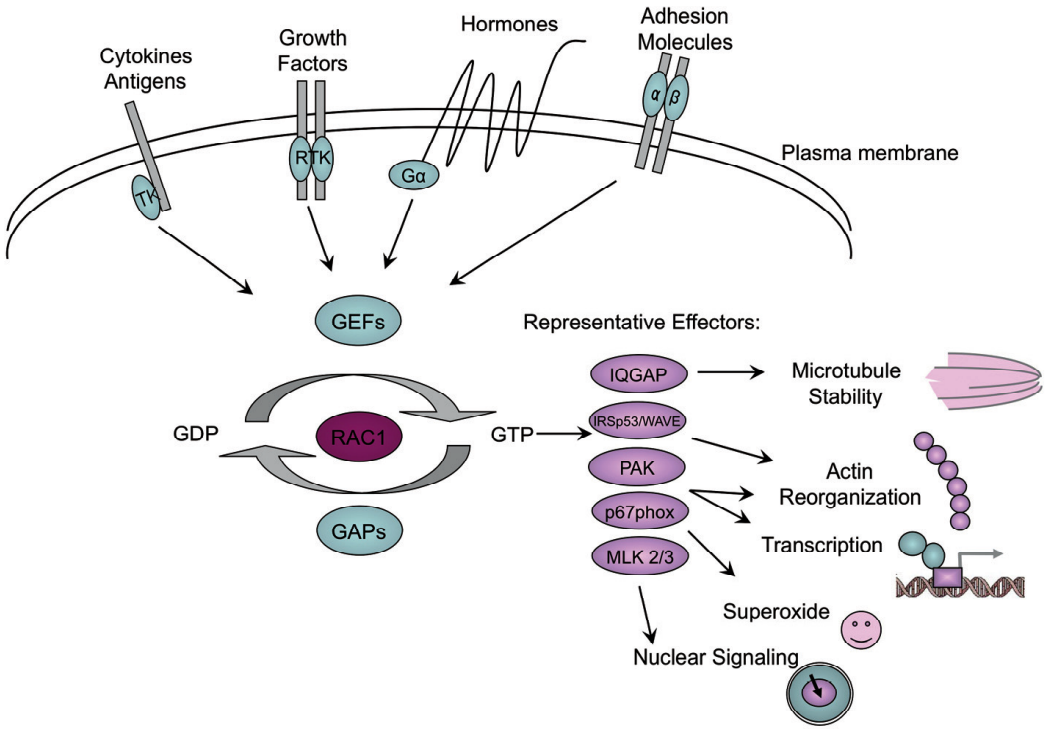

Fig. 8 Rac1 signaling model. Rac1 is a signal transducer and receive information via activated GEFs from a several extracellular stimuli such as receptor kinases, G protein-coupled receptors, and integrins. The GTP-bound Rac1 adopts an active conformation capable of binding effector molecules such as IQGAP, IRSp53/WAVE, PAK, MLK2/3, and p67phox. These effectors regulate many cellular functions, such as cytoskeleton remodeling, microtubule stability, gene transcription, and superoxide production (this picture was taken from Bosco, Mulloy et al. 2009)

1.8 Integrin regulation of small Rho GTPase Rac1

The integrin family of transmembrane receptors mediates cell–cell adhesion and cell attachment to the ECM (extracellular matrix). Integrins do not possess enzymatic activity, however, activation and/or ligand binding induces integrin clustering that leads to the recruitment of multiple signalling molecules and actin filaments (Hynes 2002). Integrins regulate multiple pathways, including Erk, PI3K (phosphoinositide 3-kinase), FAK, Src and small Rho GTPases that induce changes in cell polarity, cell migration, cell-cycle progression, gene expression and survival (Schwartz 1997; Assoian and Schwartz 2001). In addition, integrin signals are frequently required for coupling growth factor receptors to downstream effectors. Integrins regulate the activities of RhoA, Rac1 and Cdc42. These small GTP-binding proteins in turn regulate cell adhesion and changes in cell morphology by triggering dynamic changes in the actin cytoskeleton. Integrin activation of Rac1 and Cdc42 signaling induces the formation of lamellapodia and filopodia, which are necessary for cell spreading. It has recently become clear that integrin-regulated localization of Rac1 at specific plasma membrane microdomain is critical for binding to and activation of its effector PAK (p21 ctivated kinase) (Del Pozo 2004; Guan 2004). Rac is activated by growth factors present in serum in suspended and attached cells. Interestingly, Rac activation level in attached cells is higher than in suspended cells and this is due to the effect of integrins on Rac activation. Cell adhesion to fibronectin in the absence of serum induces a transient activation of Rac that is similar to growth factor induced Rac activation in non-adherent cells. Thus Rac is activated by growth factors present in serum and by cell attachment to the ECM and both stimuli are independent and accumulative. Although Rac GTP loading can be induced by growth factors in an integrin-independent manner, downstream signalling is strictly dependent on integrins. Pak is a Rac effector that is activated by serum in attached cells; however, it is not activated in suspended cells after serum stimulation, even though Rac activity is elevated under these conditions, these series of experiments indicate that adhesion to the ECM couples Rac with its effector Pak. Several observations strongly suggest that proper Rac membrane targeting regulates effector coupling and downstream signalling. Rac translocates to the membrane fraction after serum stimulation in adherent cells, but not in suspended cells. This is also the case for V12 Rac, indicating that changes in GTP loading due to integrin-mediated adhesion to the ECM do not determine Rac membrane targeting. Pak and other effectors also localize to the plasma membrane in activated cells (del Pozo,

Price et al. 2000). Binding between Rac and the lipid bilayer is regulated by RhoGDI, which keeps Rac soluble in the cytoplasm by shielding the isoprenoid moiety (Hoffman, Nassar et al. 2000). RhoGDI binds Rac in the cytosol to prevent both membrane and effector binding.the hypothesis is that integrins would locally increase the affinity of the plasma membrane for Rac, favouring RhoGDI displacement and allowing Rac effector binding (Del Pozo, Kiosses et al. 2002) in the vicinity of focal adhesions. In support of this idea, binding between Rac and RhoGDI is higher in suspended than in adherent cells. In summary, these studies (Ren, Kiosses et al. 1999; Arthur, Petch et al. 2000; del Pozo, Price et al. 2000)showed that integrins, in addition to regulating GTP loading, independently regulate GTP-Rac translocation to the plasma membrane in specific sites, CEMMs (cholesterol-enriched membrane microdomains), allowing effector binding (Grande-Garcia, Echarri et al. 2005).

1.9 Alteration of polarized phenotype by expression of human β1B integrin subunit in epithelial cells line

β1B integrin is a β1 integrin splice variant that differs from the ubiquitous β1A in the terminal portion of the cytosolic tail, in fact this domain is 9 amino acids shorter than β1A and the last 12 amino acids represent a sequence which is not present in the β1A isoform. This region of the molecule is very important for the functional association of the integrin with talin and α-actinin. Experiments conducted in the FRT (Fisher Rat thyroid) epithelial cell line in wich cells have been transfected with β1 integrin splice variant have shown that the β1B expressing cells are affected in several of their properties (Cali, Retta et al. 1998). They attached less efficiently and spread less on fibronectin, laminin or type IV collagen coated dishes, and great reduction of fibronectin fibrils associated to the basal membrane of non confluent β1B transfected cells was observed. This was paralleled by disappearance of microfilament bundles and loss of basally located focal adhesion. Furthermore, this cells showed reduced motile properties when embedded as aggregates in type I collegen gels (**Figure 9**).

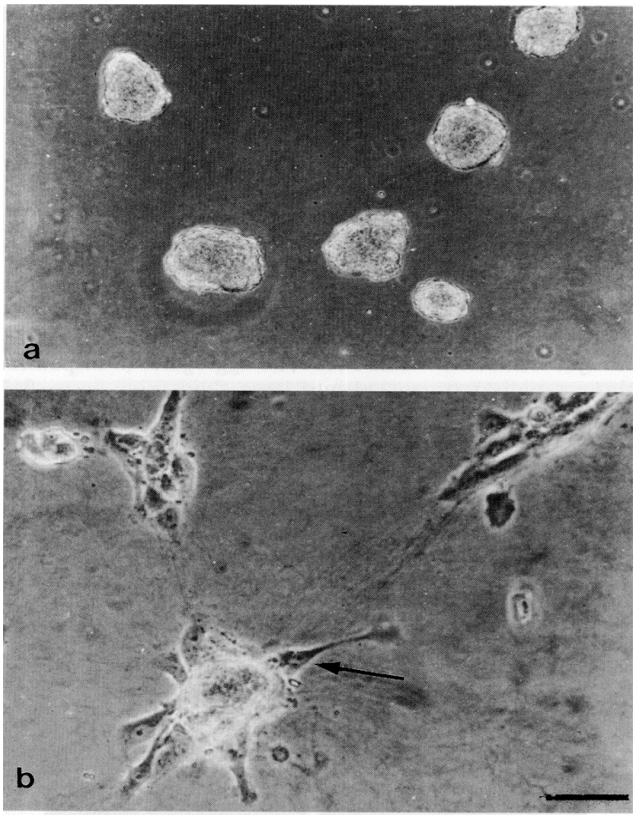

Fig. 9 Analisys of migration in a type I collagen gel. FRT (**b**) cells and β1B expressing cells (**a**) were embedded ina gel and photographed 8h after embedding. These cells have a different behaviour in fact, β1B expressing cells do not migrate and aggregates still retain their round morphology (**a**), while FRT cells migrate and have an elongated form (**b**).

FRT parental cells cultured in suspension on agarose coated dishes form solid aggregates and thereafter polarized cysts which resemble inverted thyroid follicles formed in primary cultures in the same culture conditions. FRT cells expressing β1B variant integrin aggregate regularly; the aggregates undergo compaction but do not evolve, or do it very slowly, into dilated cysts. The data indicates that integrins is an important factor in the regulation of several cellular functions as migration, spreading, adhesion to substrates, organization of ECM, and demonstrate that β1 integrins play a role in the acquisition of cell polarity. Both parental FRT and FRT cells expressing β1B variant integrin are used as model cellular system in this report focused in the role of the Rac1 protein in the control of the polarized phenotype. Several aspects od Rac1 activity regulation, localization and interactions with effectors are not fully understood. Therefore understanding the molecular basis of these complex issues will certainly improve our knoledge of the involvement of Rac1 activity in the acquisition and mantainance of the proper polarity in epithelial cells.

Materials and Methods

3. MATERIALS AND METHODS

3.1 Cell culture

The cells used as model of study during my work are epithelial FRT (Fisher Rat Thyroid) cells. They were cultured in Petri dishes from 100 to 35 mm of diameter and were kept in an incubator at 37 ° C in a humidified atmosphere containing 5% CO2 and 95% air. Colture medium was changed every three days. FRT cells were grown in F12 Coon's medium containing 5% Fetal Bovine Serum (FBS, HyClone). Growth was monitored daily by phase contrast microscopy, to assess the degree of confluence reached by the colture. When the cells reached confluence were washed once with a solution of trypsin 0.3%, glucosio 0.1% , EGTA 2 mM in PBS pH 7.3 (KCl 13.7 mM, KH_2PO_4 1.47 mM, NaCl 137 mM, Na_2HPO_4 $7H_2O$ 8.06 mM) , incubated with the same trypsin solution for 10-15 minutes at 37° C and then resuspended in the medium culture and centrifuged at 1000 rpm for 4 minutes. The pellet was resuspended again in F12 Coon's medium containing 5% Fetal Bovine Serum, and the cells were plated in Petri dishes.

3.2 **Plasmids and transfections**

FRT cells were stably transfected with the plasmid pFNR.β1B (described by Balzac, F. et all. 1993), containing a 3.5 kb fragment of the human β1B integrin subunit coding for an isoform with a cytoplasmic domain wich is 9 amino acids shorter than the one found in the β1A isoform. Furthermore the last 12 amino acids represent a sequence which is not present in β1A isoform. This region of the molecule is very important for the functional associations of the integrin with its substrates and cytosolic partners and it has been shown that the β1B functions as a dominant negative protein. Neomicin resistent stable clones were previously obtained in the last years in the laboratory (described by Calì, G. et all. 1998).

β1B-isoform expressing-FRT cells were stably co-transfected with Pcefl-AU1-ER-Rac1Q61L (a kind gift of Dr Mario Chiariello, Istituto Toscano Tumori, Siena). The DNA encoding the chimeric protein ER-Rac1Q61L consists of a fragment (inserted into Bgl II/EcoRI sites) encoding amino acids 281-599 of the mutated murine estrogen

receptor responsive to 4-OH-tamoxifen (HBD), fused to a fragment (inserted into Eco-Not I sites) encoding the protein Rac1 Q61L cloned into the vector pCEFL AU1 under the CMV promoter. The construct is in frame with the leader sequence and the tag. The tag is represented by a small peptide of six amino acids (DTYRYI) that is recognized by specific antibodies. Rac1(QL) protein is in frame with ER. Plasmid contains the internal resistance to neomycin for selection in eukaryotic cells (Figure 10). In unstimulated cells the protein ER-RAC1 (QL) is synthesized but in absence of 4-OH tamoxifen the hormone-binding domain interacts with the Hsp 90 complex, a protein complex formed by three chaperonins, Hsp 90, Hsp 70 and Hsp 56 and this interaction makes the protein inactive. When 4-OH tamoxifen is added (100 nM) the chaperonine complex detaches, and interacts with the hormone-binding domain inducing a rapid and prolonged protein activation (figure 11).

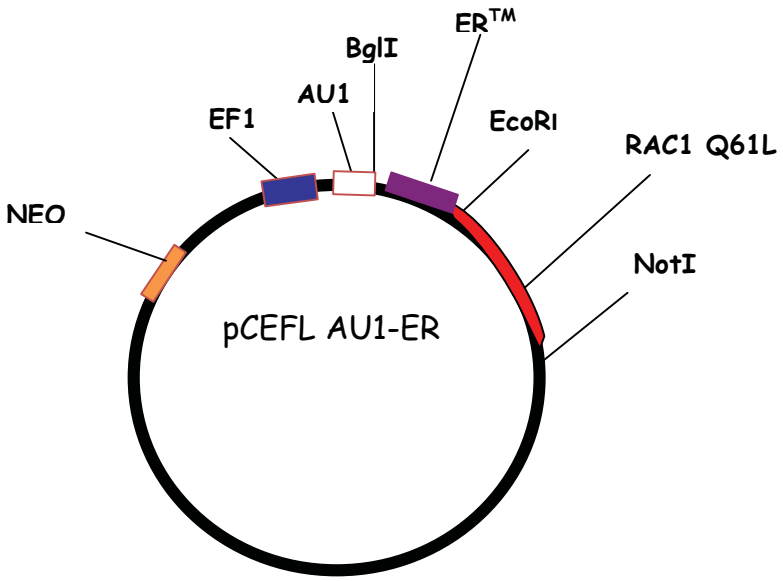

Fig.10 Schematic view of pCEFL AU1 ER. It contains a sequence of mutated murine estrogen receptor responsive to 4-OH-tamoxifen, it is fused to a fragment encoding the protein Rac1 Q61L cloned into the vector pCEFL AU1 under the CMV promoter. Plasmid contains the internal resistance to neomycin for selection in eukaryotic cells.

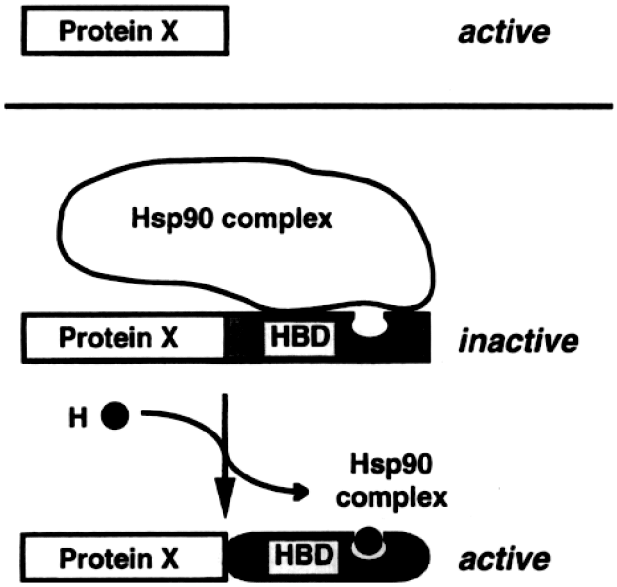

Fig.11 Schematic representation of the regulation of a heterologous protein (protein X) by fusion to the hormon-binding domain (HBD) of a steroid receptor. The interaction of HBD with Hsp complex makes the protein inactive but when the hormone (H) is added the inactivation ceases (from Picard D. 2000).

To obtain stable transfectants, the cells were co-transfected with 4 μg of the plasmid containing AU1-ER-RAC1QL cDNA and 0,4 μg of the plasmid carrying a gene that confer resistence to hygromicin (because of the FRT cells expressing β1B cDNA already were resistant to neomycin). Transfection was performed with FUGEN 6 (Roche) following the manufacturer's instructions. Hygromicin resistant clones were selected in Coon's modified Ham's F12 medium containing 5% FBS and 250 μg/ml of Hygromicin (Sigma). FRT parental cells were also transfected with the plasmid containing AU1-ER-RAC1QL cDNA and stable clones were obtained in the laboratory (by Dr A. Corteggio). Further, FRT parental cells were transfected with cDNA encoding for the human isoform of Rac1, inserted into BglII-EcoRI restriction sites of the pEGFP-C2 vector (BD Bioscience Clontech, Palo Alto, CA) and stable clones were obtained in the laboratory (by Dr A. Corteggio).

3.3 **Immunofluorescence**

Immunofluorescence studies were performed on cells seeded onto 12-mm diameter glass coverslips or on top of filters in bicameral systems (see below) in medium containing 5% Fetal Bovine Serum for a variable time. Indirect immunofluorescence staining in wich a secondary antibody labeled with a fluoruocrome is used to recognize a primary antibody was in general accomplished. Cells were fixed for 20 minutes in PBS containing 4% paraformaldehyde (Sigma-St. Luis, MO-USA), washed two times for 5 minutes in NH_4Cl 50mM, permeabilized with PBS containing Triton X-100 0,3% for 5-7 minutes and successively washed three time with PBS. Alternatively, the cell can be fixed with methanol for 1 minute at -20° C and then permeabilized with acetone for 1 minute at -20° C and washed three time with PBS. A third protocol was use in which case methanol was used as fixative and the cells were permeabilized with 0,3% Triton X-100 in PBS for 7 minutes at room temperature e then washed three time with PBS. Then the cells were incubated in humidified atmosphere with the primary antibody for 1 hour at room temperature. Cells were than washed three times with PBS and incubated in humidified atmosphere at room temperature with the appropriate rhodamine- or fluorescein- tagged goat anti-mouse or anti rabbit secondary antibody (Alexa Fluor, Molecular Probes) diluted 1:200 in PBS. After final washes with PBS, the coverslips were mounted on a microscope slide using a 50% solution of glycerol in PBS and examined with a confocal laser scanner microscope Zeiss 510LSM. The λ of the Argon ion laser was set at 488nm, that of the HeNe laser was set at 543nm. Fluorescence emission was reveled by BP 505-530 band pass filter for Alexa Fluor 488 and by BP 560-LP band pass filter for Alexa Fluor 543. Double staining immunofluorescence images were acquired simultaneously in the green and red channels at a resolution of 1024x1024 pixels.

3.4 Antibodies and reagents

The following antibodies were used: the mouse mAb anti-AU1 that recognizes a six amino acid epitope (DTYRYI) in the AU1 ER-RAC1(QL) protein. (MMS-130R, Covance); the mouse mAb anti-Rac1 (clone 23A8) against recombinant protein containing the full length Rac1 (Upstate) mouse mAb anti E-Cadherin against amino acid 735-883 in the C-terminal domain of human E-Cadherin (BD, Transduction Laboratories); rabbit anti-GM130 (Marra P., Maffucci T., et al. Nature Cell Biology vol.3.pag 1101-1113. 2001).

To inhibit endogenous Rac1 the specific inhibitor, NSC23766 (Calbiochem) at a dose of 100-150 μM was used. NSC23766 is a cell-permeable pyrimidine compound that specifically inhibits Rac1 GDP/GTP exchange activity by interfering with the interaction between Rac1 and Rac specific GEFs, Trio and Tiam1 (Gao et al.,2004).

To activate the AU1 ER-RAC1(QL) protein 4-Hydroxytamoxifen (Sigma Aldrich) was used. 4-OH tamoxifen is an active metabolite of tamoxifen which binds estrogen receptors (ER) and estrogen-related receptors (ERR) with estrogenic and anti-estrogenic effects. For nuclear chromatin staining it was used Hoechst 33258 a bis-benzimides fluorescent stains used for labelling DNA in fluorescence microscopy. It is excited by ultraviolet light at around 350 nm, and emits blue/cyan fluorescence light around an emission maximum at 461 nm.

Cell culture reagents were purchased from Gibco Laboratories (Grand Island, NY).

3.5 **SDS PAGE and WESTERN blotting**

Cells were seeded at low confluence in 100 mm diameter dishes, after 2 days, cell-culture dishes are placed on ice, and cells are washed with ice-cold phosphate-buffered saline (PBS) two times. Whole cell lysates were prepared by homogenization in modified RIPA buffer [150 mM sodium chloride, 50 mM Tris-HCl, pH 7.4, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluorite (PMSF), 1% Triton X-100, 1% sodium deoxycholic acid, 0.1% sodium dodecylsulfate (SDS), 5 μg/ml aprotinin and 5 μg/ml leupeptin] for 10 min a 4°C by shaking. Cell debris was removed by centrifugation. The lysate was cleared by centrifugation at 3000 rpm for 5 min. Protein concentration was determined with the Bio-Rad protein assay. The lysate was boiled for 5 min in 1x SDS sample buffer (50 mM Tris-HCl pH 6.8, 12.5% glycerol, 1% sodium dodecylsulfate, 0.01% bromophenol blue) containing 5% beta-mercaptoethanol, run on 10%-15% SDS polyacrylamide gel electrophoresis, transferred onto a membrane filter (Cellulosenitrate, Schleider and Schuell, Keene, NH) at 400 mA constant for 1h.The nitrocellulose was blocked by incubation with TTBS (50 mM Tris, pH 7.9, 150 mM NaCl and 0.05% Tween 20) 5% NFDM (Non Fat Dry Milk) for 1h at room temperature and successively incubated with the primary antibody diluted in TTBS 5% NFDM. The antibodies against the proteins of interest were all used diluted 1:1000

and incubated one hour at room temperature. The nitrocellulose was washed three times (5 min each) in TTBS, and the appropriate peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG, were used. Then, the membrane was washed again four times (5 min each) in TTBS. The blots were developed with the ECL system (Supersignal West Pico, Celbio, PIERCE, Rockford, IL, USA).

3.6 Bicameral culture system and TER measurements

Acquisition and the maintenance of cell polarity by epithelial cells in monolayer culture can be monitored by measuring the transepithelial reistance (TER) which is generated when cells are grown on top of filters in bicameral systems. Filters are composed of some inert materials as polycarbonate and nitrocellulose and glued on rings in plastic which are placed in conventional culture wells. Filters have a pores of 0.4 μm and a diameter of 24 mm. This system allows to create two compartments above and below the filter, and culture medium is added to both compartments of the system. The development of this technique has made it possible to study in detail mechanism governing epithelial polarity, occluding barrier function and vectorial transport in epithelial cells. Since FRT cells are derived from thyroid follicular epithelium the cell layer on the filter can be considered to represent the wall of a follicle which has been opened and laid on top of the filter with the luminal surface facing the culture medium in the upper (apical) chamber compartment as schematized in figure below (Figure 12), and the basal membrane in close contact with the filter surface.

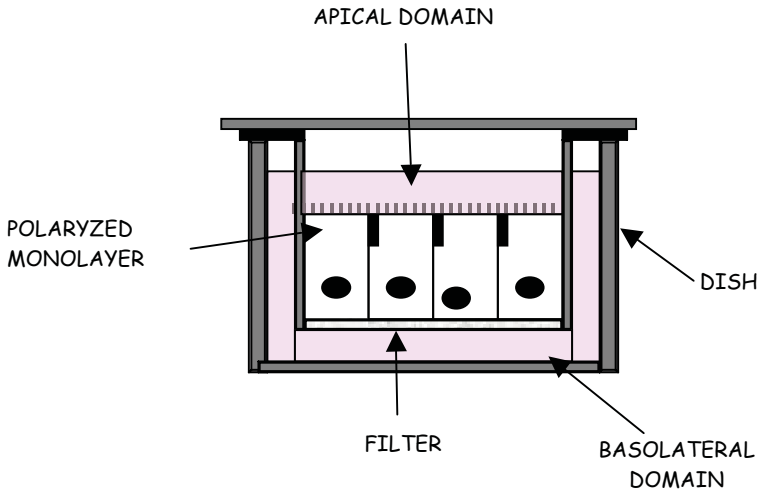

Fig.12 Drawing of the bicameral system. The cells were seeded on the filter with the apical membrane in the upper chamber compartment and the basal membrane in close contact with the filter surface.

The essentials of the method are to seed the cells on the top of the filter and allow the cells to grow to confluence and form a continuous growth-arrested monolayer. In general it can be said that the higher is the seeding density and more rapidly are reached both confluency and maximal TER values. This event correlates with tight junctions formation and great decrease in tight junction paracellular permeability. It is known that epithelial cell lines from different sources are characterized by different maximal TER values. In all experiments presented in this thesis commercial bicameral chambers were used, (Transwell^{TM} , Costar Corp., Cambrige, MA/USA). A layer of polarized, confluent cells can generates a barrier between the two compartments, apical and basolateral, that they separate and under these conditions, it is resistant to the passage of current applied through electrodes connected to a voltmeter (TER, transepithelial resistance). The TER was measured by placing an electrode in the apical compartment and another electrode in the basolateral compartment. The electrical potential difference is about of 3 mV and was measured the resistance that the monolayer opposed to the passage of electric current by another pair of electrodes connected to a voltmeter. TER of cell monolayers was measured with the aid of the Millicel-ERS resistance monitoring apparatus (Millipore). TER values are espressed as Ohms/cm2.

In this report TER measurements were performed in order to define the degree of polarization of the cells in conditions where endogenous Rac1 activity was

experimentally inhibited or where the activity of the exogenous chimeric ER-Rac(QL) protein was induced (see results section).

This system allows to create two compartments above and below the filter, the culture medium was added to both compartments of the system. In the experiments we measured the transepithelial resistance that determines The measurements were made with the Millicell-ERS apparatus (Millipore), which directly provides the values of the resistance in ohms. The measured value must be multiplied by the area of the filter to obtain the absolute value of RTE.

3.7 Suspension cultures

To prepare suspension cultures, confluent monolayers of FRT cell line were trypsinized and the single cell suspension ($2x10^2$) was plated on regular 35-mm tissue culture dishes previously coated by a thin layer of 1% agarose (Sigma) in H_2O to prevent cell attachment to the dish. Agarose was heated for 30 min at 100'C. While still hot, 1 ml of the solution was added to each plastic tissue culture dish and allowed to sit for about 10 sec. The agarose solution was then aspirated and the dishes were left at room temperature until the remaining thin layer of agarose solidified. Dishes were washed with medium before use. FRT cells in this condition first aggregate e successively form polarized three-dimensional structures, known as cysts, formed by a single layer of tightly connected cells delimiting a central cavity (lumen) that could be seen by phase-contrast microscopy. Most lumens were surrounded by a single layer of cells, but in some places additional cells were evident. The normal polarity of thyroid epithelial cells reverses when follicles are in suspension culture. During the polarity reversal, the cells remain attached to their neighbours. The surface features characteristic of the region of the cell next to the lumen (tight junction and microvilli) appear on the cell surface next to the medium and the surface features characteristic of the region of the cell next to the medium appear in the inside of the cyst. The plarity inversion involves changes first in the surface features of the epithelial cell and then in the position of cytoplasmic organelles.

3.8 Cell growth curve

$50x10^4$ cells were seeded in 60 mm diameter dishes. Each cell line was cultured in dishes to be analyzed at 24h, 48h, 72h and 96h after the initial plating. Two dishes for each experimental group were analyzed. In these experiments 4-OH Tamoxifen was added to the cultures after the first 24h . At the end of the selected time intervals, cells were collected and counted to determine the growth trend of each cell line.

3.9 Wound healing assay

The wound healing assay allows to study cell migration. It is simple, inexpensive, and one of the earliest developed methods to study directional cell migration in vitro. This method mimics cell migration during wound healing in vivo. The basic steps involve creating a "wound" in a cell monolayer, capturing the images at the beginning and at regular intervals during cell migration to close the wound, and comparing the images to quantify the migration rate of the cells. It is important that all the cultures are confluent at the start of the experiment. Therefore cells were grown to confluence in F12 Coon's medium containing 5% FBS medium and then were maintained in serum free medium for 48h. The monolayers were scratched with a sterile, disposable 1000 ml plastic pipette tip, the cells were washed 3 times and monolayers were photographed at different time intervals after the initial scratch to monitor the velocity of cell migration. In this thesis the wound healing test was adopted to monitor the migratory activity in monolayers treated with the Rac specific inhibitor or with 4-OH tamoxifen.

3.10 Expression and purification of recombinant GST-PAK-CD fusion proteins

E. coli BL21 cells transformed with the GST-fusion constructs are grown for 16-18 h in bacterical dishes with LB/agar/ with ampicillin (100 μg/ml) at 37°C. One colony was picked and was grown in 3 ml of LB/ampicillin for 6-8h. Then, 10 μl of bacterical suspension were diluted in 200 ml of LB/ampicillin and let to grown for 16-18h and successively in 2L of the same solution for about 1h to permit to bacteria to achieve the appropriate optical density value (about 600), in fact bacteria scatter light in proportion to their numbers and this is an important factor to induce recombinant proteins

expression. Expression of recombinant protein is induced by addition of IPTG 1 mM and further incubation in Luria broth (LB)/ampicillin at 37°C for 3h. Cells are harvested by centrifugation (30 min at 3000 rpm), resuspended in 40 mL bacterial lysis buffer (TRITON X100 10%, EDTA 1mM, Aprotinin 40μg/ml, 3mM PMSF), and then sonicated (3×30 s, 50% cycle, mark 4). Cell lysates are centrifuged at 4°C for 45 min at 4000 rpm, and the supernatant is incubated with glutathione-coupled Sepharose 4B beads (Amersham) for 1 h at 4°C (in a ratio of 1ml of 50% bead slurry per 20mL of supernatant). Protein bound to the beads is washed 3 times in cell lysis buffer. The amount of bound fusion protein is estimated by comparing to bovine serum albumin (BSA) standards resolved in parallel on a 12% reducing polyacrylamide gel, and afterwards stained with Coomassie blue. Protein bound to the beads were conseved at -20°C.

3.11 GTPase activity assays. GST-Pull-Down

To evaluate the rate of Rac1 activity in several experimental situations it was used a GST-Pull-Down assay. This assays is based on the use of a chimeric protein consisting of the glutathione-binding moiety of glutathione-S-transferase (GST) fused to part of an effector molecule which binds to the GTPase in its GTP-bound form. The complex of fusion protein and GTPase is then isolated from a cell lysate by immobilization ("pull-down") of the GST moiety on a sepharose substrate to which glutathione has been adsorbed. Finally, following elution from the glutathione Sepharose, the captured GTP-bound GTPase is detected by Western blotting. For the Rac activity assays, the CRIB domain of the kinase PAK was fused to GST (GST-PAK-CD). An equivalent number of cells ($3x10^6$) was analyzed for each different sample. Cell-culture dishes were placed on ice, and cells were washed with ice-cold phosphate-buffered saline (PBS) 2 times, then, cell-lysis buffer, RIPA buffer (see SDS PAGE and Western Blot) was added in the dishes (500 μL–1 mL). A cell scraper were used to harvest cell lysates, and they were incubated with lysis buffer, in tubes eppendorf, for 15 min at 4°C in shaking and were then centrifuged for 5 min at 3000rpm at 4°C. Protein concentration was determined in supernatant with the Bio-Rad protein assay. Aliquots was taken from the supernatant (100 μg of total protein) and were incubated with bacterially produced GST-PAK1-CD protein bound to glutathione-coupled Sepharose at 4°C for 30. The beads and proteins

bound to the fusion protein were washed 3 timesin an excess of cold cell-lysis buffer, eluted in Laemmli sample buffer, boiled for 5 min, and then analyzed for SDS-PAGE and Western blotting using mouse mAb anti-Rac1 diluted 1:1000 (Upstate Biotechnoloy). ECL detection followed the incubation with a HRP-conjugated secondary antibody (diluited 1.1000) (Figure 13).

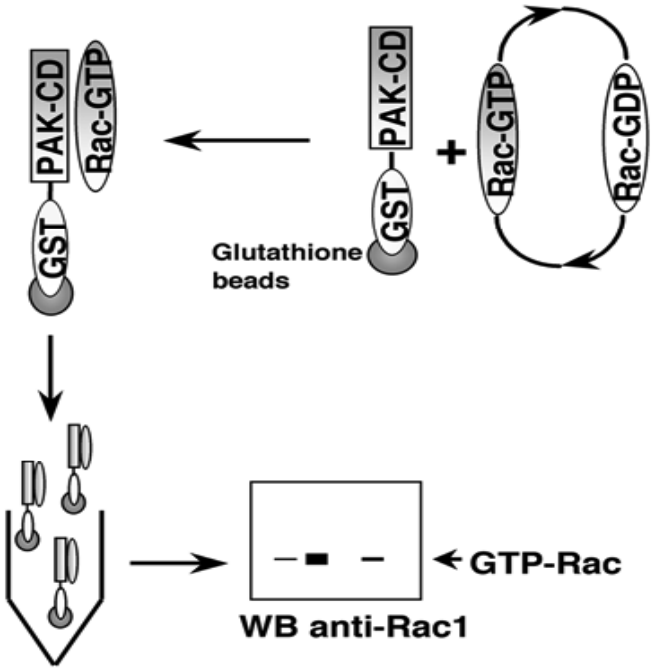

Fig. 13 Outline of the experimental scheme used to isolate Rac1 in his active GTP-bound state. Cell lysate is mixed with GST-fusion protein attached to glutathione-bearing Sepharose beads. Centrifugation of the sepharose beads is followed by washing and elution of the captured active-Rac in sample-loading buffer. Protein is then resolved by SDS-PAGE and Western Blot.

Aim of the project

3. Aim of the project

To carry out the present project interest was mainly focused in the analysis of the role of the Rac1 protein in the acquisition of the polarized phenotype in epithelial cells. To this aim a cell line (FRT) derived from the thyroid gland and expressing high levels of polarity was used.

The Rho family proteins consist of about 20 mammalian members including RhoA, Rac1 and Cdc42. This Ras-related GTPases act as intracellular molecular switches that transduce diverse signals by cicling between the active GTP-bound and the inactive GDP-bound states, leading to cellular responses crucial for cell cycle progression, apoptosis, migration., intercellular adhesion.The Rho proteins become activated through interaction with a class of positive regulators, the Dbl family of guanine nucleotide exchange factors (GEFs). The Rho family of small GTPases regulate the organization of the actin cytoskeleton and are involved in cadherin-dependent cell-cel adhesion. The process of cell-cell adhesion, during the acquisition of the polarized phenotype, can be divided into three steps: formation of new cell-cell contacts, stabilization of these new contacts and junction maturation. As will be addressed in the discussion section Rac1 is critically involved in all these processes. It is also true that several aspects od Rac1 activity regulation, localization and interactions with effectors are not fully understood and await further experimental work.

The establishment of cultures of the FRT rat thyroid cells was achieved by cloning cells of 18-month-old donor rats (Ambesi-Impiombato and Coon,1979). This epithelilal cell line have been widely used previously in studies on polarized traffic of intracellular molecules, on epithelial cells interactions with extracellular matrix and on the role of integrins in the control of the polarized phenotype. It also allowed advancements in the study of thyroid specific genes.

To study the involvement of Rac1 activity in the acquisition and mantainance of the proper polarity in FRT cells two approaches were used:

A- Elucidate the role of Rac1 in the parental FRT cells by investigating its subcellular localization and the consequences on polarized functions of these cells induced by treating them with a small molecule inhibitor (NSC23766), targeting Rac1 activation by GEFs.

In vitro this compound have been shown to effectively inhibit Rac1 binding and activation by the Rac-specific GEF Trio or Tiam1.

B- Verify the involvement of an integrin-dependent signaling to Rac1 in stable cell

lines expressing the ß1B integrin. It has been shown that β1 integrins plays a critical role in orienting the polarity of FRT cels. The FRT-β1B cells have been generated in previous years in the laboratory to directly demonstrate that ß1 integrins play a role in the acquisition of polarity, FRT cells have been transfected with the dominant-negative ß1B integrin that inactivates the endogenous ß1A integrin. An impairment of the polarized phenotype have been obseved (i.e impairment of the ability to form polarized cysts in suspension culture) (Calì et al.,1998). Furthermore, it has been shown that β1 integrin lies upstream of Rac1 in a pathway controlling orientation of polarity (Yu et al.,2005)

We wanted to test the hypothesis that in these cells, where ß1 integrin-dependent signalling is defective , the polarization defect might be related to a reduced activity of Rac1, and that it could have been possibile to rescue the normal phenotype implementing Rac1 activity, by expressing exogenous Rac1 molecules. To do this FRT-β1B cells were stably transfected with a ER-Rac(QL) expression vector that allowed cells to stably express an inducile, constitutively active, Rac1 protein. In this study on the role of Rac1 in the control of FRT phenotypical charecteristics the attention was focused on three different properties of the polarized phenotype that epithelial cell manifest in vitro: migration, transepithelial resistance and threedimensional polarized structures formation. Cell polarization is essential for the migration of individual cells or groups of adherent cells, and migration of epithelial cells is essential during tissue morphogenesis. Cells that persistently migrate exhibit a front rear polarized morphology. It has been shown that proper polarization and diretional migration of many cell types requires coordinated crosstalk between Rho GTPases and polarity proteins. In this thesis work one one of the simplest experimental procedures, the wound healing test have been used, to monitor this activity and to evaluate the influence of the Rac1 activity in the process. When epithelial cells are grown on top of filters in bicameral systems they come across sequential stages of functonal maturation that lead to junctions establishment and reinforcement. The overall process can monitored in this system measuring the increase of the transepithelial resistance. Mature fully polarized epithelial monolayer express the highest value of TER, that correspond to the minimum value of paracellular permeability and indicate solid junctions formation. Generation of polarized cysts by epithelial cells cultivated in suspension culture in the form cell aggregates may represent the end point of a morphogenetic process that cells are still able to perform in the unusual enviroment of an in vitro culture, and that requires that

cells become polarized during the complex rearrangements that are undertaken to form a stable polarized structure. Subcellular localization studies of the Rac1 molecule were performed in parallel with those regarding E-cadherin. Both proteins are in fact intimately related functionally and several studies are being carried in many laboratories trying to define in detail the nature of their direct and/or in direct interactions. Thus the present project combines the interest in defining the role of Rac1 in a model of thyroid derived-epithelium in which the functional properties of this small Rho GTPase heve not been investigated yet to novel experimenal approaches such as the use of the Rac1-GEFs-interaction inhibitor and of a novel construct used, in transfection experiments, to obtain cells that express an inducible constitutively active exogenous Rac1 protein.

Results

4. Results

As a starting point for this work the subcellular localization of the Rac1 protein in the FRT rat thyroid–derived epitelial cell line was investigated by indirect immunofluorescence. As indicated in the Materials and methods section several distinct fixation-permeabilization procedures have been utilized. Every one of them manifested advantages and disavantages, in terms of quality and/or intensity of Rac1 localization in specific cell compartments. Paraphormaldehyde fixation followed by Triton X-100 permeabilization gave a satisfactory staining of intracellular, mostly cytosolic Rac protein but failed to represent its localization at the plasma membrane level. The methanol-acetone procedure on the contrary gave the best staining for the plasma membrane-associated component but poorly showed the intracellular fraction. The best technical compromise we found was to fix the cells with methanol and permeabilize them with Triton X-100.

4.1 *Rac1 localization in FRT cells grown as monolayers on plastic or on filters*

Cells grown to subconfluency as monolayers on plastic and stained with the anti Rac1 antibodies by immunofluorescence are shown in Figure 14. The molecule is present in the cytoplasm in the form of diffuse small clusters and around the nuclear envelope (a). A strong staining can be evidenced in the cell plasma membrane at sites of cell-cell contacts (a and b).

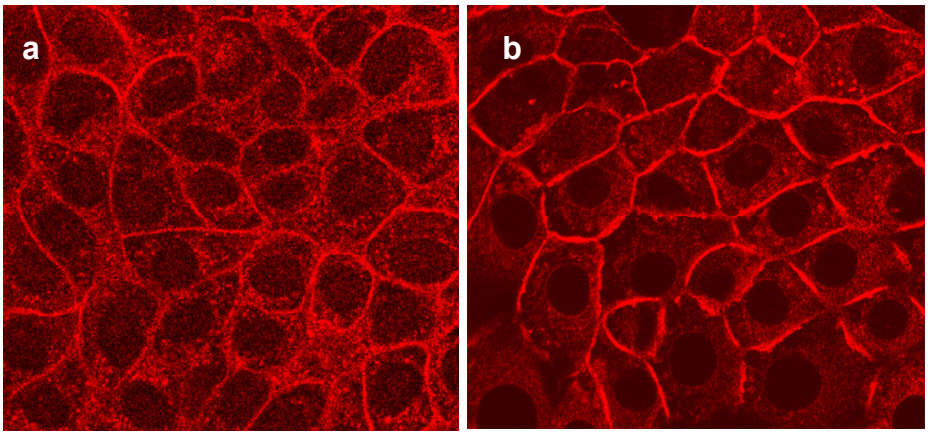

Fig.14 Subcellular localization of the Rac1 protein in confluent monolayers of FRT cells evidenced by immunofluorescence. Cells grown on monolayers were fixed with methanol/Triton X100 (**a**) and with methanol/acetone (**b**) and were stained with anti Rac1 antibody. Both procedures stain well Rac1 on the cell plasma membrane at sites of cell-cell contacts, The cytosolic localization into cytoplasm in the form of diffuse small clusters is better shown in **a**. The methanol-acetone procedure gave the best staining for the plasma membrane-associated component but poorly showed the intracellular fraction (**b**).

A similar distribution pattern is observed also when cultures are not confluent and form colonies af different sizes (Figure 15). It is interesting to underline that, according to a generally accepted point of view, the plasma membrane-located fraction of Rac1 may be considered the activated form of this protein.

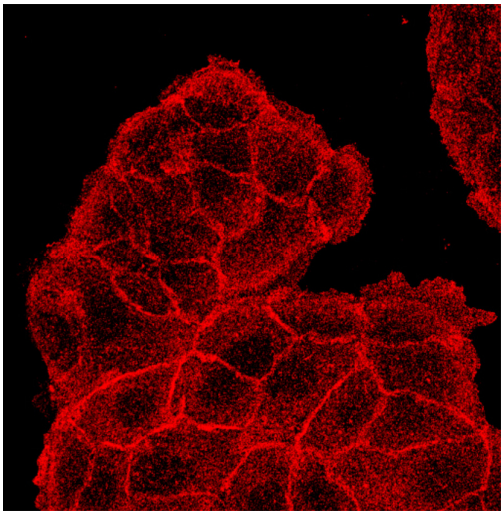

Fig.15 Immunolocalization of Rac1 protein in not confluent FRT cells. FRT cells were fixed at the stage of small colonies and were stained with anti Rac1 antibody. Rac1 is localized on the plasma membrane and in the cytoplasm similarly to cultures in a more advanced state of confluency

In this not confluent culture condition few cells that are positioned at the colony periphery generate lamellipodia. These structures have to be intended in connection with the migratory activity of the cells, that eventually ends up with fusion of the colonies. As shown in Figure 16 Rac 1 accumulates in lamellipodia.

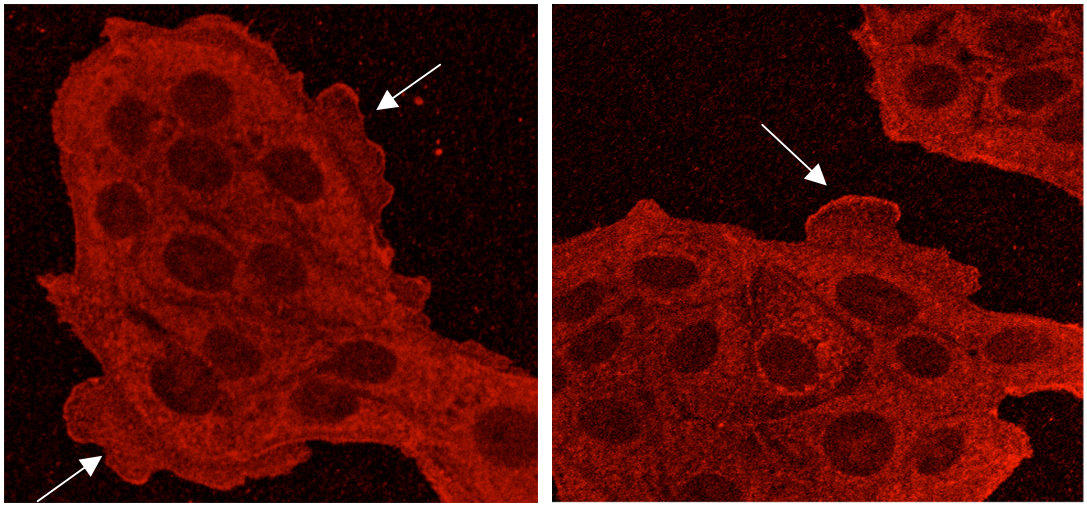

Fig.16 Rac1 protein is localized in lamellipodia in not confluent cells. FRT cells were analyzed for the Rac1 localization. They were fixed when cultures are not confluent and stained with anti Rac1 antibody. At this stage few cells that are positioned at colony periphery generate lamellipodia and these structures are positive for Rac1 staining (see arrows in pictures).

We then analyzed the Rac1 localization in cells that were grown as monolayers on filters in bicameral system, a condition that allows cells to achieve a high degree of morphological and functional polarization. Confocal microscopic analysis of cells in this cultural setting shows that in FRT cells Rac concentrates in the lateral membranes in a region that is close to the apical domain.(Figura 17)

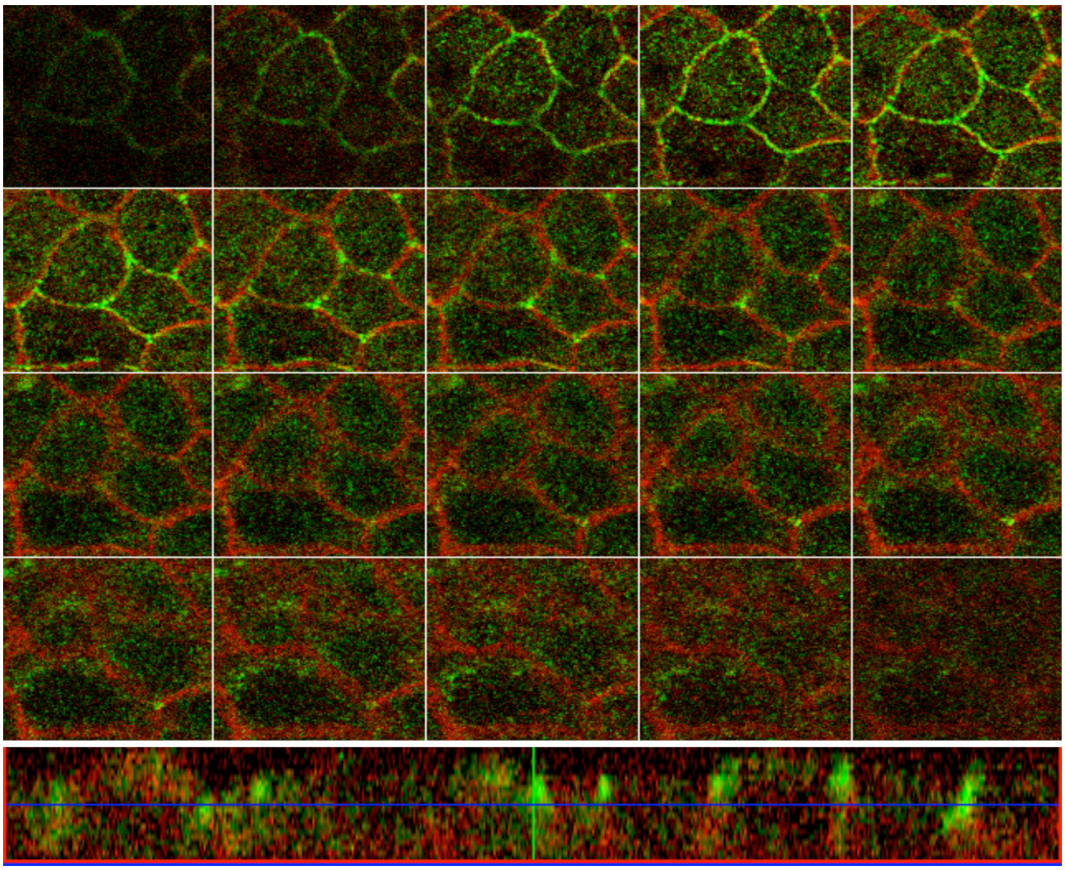

Fig.17 Rac1 localization in monolayers on filter in bicameral system. Growing cells on filters in a bicameral system allows cells to achieve a high degree of morphological and functional polarization. In this cultural setting, multiple sequencial sections obtained by confocal imaging shows that in FRT cells Rac1 concentrates in the lateral membranes in a region that is very close to the apical domain (see green signal), while Na^+ /K^+ ATPase (red signal), a marker for the basolater domain , do not colocalize with Rac1.

We also investigated the distribution of the Rac1-GFP in FRT clones stably expressing this chimeric protein that were obtained in our laboratory (Corteggio A. et al., unpublished results). As shown in Figure 18 the same type of distribution, compared to wild type FRT cells, is found in FRT cells expressing the chimeric Rac-GFP protein. In our work cells expressing Rac1-GFP were mostly used in studies in which a contemporary analysis of Rac1 and E-cadherin localization had to be attempted, since

suitable primary antibodies for detection of these proteins were both of the monoclonal type.

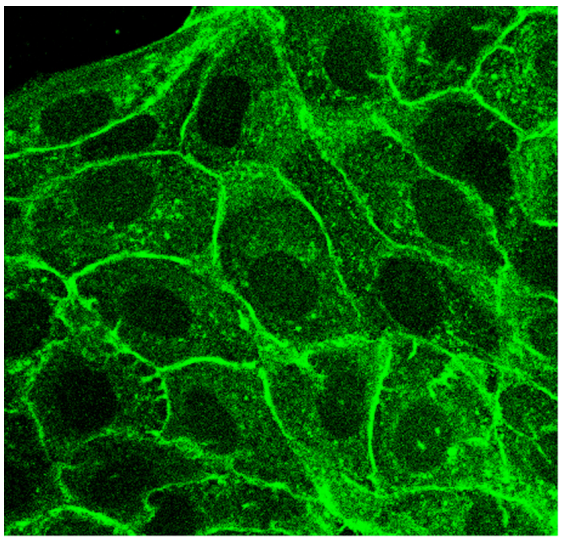

Fig. 18 Analisys of Rac1 distribution in clones stablly expressing a chimeric Rac1-GFP protein. Rac1-GFP expressing cells were fixed and analyzed for Rac1-GFP distribution. In these cells a large fraction of the protein is present on plasma membrane like endogenous Rac1 in parental FRT cells.

4.2 *Rac1-GFP colocalizes with E-cadherin at plasma membrane at regions of cell-cell contacts*

FRT cells were stained by immunofluorescence to determine E-cadherin distribution in the cells. As expected, due to its localization in the adherent junctions cadherin appeared to be concentrated at the plasma membrane at regione of cell-cell contacts, a localization that is strongly reminiscent of that of Rac1. Furthermore a significative amount of this protein was localized in the perinuclear region, in a compartment with morphological features of the Golgi complex (Figure 19). Colocalization of Rac1 with E-cadherin was investigated in Rac-GFP expressing cells using the monoclonal anti E-cadherin antibody.

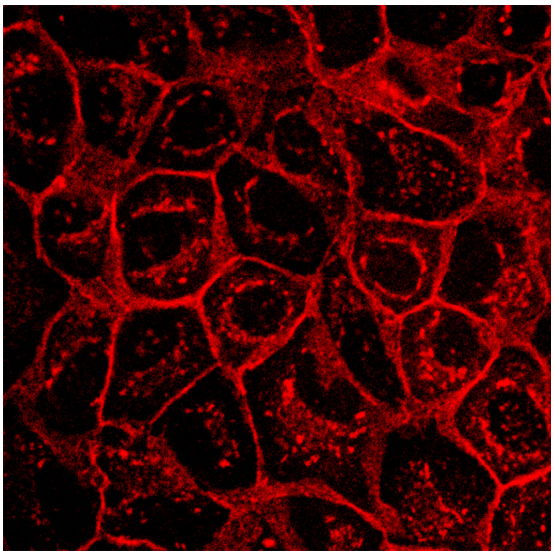

Fig.19 Cellular staining for E-Cadherin. FRT cells were fixed and stained with anti-E-cadherin antibody. Cadherin appears to be concentrated at the plasma membrane, like Rac1, and a significative amount of this protein is localized in the perinuclear region in a compartment with morphological features of the Golgi complex.

As shown in Figure 20 the GFP fluorescence matched that of E-cadherin, suggesting that Rac1 is concentrated at the plasma membrane, in association to the adherens junctions. It is interesting to observe that no relevant staining of Rac-GFP corresponded to the Golgi compartment.

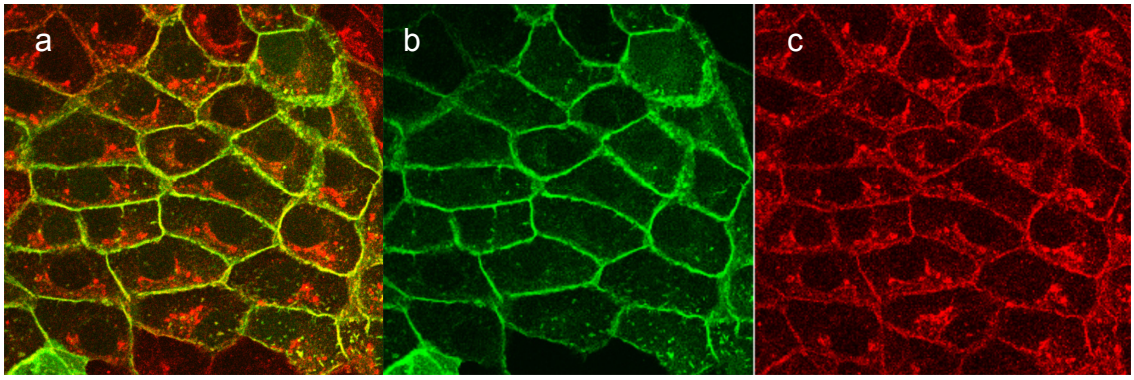

Fig.20 Parallel analysis of Rac1-GFP and E-Cadherin distribution in Rac-GFP expressing cells. The cells were fixed and stained only with monoclonal anti E-cadherin antibody. Rac1-GFP (**b**) and E-cadherin (**c**) localize on plasma membrane and the merge in **a** suggest that at this level both proteins colocalize. Furthermore E-cadherin is localized also in a compartment that corresponds to the Golgi apparatus but the GFP fluorescence is not detectable in this area .

To carry out the present project the interest was mainly focused on the role of Rac1 in the control of polarized functioning of FRT cells. We considered that a useful approach to uncover Rac1- dependent phenotypical properties could have been to treat cells with a specific inhibitor of Rac1 activation and monitor Rac1 distribution in the cells and effects on polarized functions, such as cell migration, acquisition of transepithelial

resistance by cells grown on filters in bicameral systems and formation of polarized structures in suspension culture. The NSC23766 molecule used for this purpose have been described in the paper by Gao et al., (2004). Its effects rely on its capacity to fit into the GEF-recognition groove of Rac1 to act as an activation-specific inhibitor.

4.3 *The treatment with the Rac1 inhibitor induces changes in the overall morphology of FRT cells observed at semiconfluence by phase contrast microscopy*

Figure 21 shows that when FRT cultures are treated with the inhibitor changes are observed in the morphology of the colonies. In control cultures colonies have mostly a rounded aspect, while after NSC23766 treatment they have a deformed, mostly elongated appearance, probably due to changes in morphology of component cells. Cells were smaller and had a less flat aspect compared to control cultures. These changes are gradual and are better manifested after the first 24 hours of treatment. Changes in cell morphology are less evident in the case of more confluent cultures.

As indicated in Figure 22 a significative reduction in the cell number in treated cells is also observed.

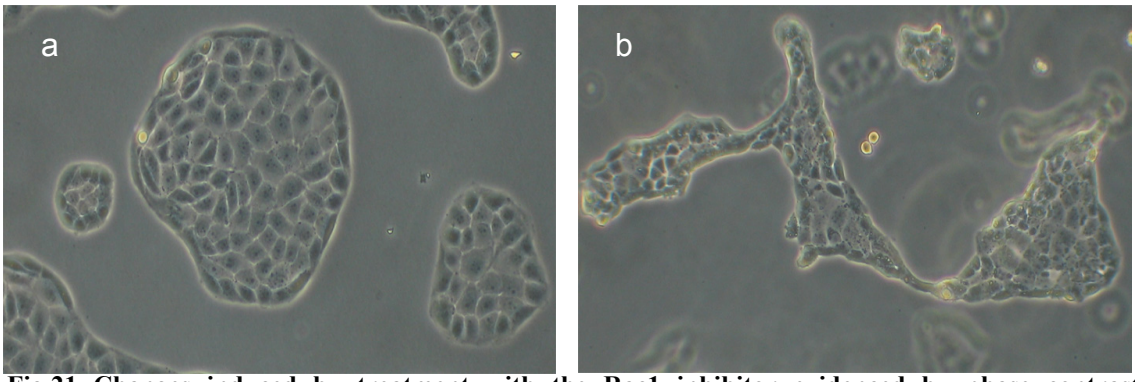

Fig.21 Changes induced by treatment with the Rac1 inhibitor evidenced by phase contrast microscopy of living cells. FRT cells were plated on plastic dishes and kept overnight in regular medium. Then NSC23766 was added. Untreated FRT cells (**a**) and inhibitor-treated cultures (**b**) were photographed 24 hours after the inhibitor addition. Following Rac1 inhibition colonies assumed very irregular shapes and increased the tendency to fuse. Compared to the control component cells were smaller and less spread.

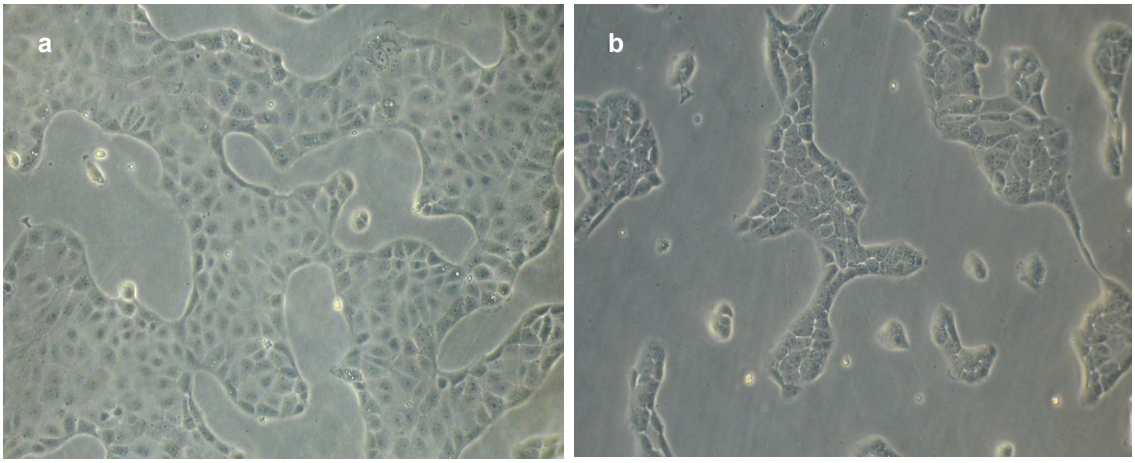

Fig.22 Treatment with the Rac1 inhibitor reduces the growth rate. FRT cells were plated at the same cell density on two different plastic dishes and kept overnight in regular medium. Then medium was changed in all dishes and to one dish NSC23766 was added . Untreated FRT cells (**a**) and inhibitor-treated cultures (**b**) were photographed 48 hours after the inhibitor addition. It is clearly evident that a smaller number of cells is present in dhe dish treated wiyh yhe inhibitor

4.4 *The disappearance of Rac1 molecules from the plasma membrane follows the treatment with the Rac1 inhibitor*

The traslocation of the GTP-bound Rac to the plasma membrane is essential for Rac 1binding to effectors. Although the molecular mechanism that control the targeting of GTP-Rac to the plasma membrane remains largely unknown it appears that it is integrin-regulated and depends from cell adhesion to ECM. According to this point of view the plasma membrane localized Rac1 molecules should represent the fraction of active protein inside the cells. The consequences of Rac1 activity inhibition on its subcellular localization would be therefore an issue interesting to be investigated. This was checked by treating adhaerent cells with the NSC23766 inhibitor and monitoring Rac1 localization in fixed cells by immunofluorescence. Cells were plated in regular medium and allowed to adhaere to the substratum for 24 hours, a sufficient time for the cells to spread and to target Rac to the plasma membrane. Then the inhibitor was added and kept in the medium for 48 hours. Cultures were fixed at the intervals of 12, 24 and 48 hours after the addition of the inhibitor.

The main observation related to this type of experiment is that Rac1 staining at the plasma membrane, in the regions of cell-cell contacts, became progressively fainter. (Figure 23).

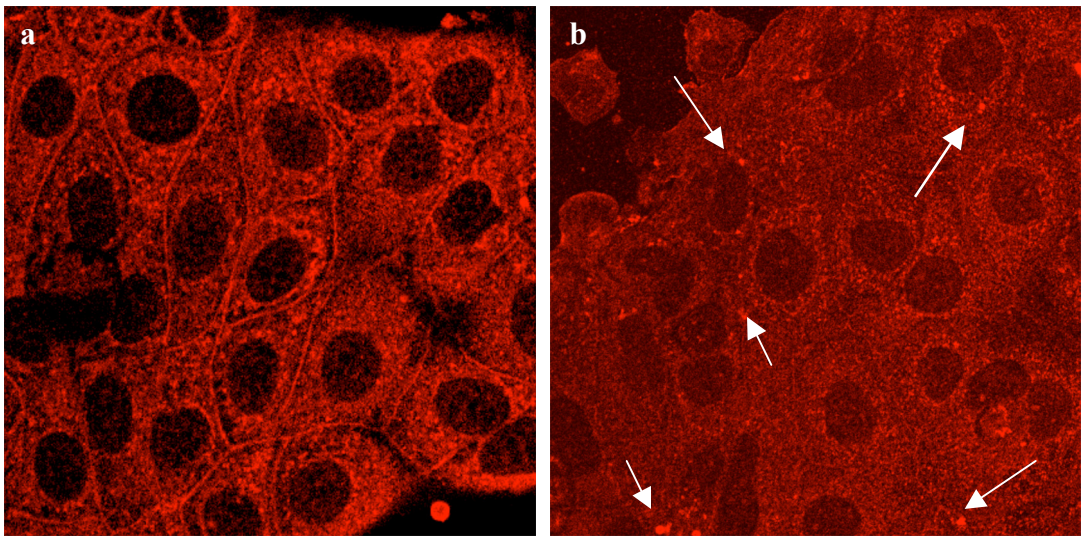

Fig.23 Reduced plasmamembrane localization of Rac1 in FRT cells treated with the Rac1 inhibitor. Cells were treated for 24 hours with NSC23766 and then fixed and stained by immunofluorescence with anti Rac antibodies. Compared to the control (**a**) a reduced amount of the molecule is visibile associated to the cell plasma mebrane (**b**). This finding is in agreement with the generally accepted idea that Rac1 is membrane-associated in its active conformation. In the cytoplasm of treated cells clusters of Rac1 molecules were apparent (arrows). They had different sizes and their number increased prolonging the treatment with the inhibitor (see Figure 10).

On the contrary to our expectations however this was not a rapid effect (morphological changes are recognizable following the first 24 hours of treatment) and was prevalently observed in colonies in subconfluent monolayers. In addition when cells were fixed and permeabilized with metanol/acetone large clusters (vescicles ?) of variable sizes with a positive staining for Rac1 appeared in the cytosol. Their number increased with time, suggesting that this phenomenon was not an artifact due to the specific reagents used in the fixation (Figure 24).

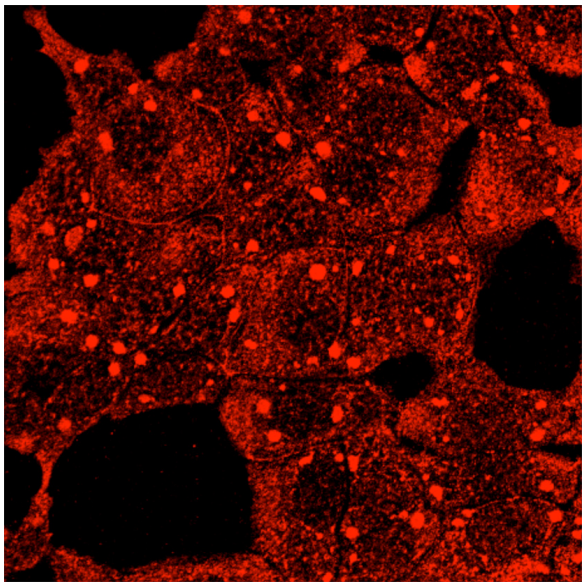

Fig.24 Rac1-positive clusters in the cytoplasm of FRT cells treated with the Rac inhibitor. Cells were treated for 48 hours with NSC23766 and then fixed and stained by immunofluorescence with anti Rac antibody. This field was selected in the virtue of the great number of cells that contain the peculiar Rac-positive granules in the cytoplasm.

Interestingly these structures were very rarely observed when NSC23766-treated FRT-RacGFP cells were investigated (Figure 25).

Differently from control in FRT-RacGFP cells, in addition to a significative reduction in plasma membrane staining, a diffuse, although pale, staining for the molecule was observed in the cytosol of cells treated with the inhibitor. We do not have an explanation for this discrepancy.

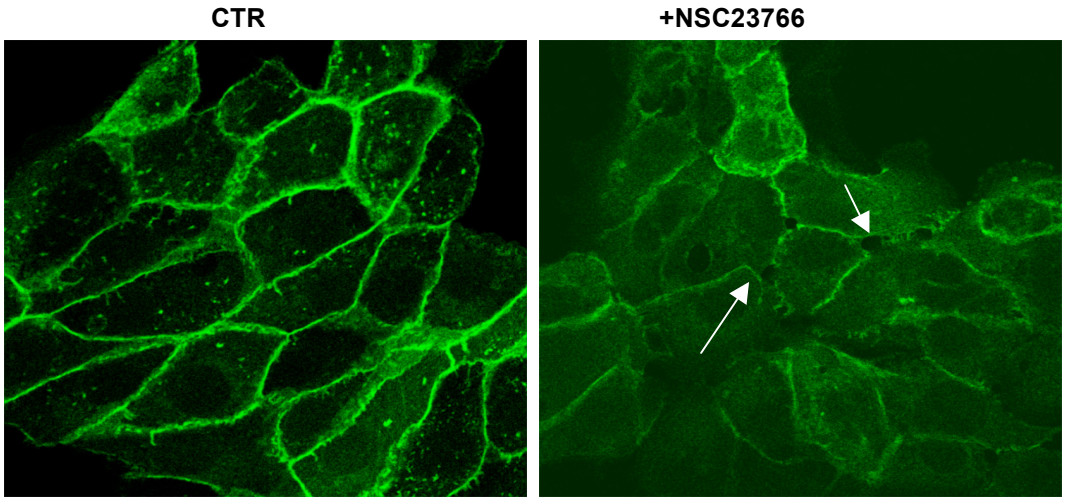

Fig.24 Reduced Rac plasma membrane staining in FRT Rac-GFP-expressing cells. Cells were treated for 48 hours with NSC23766 and then fixed. Staining is very pale at the plasma membrane. Arrows point to limited surface areas where cells appear to be at the point to separate one from the other.

Figure 25 shows FRT cells expressing the Rac GFP protein stained by immunofluorescence with anti Rac1 antibody to localize endogenous Rac1 molecules in both control and inhibitor-treated cells. For both the endogenous and the chimeric Rac-1 molecules a significative reduction of plasma membrane staining is visibile. Endogenous Rac is almost completely undetectable while Rac-GFP is still visibile. One possibile explanation is that the chimeric protein is expressed into a large excess compared to endogenous Rac1 (compare **b** to **c** in the figure 12)

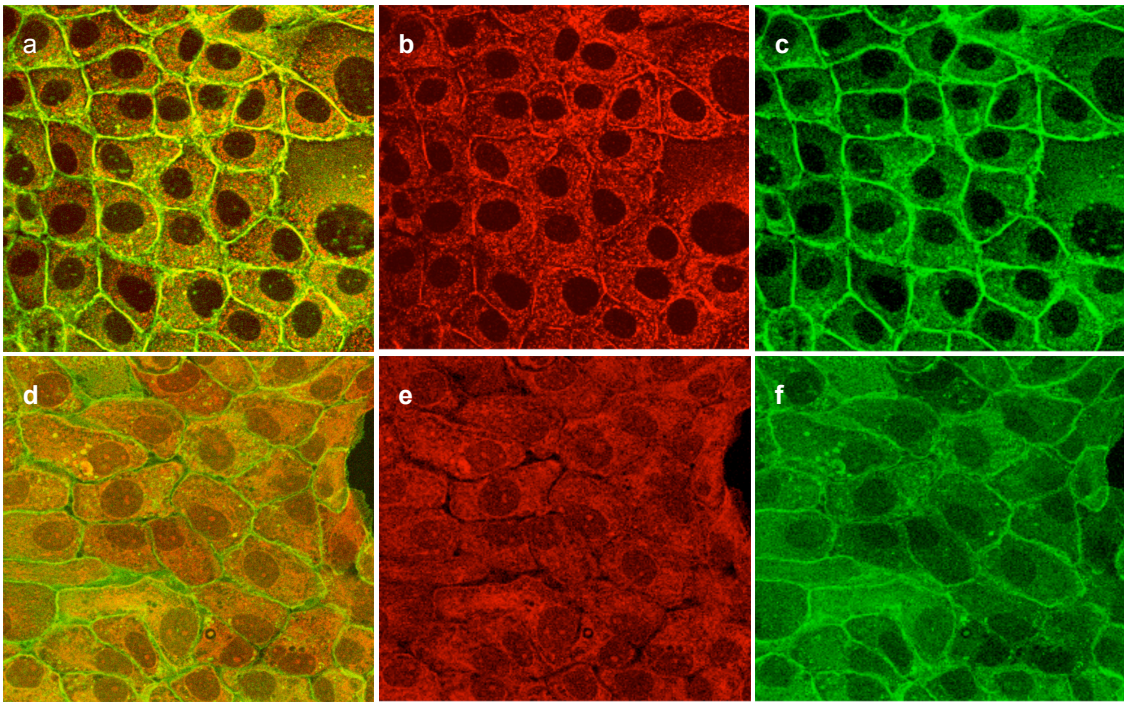

Fig.25 Comparison between endogenous Rac1 staining and Rac-GFP staining in cells treated with the Rac inhibitor Control (**a**,**b**,**c**) and treated cells (**d**,**e**,**f**) observed 30 hours after the beginning of the treatment. Cells were immunostained with anti-Rac antibody to localize endogenous Rac1 (**b,e**). Rac-GFP fluorescence (**c, f**). Only negligeable amounts of endogenous Rac1 is visible while Rac-GFP staining although greatly reduced can be detected on the plasma membrane of several cells. The simplest explanaton for the finding might be that FRT-Rac-GFP cells express the chimeric protein at very high levels and therefore it takes a longer time to be dissociated from the membrane. **a** and **d** merge

4.5 *Inhibition of Rac1 activity impairs acquisition of transepithelial resistance*

Many cells , especially epithelial cells, do not migrate as single cells but rather migrate as sheets or clusters. *In vitro*, on scratching of a wound, a cell monolayer induces the synchronized movement of sheet of cells. As with single cells, the migrating sheets detect the direction of migration and polarize with the protrusive activity constrained to the front. Interaction with neighbours can provide additional directional cues to cells in monolayer. To study the migration of cell monolayers the Wound Healing Assay was performed (see materials and methods). The test was executed both in the absence and in the presence of NSC23766 inhibitor. The repair of the wound was monitored for several hours following the scratch, made with a pipette tip, and photographs were taken at different intervals. The time necessary to close the wound depends on the original size of the wound itself. The inhibition of Rac1 activity determined a significant delay in closing the wound areas (figure 26). According to results in different experiments the FRT-treated cells have a loss in the efficiency in wound healing ranging from 35% to 47% compared to control cells.

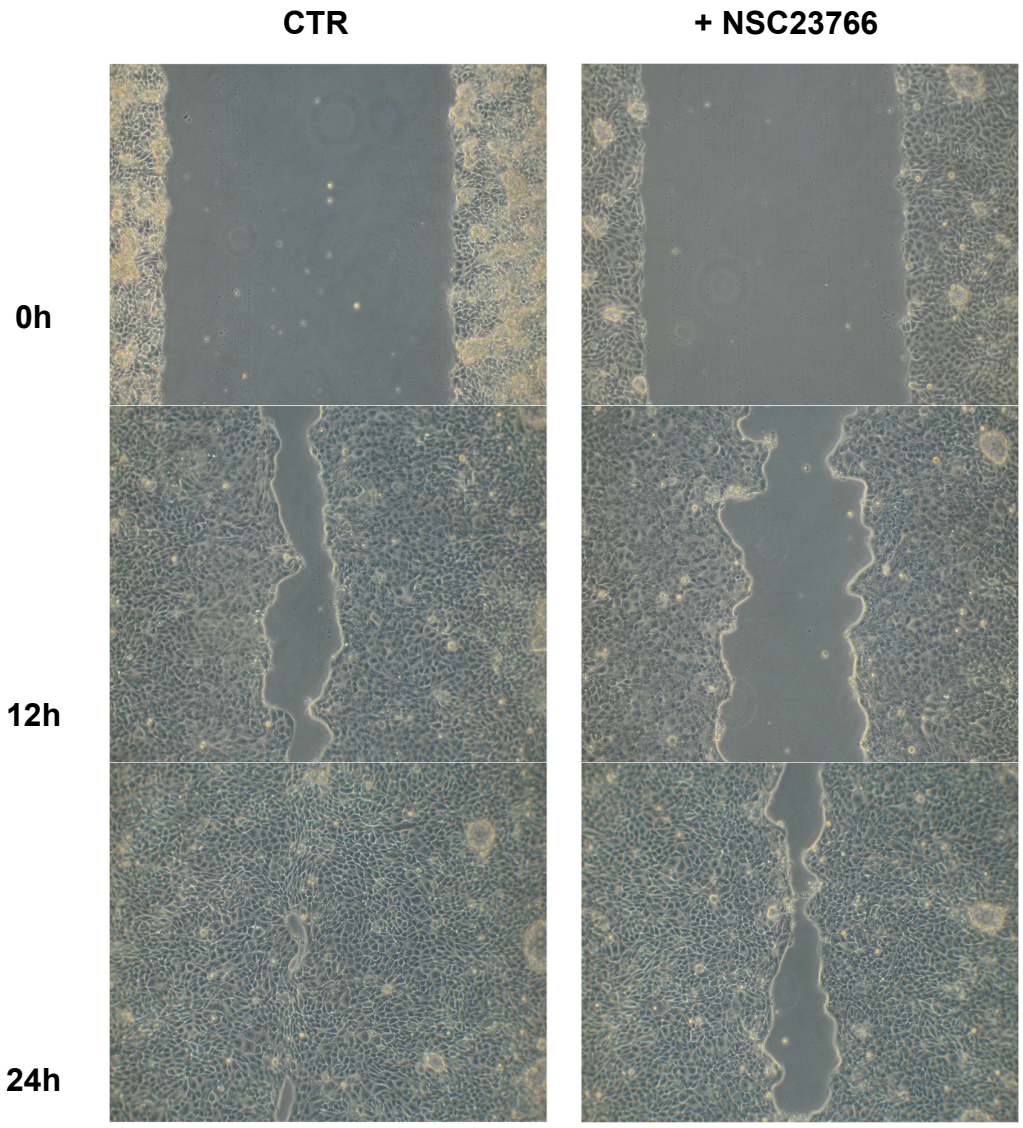

Fig.26 Wound healing test. Comparison between FRT parental cells and cells treated with Rac1 inhibitor. Cells were photographed by phase contrast microscopy immediately after the scratch (time 0) and at different consecutive intervals. It is immediately apparent that Rac1 inhibition reduced the migratory ability of treated cells.

4.6 *Inhibition of Rac1 activity impairs the acquisition of transepithelial resistance*

Transepithelial resistance (TER) measurements have become universally established as the most convenient, reliable and non-destructive method to evaluate and monitor the growth of epithelial tissue cultures, grown on filters in culture inserts. The confluence of the cellular monolayer is quickly determined by a sharp increase in TER. This event correlates with tight junctions formation and great decrease in tight junction paracellular permeability. FRT cells can reach high values of TER compared to other different epithelial cell lines (i.e MDCK, Caco2, etc…). The effect of the Rac1 inhibitor was tested with two different approaches: in one case the inhibitor was added at an early time (few hours after plating) to the culture medium, when cells were still poorly

polarized and exhibited low TER values; in the second case the inhibitor was added when the monolayer have already reached the plateau value of TER. Transepithelial resistance of the cell monolayers was measured by the use of the Millicel-ERS resistance monitoring apparatus (Millipore, Milan). TER values are epressed as Ohms/cm2. When FRT cells are plated in the presence of NSC23766, although the capacity to express a measurable TER is not completely lost, values are significantly lower compared to the control (Figure 27 a). This effect is reversibile: removal of the inhibitor allows cultures to recover and gain higher values of TER (Figure 27 b).

This result suggests that prolonged Rac1 inhibition does not allow cells to reach their final mature polarized phenotype. Rac1 inhibition could interfere with the sorting machinery and/or with the formation of stable junctional complexes at the plasma membrane (see also below for effects on E-cadherin localization in treated cells). An unlike result was obtained when Rac1 activity was inhibited in monolayers kept on filters for few days and characterized by high TER values. In this case the addition of the inhibitor did not evoke detectable changes in the TER (Figure 27 c).

In can be assumed that in this type of experiment cells could have been fully polarized at the moment they were confronted with the inhibitor. The results suggest that in this type of culture setting (i.e cells grown on filters) Rac1 activity is predominantly involved in the process of acquisition of cell polarity, probably through its effects on stabilization of junctional complexes.

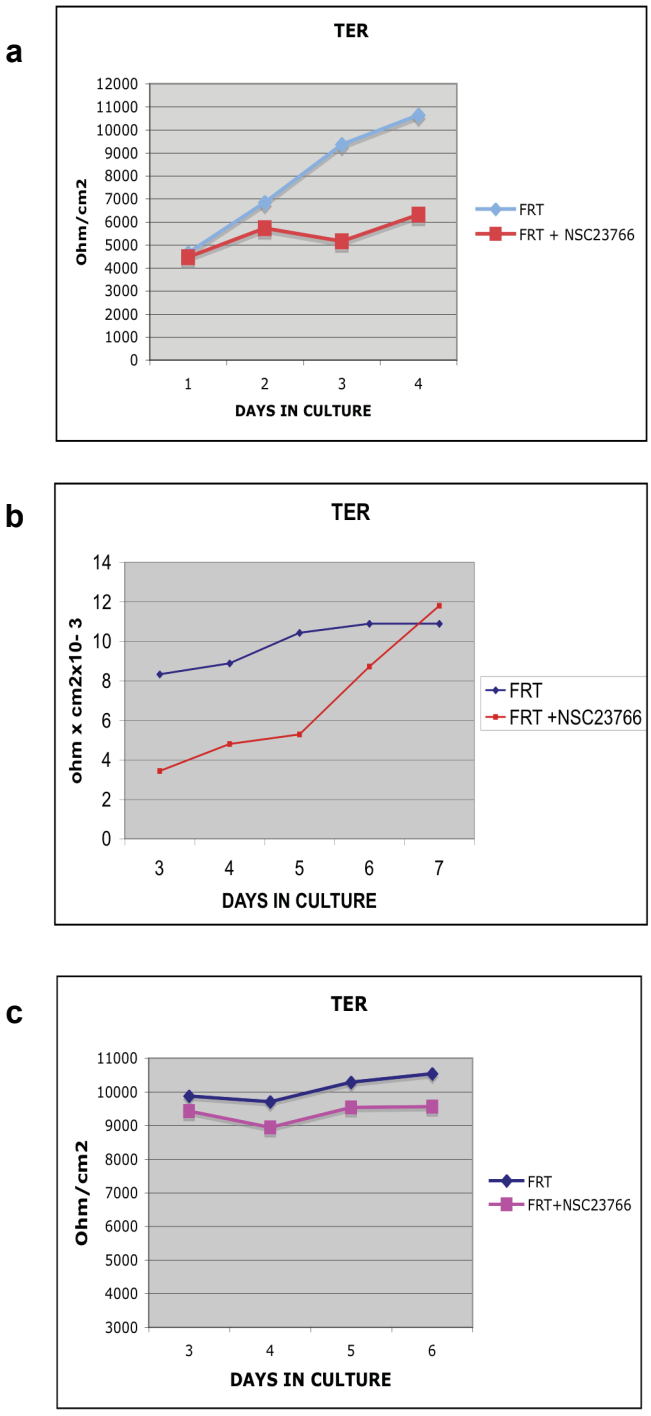

Fig.27 Rac inhibition affects TER in FRT cells grown on bicameral systems. In the top panel an exemplicative experiment is shown where the rise in TER is monitored for several days after the initial plating. High values of transepithelial resistance (TER), generated when cells are grown confluent on the surface of filter, correlate with the acquisition of a mature condition of cell polarity. When the inhibitor is added at an a early time (few hours after plating) FRT cultures (red line in **a**) reach significantly lower values of TER compared to the control. The middle panel (**b**) shows that the effect is reversible. Removal of the inhibitor (in this particular experiment at day 5) allows cultures to gain higher TER values, comparable to those generated by controls (**b**). In the panel **c** the TER curves refer to cells that were plated on filters at high density and that have already reached plateau values at the 3th day. Inhibition of Rac1 in this culture condition is very much less effective in eliciting changes in TER

4.7 *Inhibition of Rac1 activity interferes with the generation of polarized cysts*

A characteristic morphogenetic event that can be observed when FRT epithelial cells are grown as aggregates in suspension culture is that aggregates undergo compaction and then evolve into polarized three-dimensional structures, indicated as cysts (or inverted follicles). The ability to form cysts is linked to the acquisition of a polarized phenotype: cells are connected by tight junctions, segregate membrane proteins in distinct plasma-membrane domains, and vectorially pump liquid inside the follicular lumen. We decided to investigate the effect of Rac1 inhibition on the morphogenetic ability of FRT cells to develop such polarized structures. Cells were plated in suspension on agarose-coated dishes (see Materials and methods) in the presence or in the absence of NSC23766. Crucial in this type of experiment is the time when the inhibitor is added to the cultures. We proceeded in two different ways: a) adding the inhibitor at the very onset of the culture to the suspended cells, b) delaying the administration of the inhibitor about 6-8 hours from the time of initial plating. Cysts formation was monitored in the following 48 hours and pictures taken at 24 and 48 hours. In the first case the cells hardly formed aggregates and remained in suspension as single cells or small clusters, made of very few cells. In the second case cells aggregated, however, as shown in Figure 28, following the inhibition of Rac1 aggregates, although vital, did not form cysts.

These results suggest that Rac1 activity is required both in mantaining the association between cells in the building of an aggregate and also during the establishment of the polarized phenotype that precedes the organization of the follicular cysts.

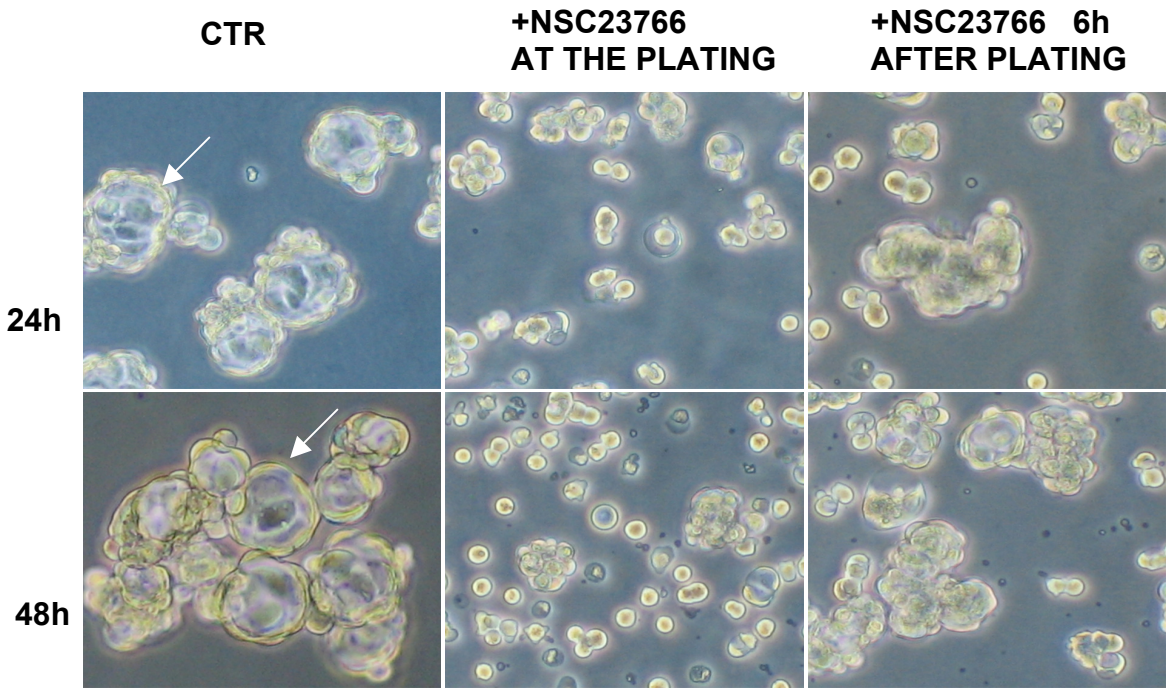

Fig.28 Rac inhibition affects cell aggregation and polarized cysts formation. Cells were grown in suspension as indicated in materials and methods and cultures were monitored at intervals for the presence of cysts with lumen. By 24 hours cysts already formed in control cultures. They are stable structures and can be kept in suspension for very long periods of time. When the Rac inhibitor is added to the cells at the onset of the experiment the major effect is on cell aggregation. Cells remain in suspension mostly as single cells, and a very limited number of small aggregates form. If cells are allowed to form aggregates before NSC23766 administration aggregates stay as they are and did not complete the morphogenetic process that leads to reorganization into a polarized cyst.

4.8 *Reduced plasma membrane localization and disappearance of the Golgi complex-associated E-cadherin in cells treated with the Rac1 inhibitor*

Due to the role of the Rho small G-proteins in the dynamic organization and mantainance of the E-cadherin based adhaerens junctions, the E-cadherin localization was investigated in FRT cells treated with the Rac1 inhibitor. As above mentioned this condition results in a progressive loss of plasma-membrane-associated Rac1 molecules. Immunofluorescent staining of the cells showed that a comparable loss of E-cadherin follows the treatment with NSC23766 (Figure 29)

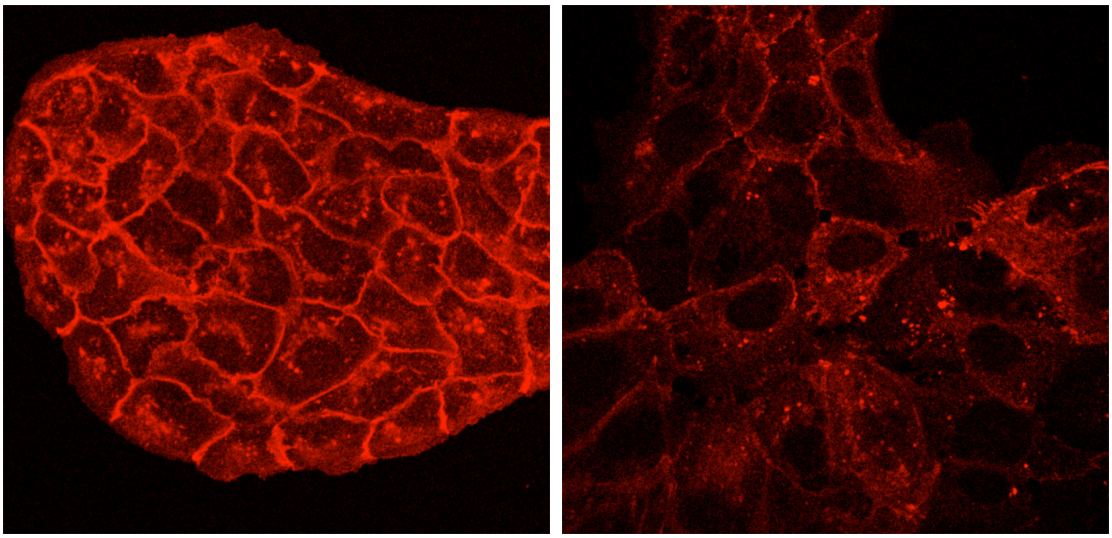

Fig.29 E-cadherin immunostaining in control and Rac inhibitor-treated FRT cells. E-cadherin localizes at the plasma membrane at sites of cell.cell contacts in control cells (**a**) A significative amount of the protein is also present in a compartment close to the nucleus. As a consequence of Rac1 inhibition a quite relevant disapperance of E-cadherin can be observed both at the plasma membrane and at the perinuclear region (**b**) Incubation with the inhibitor lasted for 48 hours.

Furthermore this effect is accompanied by the disappearance of the staining from the Golgi area and the generation of cadherin-positive granules of different sizes dispersed in the cytosol (Figure 30).

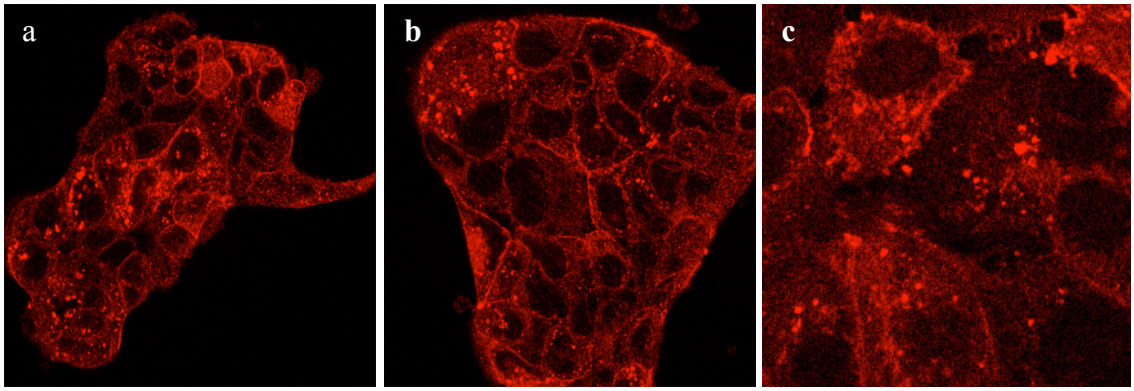

Fig.30 Cadherin positive granules in FRT colonies after the treatment with the Rac inhibitor. Cells were treated for 48 hours in the presence of the inhibitor, fixed and stained for E-cadherin. Several cells contain E-cadherin positive granules (or equivalent structures). These structures are better seen at a higher magnification in cells shown in **c**.

In several areas cells appeared to have lost their connection in the virtue of the loss of E-cadherin (arrows in Figure 31).

Although the overall morphology of the colonies changed (see phase-contrast pictures of living cells) detached cells did not disperse in the dish. One possibile interpretation is

that the cells still continue to mantain their adhesion to the extracellular matrix substratum.

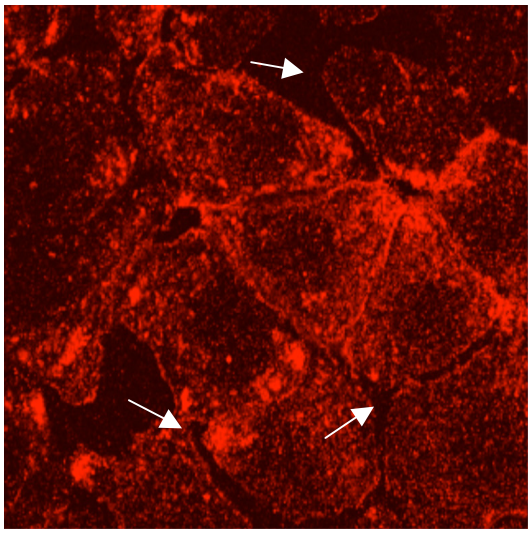

Fig.31 Loss of plasma membrane E-cadherin and loss of intercellular contacts in FRT cells after the inhibition of Rac1 activity (arrows). 48 hours of incubation with NSC23766

The extent of the cadherin disappearance from the membrane parallelled that of Rac1. WhenE-cadherin immunofluorescence was done in FRT-Rac-GFP cells after the treatment with the inhibitor it was frequently observed that regions in the plasma membrane that were devoided of one molecule were devoided of the other as well (Figure 32).

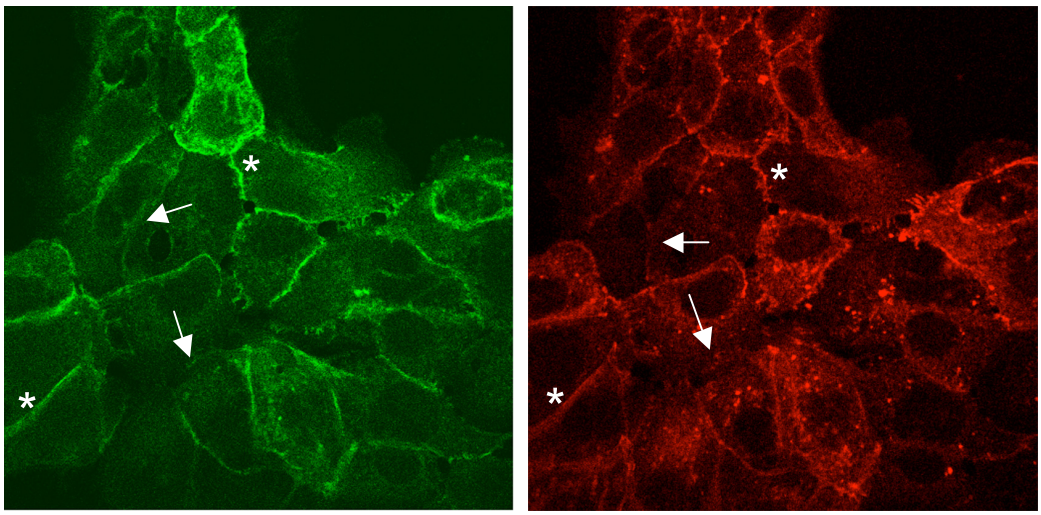

Fig.32 Loss of plasma membrane E- cadherin parallelles the loss of Rac1. FRT Rac-GFP cells treated for 48 hours with the Rac1 inhibitor were fixed and immunostained for E-cadherin. Where Rac1 is dissociated from the membrane also E-cadherin is absent (arrows). On the other hand in GFP-positive membrane regions also E-cadherin is present (asterisks). Note the numerous E-cadherin-positive granules dispersed throghout the cytosol in many cells.

As mentioned one intriguing observation was that similarly to endogenous Rac1 also E-cadherin appeared in cytosolic vescicles following the treatment with the inhibitor. It might have been of interest for us to establish if the same intracellular compartment and/or structure contains both type of molecules. This could have allowed us to have an hint to interprete the fate of the molecules that were dissociated at the plasma membrane level. Surprisingly enough in fact in our experimental conditions the total amount of the E-cadherin did not change, as indicated by Western blot analysis of control and inhibitor-treated samples (Figure 33). However we are at the moment hampered in clarifying the issue since GFP-expressing cells do not tend to form Rac1 clusters and we do not have suitable antibodies to investigate a colocalization between endogenous Rac and E-cadherin.

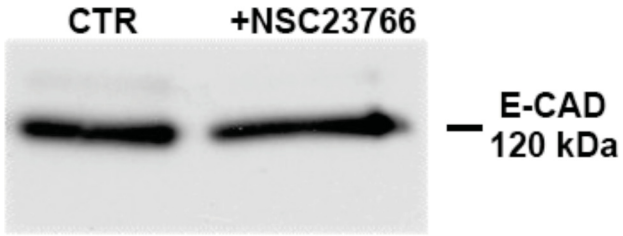

Fig.33 Analysis of the effect of Rac1 inhibitor on E-cadherin levels by western blot. Parental FRT cells were plated in dishes with 10 mm of diameter and after 24h from plating, same of they were treated with Rac inhibitor (100 μM) for 48h; then cells extracts were obtained and western blot analisys was performed. In the sample treated with inhibitor and in the control the total amount of the E-cadherin did not change.

4.9 *Rac1 inhibition leads to Golgi apparatus partitioning*

One question we wanted to answer to was: is E-cadherin disappearance from the Golgi area, observed after Rac1 inhibition, related to some kind of alteration in the general organization of this cellular compartment ?

To properly address this issue the anti-GM130 polyclonal antibody (see Materials and methods) was used in association to anti E-cadherin monoclonal antibodies in indirect immunnofluorescence experiments. In control FRT cells the anti GM-130 antibody nicely stained vescicular structures concentrated at the perinuclear area. Cadherin staining was to a large extent superimposable to that of GM130 (Figure 34 **a**,**b**,**c**). In some cases E-cadherin appeared to be in close association but not superimpose to

GM130-containing structures. This probably reflected the presence of a fraction of E-cadherin in a different Golgi region. It has to be evidenced in fact that the GM130 antigen is a molecular marker for the cis Golgi membranes (and ERGIC compartment).

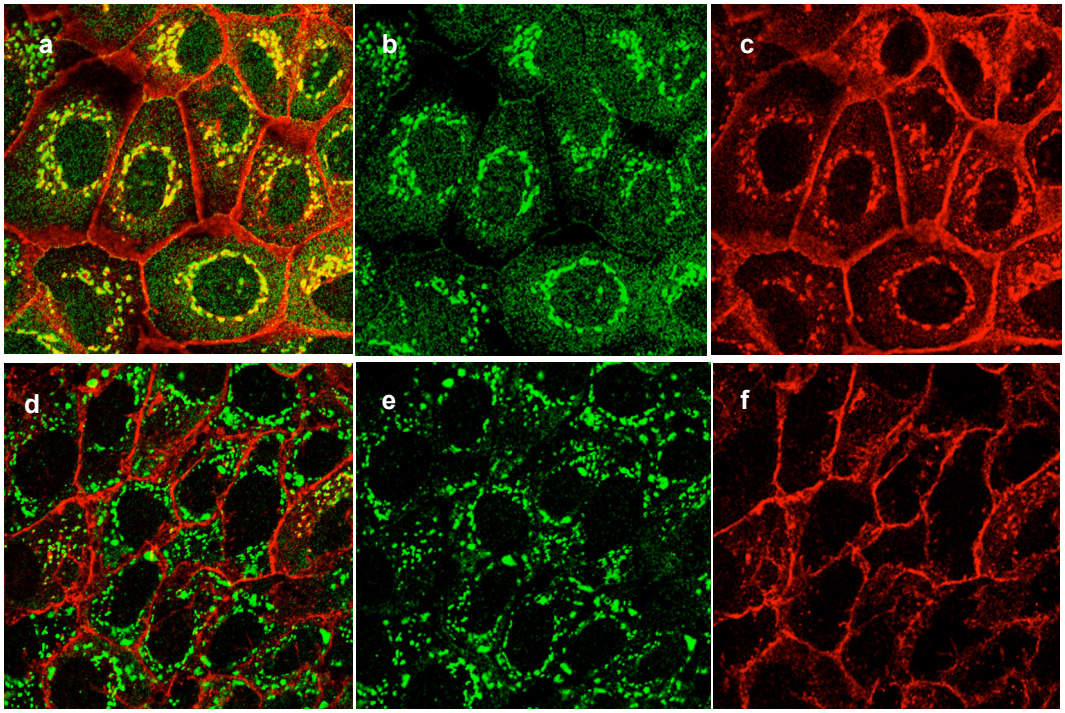

Fig.34 Changes in the Golgi apparatus organization accompanies E-cadherin disappearance from this compartment. After 30h of treatmnt with the inhibitor FRT cells were analyzed by immunofluorescence with anti GM130 and anti E-cadherin antibodies. In control cells the two proteins have the same intacellular distribution pattern and colocalize to a large extent (see **b,c** and the merge in **a)**. In treated cells Golgi elements appeared more irregular in shape and dispersed in the cytosl (**e**). No E-cadherin staining is detactable at this stage in association to GM130-containing structures (**f** and merge in **d**).

As mentioned in the previous section the treatment with the NSC23766 inhibitor made the staining of the E-cadherin at the Golgi undetectable in the vast majority of the cells. GM130 staining was however clearly visibile and allowed to evidence the partitioning of the Golgi and dispersal of GM130 positive vescicles throughout the entire cytosol of the cells (Figura 34 **e**). Furthermore Figure 35 clearly shows that E-cadherin-positive large vescicles, accumulated in treated cells, are distinct from these cis-Golgi elements stained by the anti-GM130 antibodies.

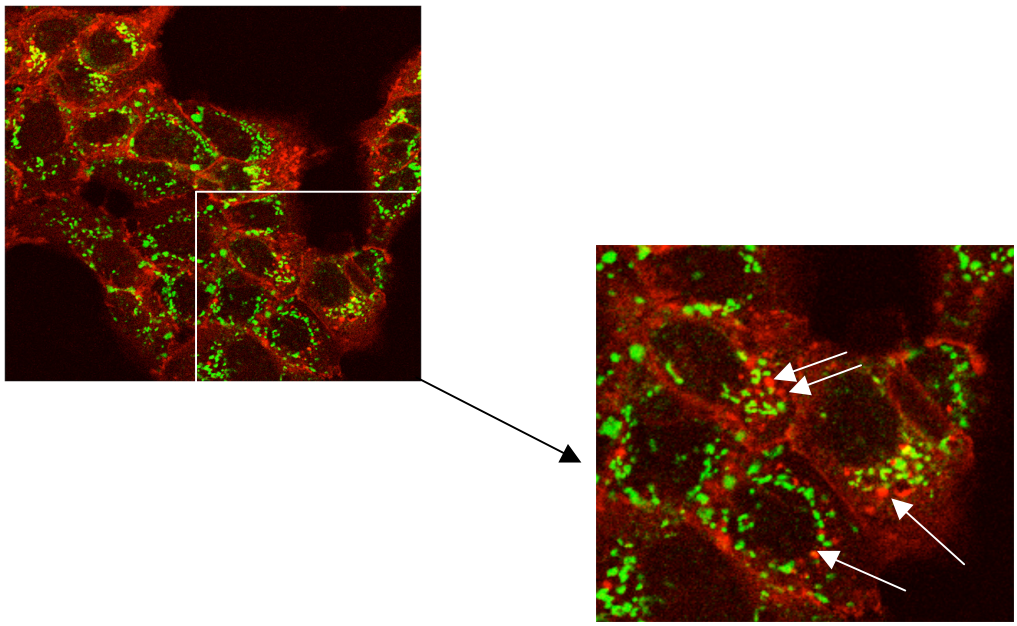

Fig.35 E-Cadherin containing granules do not colocalize with dispersed Golgi elements in cells treated with Rac1 inhibitor. As shown in the insert and indicated by arrows E-cadherin positive structures that accumulate in cells treated with NSC23766 do not superimpose to GM130-positive structures.

This interesting observation raise a number of interesting questions such as: by which mechanism the Golgi complex became depleted of E-cadherin?, is this a general consequence of Rac1 inhibition, affecting the traffic of other proteins in the cell ?, is the Golgi complex still able to function in these conditions? Our preliminary results suggest that the effect is reversibile, and that Golgi structures reassemble upon removal of the inhibitor from the culture medium .

In summary the results obtained with NSC23766 show that Rac1 inhibition in FRT cells:

- causes Rac disappearance from the plasma membrane
- inhibits oriented cell migration
- prevents the acquisition of a high value of transephitelial resistance
- impairs the formation of polarized cysts in suspension culture
- disrupts E-cadherin association to plasma membrane and Golgi stacks.

The above mentioned observations define the molecular basis for alterations observed in the polarization process in FRT cells treated with the Rac1 inhibitor. Furthermore they seem to establish a relationship between Rac1 activity and Golgi complex integrity, a cell biology issue not investigated so far.

4.10 *FRT cells expressing the dominant negative version of the β1 integrin: a model to study Rac1 activity*

As pointed in the introduction FRT cells expressing the dominant negative β1B form of the human β1A integrin subunit show phenotipical changes very similar to those observed in parental FRT cells treated with Rac1 inhibitor. β1B is a β1 integrin splice-variant that differs from the ubiquitous β1A in the terminal portion of the cytosolic tail. The transfected integrins associated with the endogenous alpha subunits and are delivered to the plasma membrane. β1B-expressing cells attach less efficiently and spread less on fibronectin, laminin or type IV collagen coated dishes. Expression of β1B do not significantly modify the ability to manifest the polarized phenotype when cells are grown to confluence on filters in two-chamber-systems. Moreover, formation of polarized cysts in suspension culture is impaired. β1B-transfected cells show reduced motile properties when embedded as aggregates in type I collagen gels (see Figure 9 in the Introduction section). When immunestained for Rac1 β1B-expressing cells show reduced amounts of Rac1 at the cell plasma membrane (Figure 36).

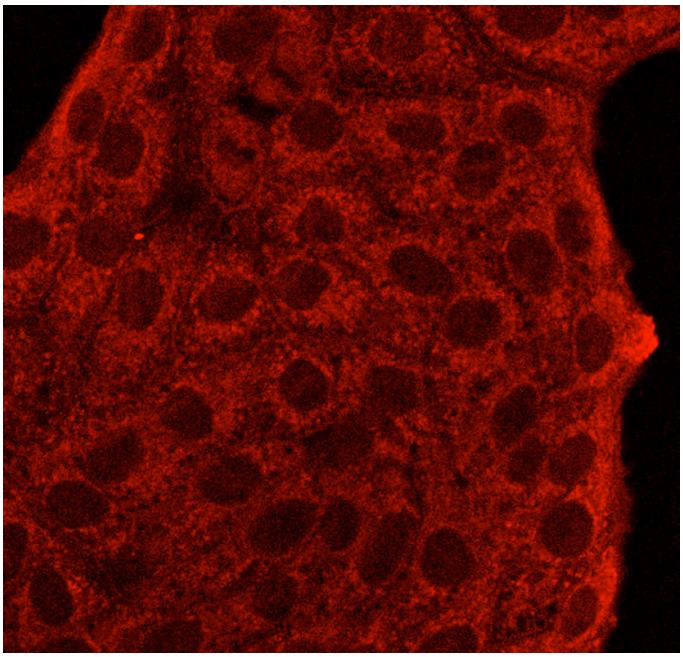

Fig.36 Rac1 staining in FRT β1B-expressing cells. Rac1 localization was analyzed with anti Rac1 antibody by immunofluorescence. The amount of the protein in the plasma membrane is lesser than parental FRT cells.

Figura 37 shows the different behavior of cells stably espressing the β1B integrin subunit (FRT-β1B cells) compared to the parental FRT cells (TER acquisition, cell

migration, and polarized cyst formation were monitored). As a general comment to these experiments it should be said that these cells are only partially affected in their ability to express the properties that have been analyzed. Therefore it was considered to be plausibile to attempt to fully restore their normal behaviour by rescuing the activity of molecular regulators implicated in the generation of the polarized phenotype.

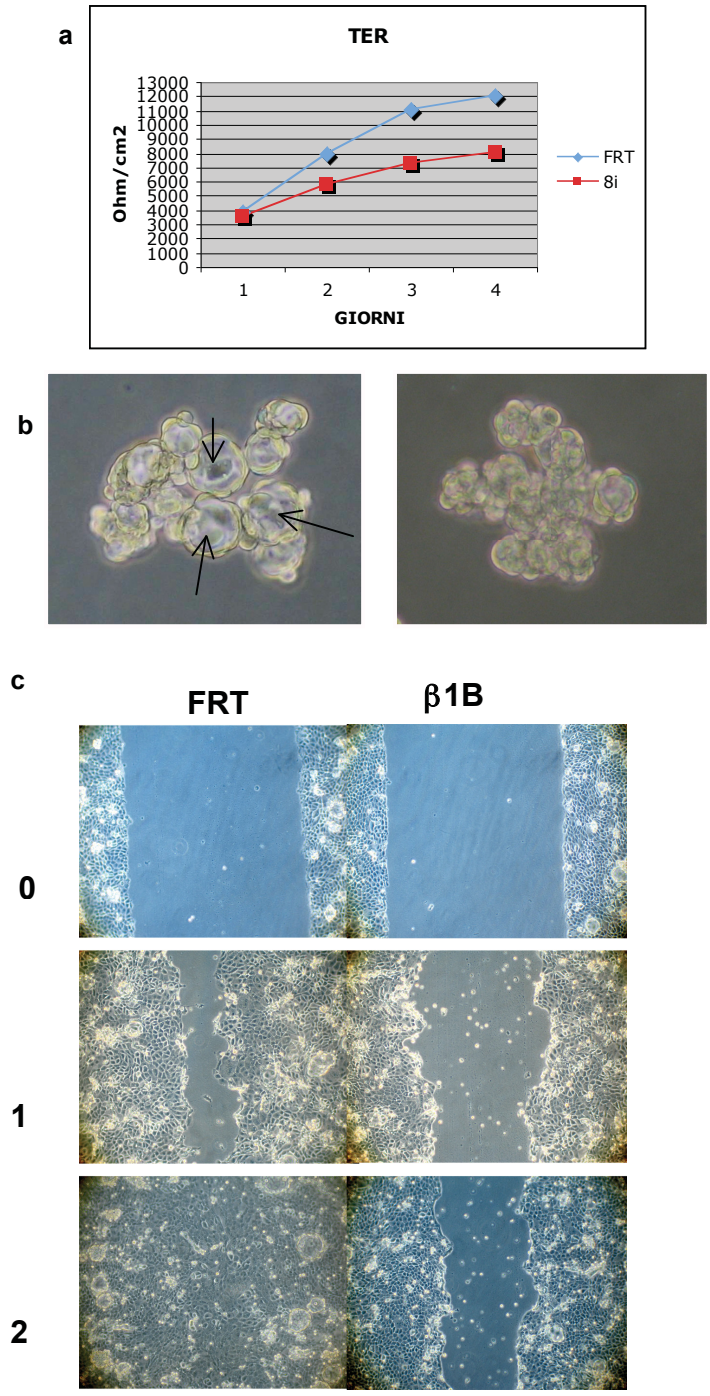

Fig. 37 Different behavior of cells stably espressing the beta 1B integrin subunit (FRT-β1B cells) compared to the parental FRT cells.A-Transepithelial acquisition resistence**,B-** Inverted cysis formation in sospension culture**, C**-wound healing test. In all cases FRT-β8i cells show an impairment in fully expressing the polarized phenotype. **Note in B** that FRT parental cells form numerous liquid filled cysts while FRT-β 8I cells do aggregate but only form few small cysts.

We tested here the hypothesis that an active Rac1 might correct the FRT-β1B cells polarity defect. Starting from the 8i clone of the FRT-β1B cells FRT-β1B 8i cells were obtained that stably expressed an inducible constitutively active Rac1 protein, ER-Rac1(QL). As indicated in the Materials and methods section the inducibility was obtained by fusing the Rac1(QL) downstream of a tamoxifen (4-OH-Tamoxifen)-sensitive mutant of the estrogen receptor ligand binding domain.

A representative Western blot is shown in Figure 38 with three clones expressing different levels of the chimeric gene. Clone 32 and 46 were selected for this study. From now on the cells will be indicated as 8i ER-Rac1(QL).

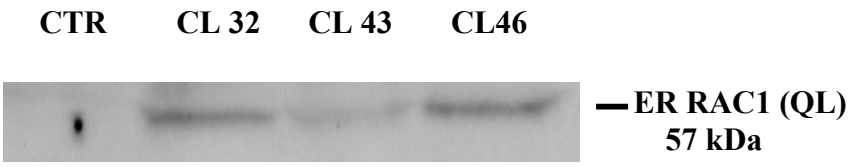

Fig.38 Western blot analisys of ER Rac1(QL) expressing cells. FRT β1B cells were transfected with ER Rac1(QL) construct and three stable clones were obtained (CL32, CL43, CL46). The presence of transfected protein was found by western blot using anti AU1 antibody that recognize a small tag on the chimeric protein. Three clones expressed different levels of ER Rac1(QL). As it can see in the picture, CL32 and CL46 expressed a higher level of the protein and were selected for this study.

4.11 *ER-Rac 1(QL) localization. Effect of tamoxifen treatment*

Cells grown on monolayers were fixed and stained with the AU1 antibody (see materials and methods). Figure 39 shows that the transfected Rac protein diffusely distributed in the cytosol in the absence of tamoxifen. Upon tamoxifen treatment the ER-Rac1(QL) protein became active, localized in the plasma membrane, and in confluent cells it was mostly found on the lateral plasmamembrane at sites of cell-cell contacts. From our observations it became apparent that a 30 min. incubation with tamoxifen is sufficient to transfer a detactable fraction of the Rac protein to the plasma membrane.

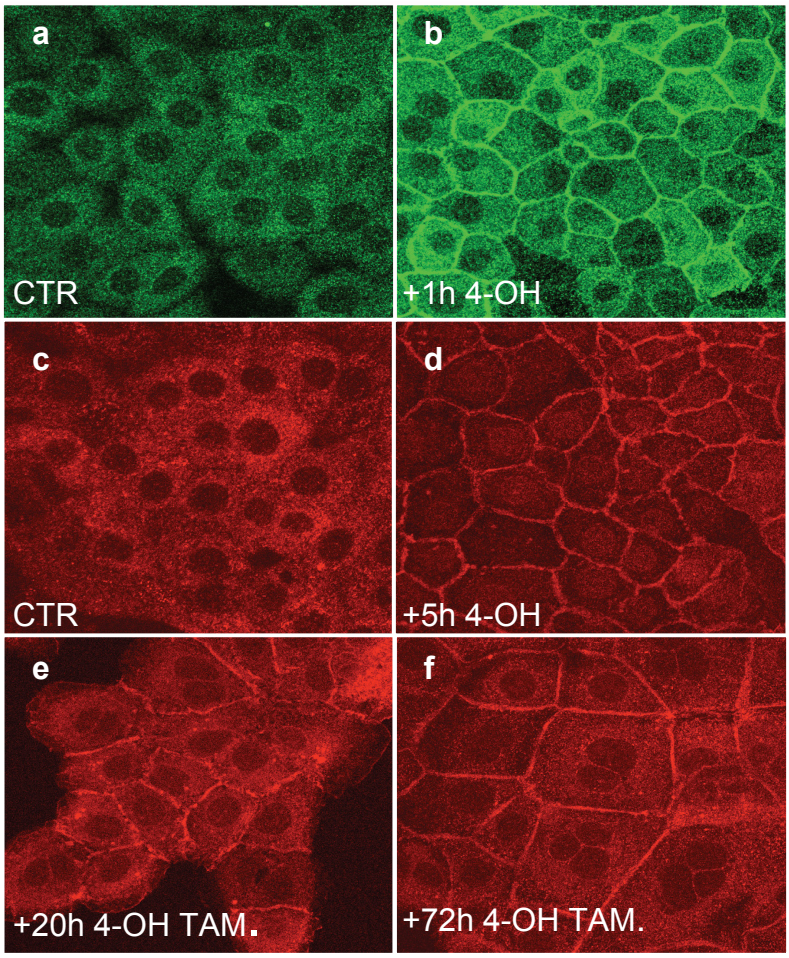

Fig. 39 An inducible constitutively active Rac1 protein expressed in FRT-β 8I cells. (clones stably espressing the ER-Rac1(QL) construct were selected for these studies.) Cells grown on monolayers were fixed and stained with the AU1 antibody that recognizes a specific element in the exogenous protein**.** In the absence of 4-OH tamoxifen the Rac1 protein is diffusely distributed in the cytosol (**a** and **c**). Upon 4-OH tamoxifen treatment the active ER-Rac(QL) protein localizes to the plasma membrane at sites of cell-cell contacts. In **b** cells were treated with 4-OH TAM. for 1 hour before fixation. In **d** e **f** cells were incubated in the presence of 4-OH TAM for longer periods (5, 20 and 72 h) and then fixed and stained. **Note** at 72h several binucleated cells.

Incubation with tamoxifen did not strikingly change cell morphology. However for prolonged incubation periods (i.e 48,72h hours) a number of large binucleate cells could be detected in the monolayer culture. (Figure 39 f and Figure 40 b and c). This late observation was suggestive of an impairment in the cytokinesis and the growth rate of the cells was investigated by generating a growth curve for cells in the presence and in the absence of tamoxifen. From this experiments it emerged that prolonged expression of constitutively activated Rac reduces the cell proliferation rate .

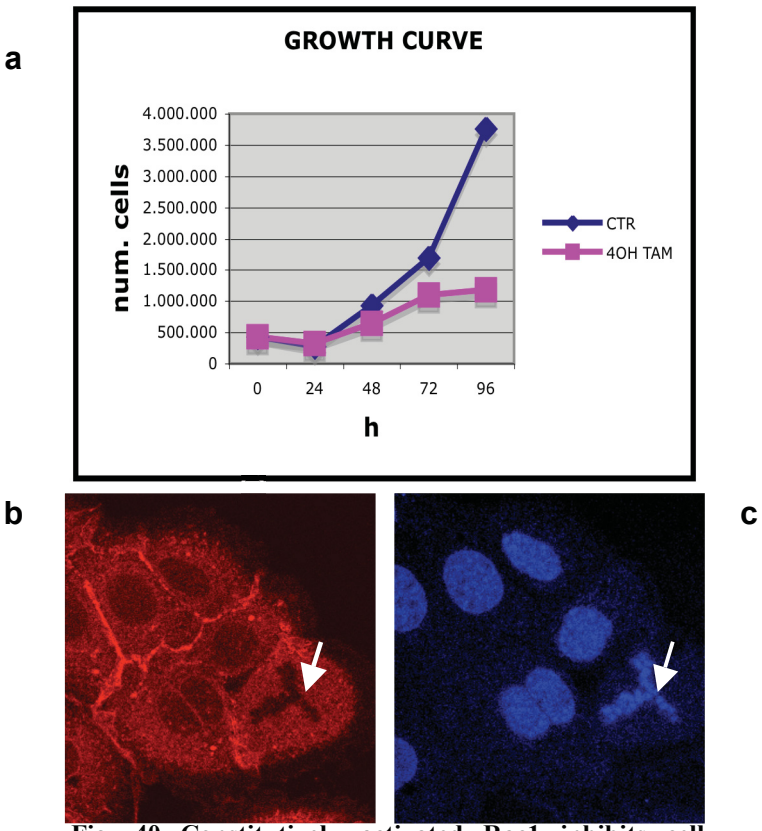

Fig. 40 Constitutively activated Rac1 inhibits cell proliferation (growth curve, a) and impaired cytokinesis. Upon **4-OH tamoxifen treatment** between 48h and 72h a significative proportion of cells in the monolayer showed two or more nuclei. Staining of chromosomes with Hoechst evidenced abnormalities in chromosomes distribution during mitosis (see arrows in **b** and **c**).

Since our interest was mainly centered on the effects of the constitutive Rac1 activation on the polarized phenotype of the FRT-β1B cells the conseguences of tamoxifen treatment on cell migration, TER acquisition and cysts formation in suspension culture were evaluated in 8i ER-Rac1(QL) cells. To be sure that recorded data were not dependent upon the characteristics of the clones selected for the investigation, identical experimental procedures were concomitantly carried on 8i ER-Rac1(QL) cells and in FRT parental cells that express the ER-Rac1(QL) construct (also available in the laboratory, thanks to A. Corteggio).

4.12 *Activation of ER-Rac1(QL) reduced the wound healing efficiency*

8i cells expressing the β1B integrin subunit show reduced migratory potential both in monolayer culture and in a threedimensional environment when embedded in collagen gels. To evaluate the effect of Rac1 activation on the migratory properties of the cells the wound healing efficiency in the scratch test was tested. Activation of ER-Rac1(QL)

by 4-OH-TAM. was induced in both 8i ER-Rac1(QL) and FRT parental cells expressing te same construct. Scratched monolayers were monitored for 48 hours and pictures taken every 12 hours interval (Figure 41).

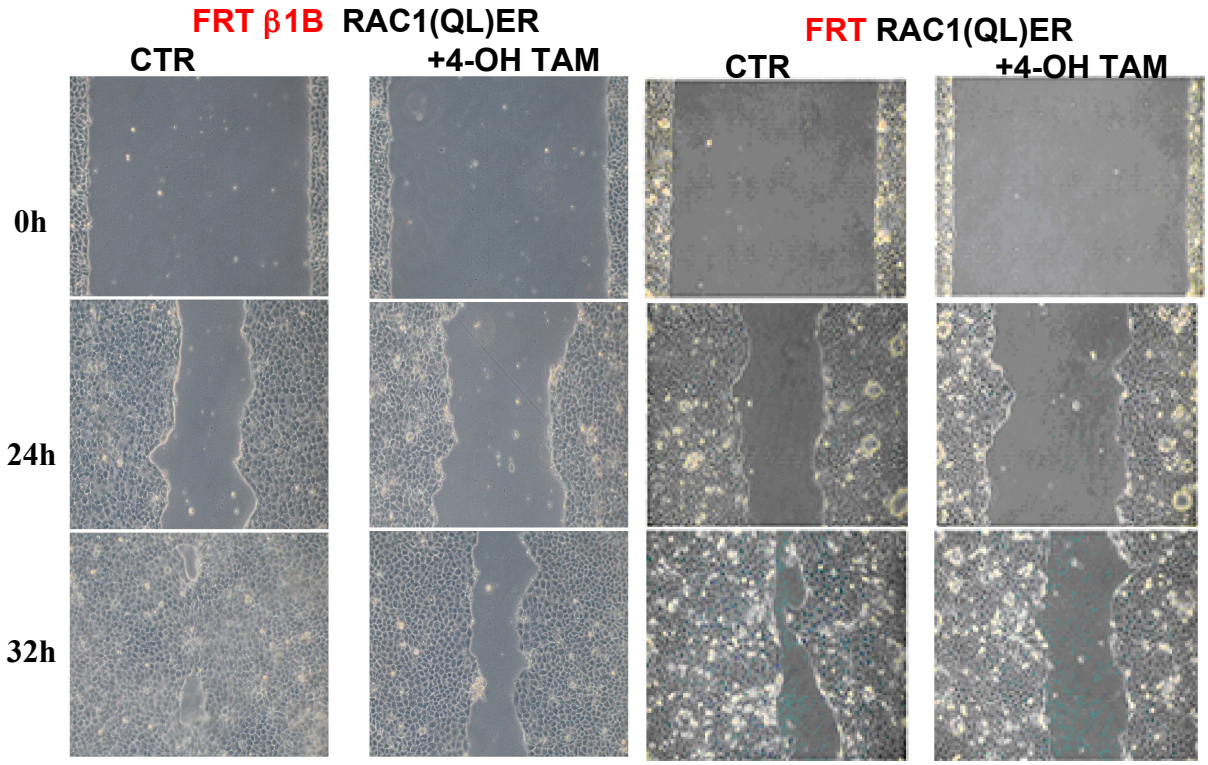

Fig. 41 Evaluation of the effect of Rac activation on the migratory properties of the cells. Activation of ER-Rac(QL) by 4-OH-TAM in FRT-β 8I (**a**) and FRT parental cells (**b**) that were transfected with the same construct reduced cell motility and delayed closing of the wound. Representatives fields of the scratch were shown for each time point.

Differently from our expectations 8i ER-Rac1(QL) manifested a reduced migratory activity and a delay in closing the wound was documented. It is interesting to note that the same behavior characterized also the FRT parental cells that express the ER-Rac1(QL) construct suggesting that this type of result does not correlate with the type of cells but rather represent a cellular response to a sustained Rac1 activation.

4.13 *Activation of ER-Rac1(QL) interferes with the acquisition of transepithelial resistance by confluent cells grown on filters*

We then asked wether a constitutively active Rac1 could influence the polarization process as monitored by the raise of the transepithelial resistance (TER). To check this issue, cells were grown as monolayers on filters in bicameral systems. Suspended cells after trypsinization were plated on the surface of filters in bicameral systems and allowed to attach. 4-OH Tamoxifen was added to the cells 24 h after the plating. Transepithelial resistance (TER) measurements were conducted every 24 h. TER graphics in Figure 42 **b** and **c** clearly evidences that activation of ER-Rac1(QL) interferes with the acquisition of transepithelial resistance. It can be excluded that the effect should be related to tamoxifen itself, since the treatment of control FRT-β 8I cells with tamoxifen did not result in changes in TER acquisition rate (Figure 42 **a**).

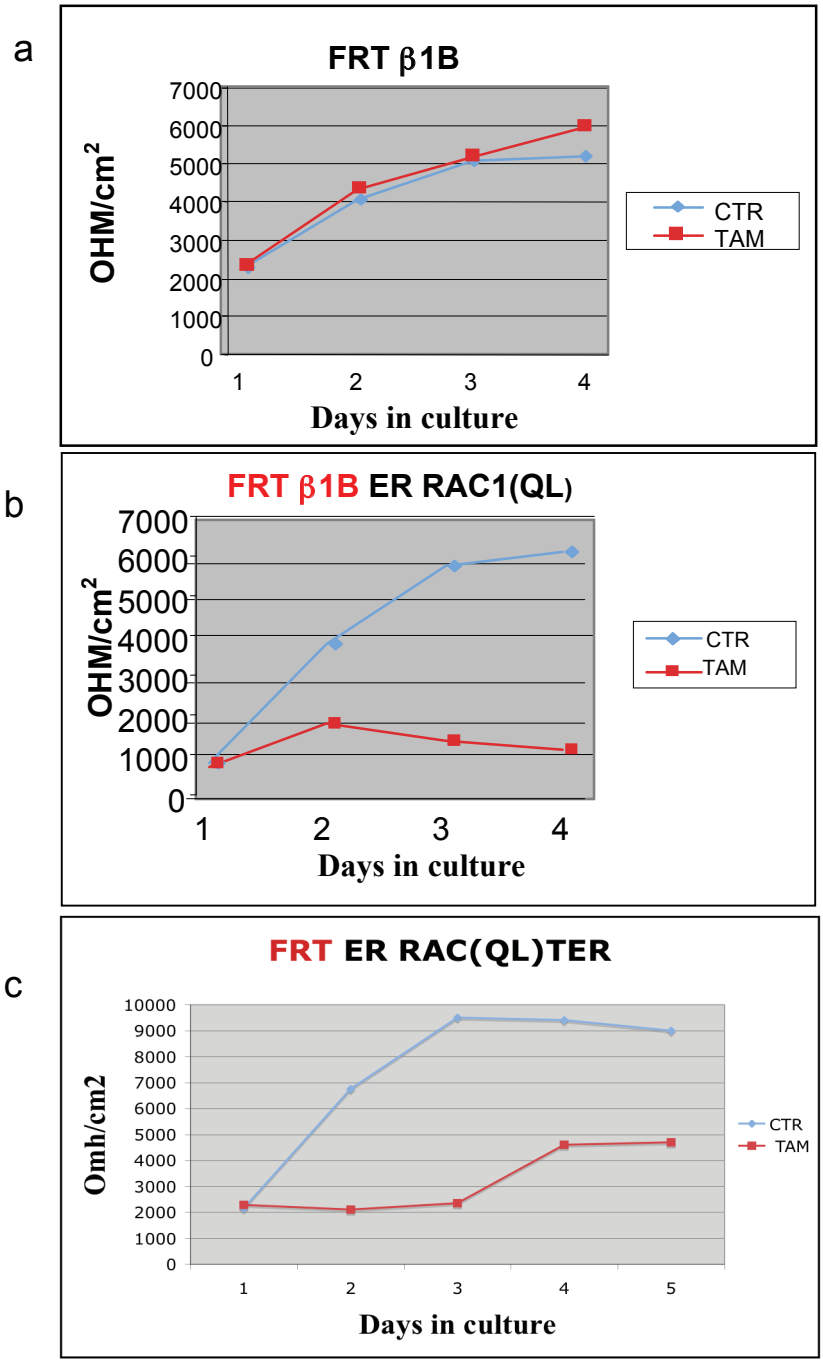

Fig.42 Graphics of TER acquisition. Activation of ER-Rac(QL) by 4-OH Tamoxifen in FRT-β 8I (b) and FRT parental cells (c) that were trasfected with the same costruct hampered TER acquisition. Suspended cells after typsinization are plated on the surface of filters in bicameral systems and allowed to attach. 4-OH Tamoxifen was added to the cells 24 h after the plating and TER measurements were conducted every 24 h. It can be excluded that the effect should be related to tamoxifen itself since the treatment of control FRT-β 8I cells with tamoxifen did not result in changes in TER acquisition (**a**).

4.14 *Activation of ER-Rac(QL) impaired polarized cysts formation by cells in suspension culture*

Integrins may be involved in the control of the processes that have to do with complex morphogenetic events , i.e, formation of epithelial cysts and tubules. It is well known that integrins regulate several aspects of Rac1 activity (for example Rac targeting to the plasma memmbrane and GTP-Rac localized effector interactions). Furthermore Rac1 is necessary to orient epithelial polarity when epithelial cells polarize and it has been shown to promote tubulocystic structures in the MDCK cellular system. As reported in the Introduction section the FRT cells expressing the mutated β1B integrin subunit are affected in their ability to form polarized cysts. They aggregate regularly and aggregates undergo compaction but do not evolve,, or evolve very slowly, into dilated cysts. We reasoned that Rac1 was necessary for the FRT cells to express their polarized phenotype and that the property to form polarized structures could have been rescued in FRT-β 8I upon activation of exogenous ER-Rac1(QL).To do this 8i ER-Rac1(QL) cells were plated in suspension on agarose coated dishes in the presence of 4-OH Tamoxifen to induce Rac1 activation. Cells formed aggregates but aggregates did not develop into polarized (fluid filled) cysts with time, but rather tended to fuse into lager and more complex solid structures (Figure 43). Likewise wild–type FRT cells expressing the same ER-Rac1(QL) appeared to be similarly hampered in polarized cyst formation.

Again also in this experimental setting a prolonged Rac stimulation, as the one obtained by the permanent presence of tamoxifen in the culture medium, did not achieved the expected results but rather some kind of inhibition of the machinery involved in cell polarization.

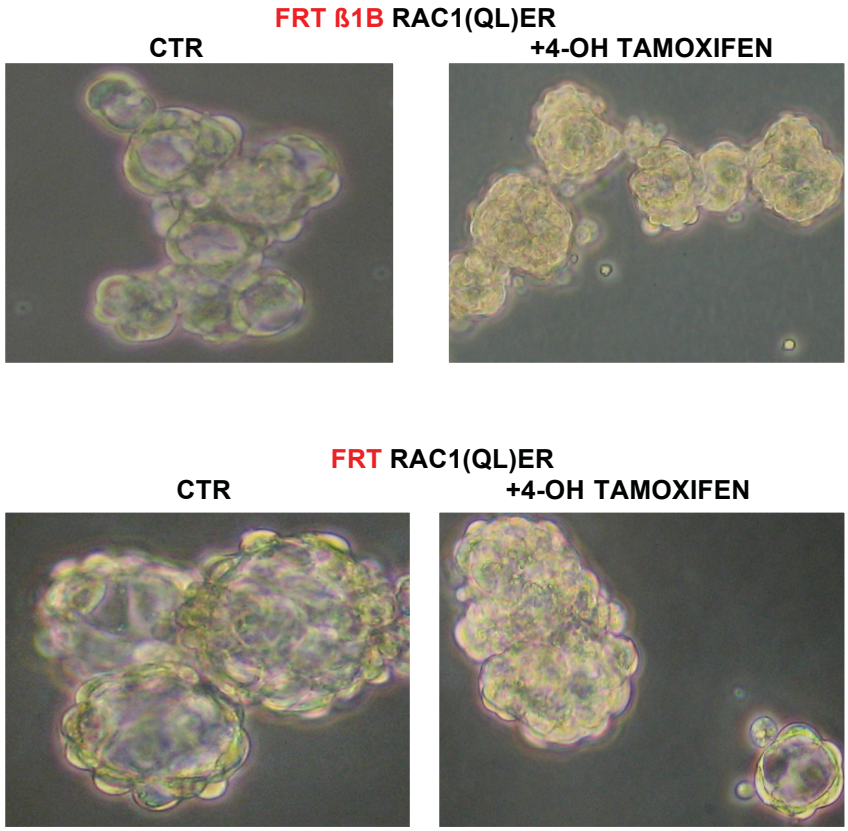

Fig.43 Suspension cultures on agarose coated dishes and evaluation of the formation of polarized epithelial structures (i.e. inverted cysts). Cells plated in suspension in the presence of 4-OH TAM. form aggregates but this aggregates do not develop into polarized (fluid filled) cysts with time. This result apply bothto FRT-β 8I and FRT parental cells that express the ER-Rac(QL) construct.

In summary the effects obtained through the activation of ER-Rac1(QL) in FRT cells expressing the β1B integrin can be described as follows:

- reduction in the growth rate and alterations in the cytokinesis
- impairement in acquisition of TER (transepithelial resistance)
- reduced migratory activity (in the wound healing test)
- unsuccesfull development of polarized structures starting from aggregates cultivated in suspension

The data indicate that sustained but not regulated acivation of Rac1 impairs the acquisition of cell polarity and this might explain why the attempt to correct the phenotype in FRT expressing the β1B integrin remained unsuccesful. Therefore the issue that Rac activation should be spatially and temporally controlled is a major theme to be discussed.

Discussion

5.DISCUSSION

The work for this thesis is mainly focused on the analysis of the role of the Rac1 protein in the acquisition and mantainance of the polarized phenotype in thyroid epithelial cells FRT. The work represenst a part of a more broad project that is devoted to the characterization of the signal transduction pathways that are activated by cell-cell and by cell-ECM interactions during the acquisition of epithelial cell polarity. The project is focused on two pathways: the one activated by the apical PAR3/PAR6/α PKC complex and the one activated by the basolateral β1integrins, both of which apparently rely on GTPases signaling. Polarity is an essential property of epithelial cells that is acquired upon cell-cell interaction. The orientation of polarity depends, instead, upon cell interaction with the extracellular matrix (ECM). As a general rule, the side of a cell that is in touch with the ECM is, or will become, a basal surface. Loss of epithelial cell polarity during the progression of carcinomas is a major example of its relevance in human pathology. Many progresses have been made toward the understanding of the molecular mechanisms underlying the acquisition and the maintenance of cell polarity, and many proteins have been identified that play a pilot role in these processes. Some of them, that are very conserved throughout evolution, are organized in complexes, like the Par3/Par6/aPKC complex, which is mainly involved in the definition of the apical cell domain. It is interesting to note that this complex not only plays a role in the process of definition of the epithelial cell polarity but it is also involved in the process of polarization of other cells, like those that express the ability to migrate.

The acquisition of cell polarity, which includes the establishment of the tight junction barrier, the polarized assembly of the cytoskeleton and the appropriate organization of membrane traffic, requires an external cue that in epithelial cells is represented by the interaction of cells with their neighbors. This is mainly mediated by cell adhesion molecules, such as the cadherins and the nexins.The Rho family of small GTPases,whose prototypes are RhoA, Rac1 and Cdc42 regulate many biological processes including cell cycle progression, apoptosis, migration,intercellular adhesion. Rho-GTPases and their effectors are also key regulators of microfilament and microtubule dynamics and, consequently, are crucially involved in polarity signaling.

Unraveling how signaling transduced via Rac are translated into oriented distribution of molecules in epithelial cells is a central issue to fully understand the processes of acquisition/maintenance of cell polarity. Compartimentalization of the Rac signaling pathways, and of its activators GEFs, as well as the interdependence of the signaling

derived from the PAR3/PAR6/aPKC complex and the one derived from the beta1 integrins, are also of major relevance

5.1 FRT CELLS A USEFUL MODEL TO STUDY CELL POLARITY

Fibroblast are a convenient model to study cell migration and formation of motion-related structures such as lamellipodia, filopods etc…The MDCK canine kydney-derived cell line is the most widely model for studies on epithelial cell polarity. A substantial amount of data have been collected on several issues, i.e polarization of cell monolayers, intracellular sorting of proteins and lipids, generation and stabilization of junctional complexes, cadherin turnover and the functional interplay between Rac1 and other Rho GTPases. However from time to time conflicting results on certain issues are reported in the literature, i.e Akhtar and Hotchin 2001 on E-cadherin endocytosis (Akhtar and Hotchin 2001). This is not surprising because the role of Rac1 as been shown to be dependent on both cell type and cell context (Braga et al. 1999; Hordjik et al. 1997; Potempa and Ridley 1998; Takaishi et al 1997). The FRT cell line was originally established and characterized by Ambesi-Impiombato et al. (Ambesi-Impiombato et al. 1979) and (Nitsch et al. 1985). FRT cells are metabolically dedifferentiated, but still express a thyroid-specific transcription factor, Pax8 (Mascia et al. 1997) and are polarized and connected by a continuous belt of tight junctions. FRT cells express a set of integrins that partially differs from that expressed in MDCK cells, wich include in addition to $\alpha 3\beta 1$ integrin, necessary for the laminin assembly, the $\alpha 5\beta 1$ fibronectin receptor, involved in extracellular assembly of the fibronectin matrix (Calì et al., 1998). In culture, FRT cells have the ability to form domes that correspond to domains of the epithelial layer where cells detached from the culture dish, due to transepithelial transport of ions and water, and accumulation of fluid underneath the cell layer (Nitsch et al. 1985; Garbi et al. 1996). In suspension culture they form hollow cysts where a polarized monolayer of cells has the apical surface facing the outside. When grown as monolayers on filters in bicameral systems they develop very high values of transepithelial resistance (compared to lower values of MDCK).

5.2 PRINCIPAL ASPECTS INVESTIGATED AND RESULTS OBTAINED

As mentioned the objective of this thesis work was to analyze the role of Rac1 in the acquisition of the polarized phenotype in thyroid epithelial cells FRT and in FRT cells

transfected with a protein that acts as a dominant negative of the beta1 integrin. FRT cell derived from rat thyroid express the polarized phenotype, while in the FRT-β1B, in which beta1 integrin signalling is defective, an impairment of the polarized phenotype have been obseved. We have tested the hypothesis that this may be due to a reduced Rac1 activity. The observation that these cells manifest properties similar to those evidenced in FRT cells where Rac1 is inhibited gave origin to this hypothesis.

The experimental part of the work have been focused on:

A- Identification and localization of Rac1 in FRT parental cells and in clones of FRT-β1B.

B- Evaluation of the effects induced by the tretment of FRT cells with the inhibitor of Rac1 activation NSC23766.

Cell migration, acquisition of transepithelial resistance and formation of polarized structures in suspension cultures have been studied in detail.

C- Generation of subclones derived from FRT-β1B cells that express after transfection a Rac1 construct whose activity rely on treatment with 4-OH-tamoxifen

D- Evaluation of the effects induced by the tretment of FRT-β1B cells with 4-OH-tamoxifen. Again cell migration, acquisition of transepithelial resistance and formation of polarized structures in suspension cultures have been checked in detail.

The evaluation of the effects induced by the pharmacological inhibition of Rac1 represents the more substantial part of the experimental activity performed.

In this set of experiments subcellular localization studies of the Rac1 molecule were performed in parallel with those regarding E-cadherin. The principle results that have been obtained can be summarized as follows:

A- Blocking the activation of Rac by the GEF inhibitor NSC23766 determines:

- Changes in the morphology of cells and cell colonies as demonstrated by in vivo observations made by phase-contrast microscopy
- Dissociation of Rac1 from the plasma membrane as evidenced by immunofluorescent staining of fixed cells
- Disappearance of E-cadherin from regions of cell-cell contacts and appearance of E-cadherin-positive large vescicles in the cytosol
- Complete loss of the E-cadherin localization at the level of the Golgi apparatus
- Structural alterations of Golgi apparatus evidenced by immunofluorescence
- Inhibition of cell migration
- Great reduction in values of TER
- Impairment in cell aggregation and development of polarized cysts

The data indicate that Rac1 is a major regulator of the polarization process in FRT cells. Since we only observed minor effects on TER when the inhibitor was used in monolayers that have already reached maximal values of transepithelial resistance we hypothesize that the protein is principally involved during the polarity acquisition phase and is likely dispensable when the fully mature phenotype is manifested. In addition to the control of cell-cell adhesion the presented data suggest that Rac1 may be involved in the control of intracellular traffic of proteins through the control of the Golgi apparatus integrity. This is a relavant and exciting new finding that deserves further investigation.

B- Inducing the costitutive expression of activated Rac1 molecules by tamoxifen administration determines:

- reduced cell growth and alterations in cytochinesis
- impaired TER acquisition
- reduced migratory activity
- stimulation of aggregation but deficient maturation of aggregates into cysts

These results indicate that costitutive activation obtained expressing the ER_Rac(QL) molecule is not sufficient to resume the normal phenotype in FRT-β1B cells expressing the dominant-negative version of the β1 integrin. We tend to exclude that the results may arise from some special feature that characterize clones we have selected for this study. In fact the same results were obtained with the parental FRT cells expressing the same construct. All the observations refer to long term experiments where Rac1 activity was sustained for long time. Rac1 activity therefore could not have been adeguately modulated. Altoghether results confirm that the formation of apico-basal polarity in epithelial cells should be very carefully controlled. The unbalanced expression of only one of the many actors involved in the generation of the polarized phenotype is able to impair the cell polarization process.

The data presented in this data will be discussed relative to relevant data on the subject available in the literature.

5.3 THE NSC23766 MOLECULE INHIBITS RAC1 ACTIVATION

To carry out the inhibition af Rac1 activation we relied on the use of the NSC23766 molecule, a chemical compund, which is now commercially avilable, and that was originally identified in a screening to search for a Rac-Gef interaction-specific inhibitor

(Waszkowycz et al., 2001). The characterization of its biological effects on Rac1 activity have been described by Gao and coll (Gao et al., 2004). In brief the molecule fit into a surface groove of Rac1 known to be critical for GEF specification. In this condition Rac1 binding and activation by Tiam1 and Trio are affected in a dose-dependent manner. NSC23766 represent a novel experimental tool available to study the role of Rac1 in various cellular functions and by a prospectical point of view to investigate the reversibility of tumor cell phenotypes associated with Rac deregulation. A novel inhibitor of Rac family small GTPases has been recently decribed that inhibits Rac1 activity (and that of Rac1b, Rac2 and Rac3) (Shutes et al.,2007)

We used the inhibitor molecule in the 100 and 150 micromolar concentration range. This allowed us to induce specific biological effects while keeping the cells alive. The main trouble that was experienced during the of the experimental activity is related to the impossibility to obtain a quantitative measure of the degree of Rac1 activity inhibition obtained. Our attempts to set ideal conditions to measure Rac activity biochemically failed. Standard protocols to measure the Rac1 GTPase activity were utilized as indicated in Materials and methods section, however the numerous experiments performed did not gave reproducible results. We are hardly trying to solve the problem and also would like to set protocols to measure Rac1 activity indirectly (ERK activation, Pak phosphorylation etc.). Prevoius studies on biological functions of RhoA proteins in epithelial cells have been conducted through constitutive expression of Rho mutants, utilizing expression vectors driven by a viral promoter. Short and long-term experiments were also performed expressing RhoA and Rac mutants under the control of the tetracycline-repressible transactivator. The use of the Rac1 inhibitor could represent an alternative experimental approach, especially in long-term experiments.

5.4 CHANGES IN CELL MORPHOLOGY INDUCED BY INHIBITION OF RAC1 ACTIVATION

Following Rac1 inhibition cells appeared smaller in size and colonies assumed very irregular shapes and increased the tendency to fuse. This changes indicated that the cells were reponsive to the treatment. Changes were gradually manifested with time and better seen after the first 24 hours of tratment.

A number of analogies can be drawn, in spite of differences in the cell system and in the experimental approaches, with observations made by Jou and Nelson (Jou and Nelson,1998). Using tetracycline-repressible expression they examined short-term or

prolonged overespression effects of known amounts of Rho proteins mutants before, during and after the development of cell polarity in MDCKII cells.

In low density MDCK cultures after 36 hours in the presence of DNRacN17 (dominant negative) cells have been shown to become less spread on the substratum and rounded-up, and the colonies to become increasingly compacted. We noticed that after inhibition of Rac1 activity cell growth was negatively affected in FRT cells. A similar effect was suggested to take place also in RacN17-expressing MDCK cells. Furthermore when the morphology of cells expressing the CARacV12 (constitutively activated) molecules was analyzed by Jou and Nelson the cells were described as being flat, with prominent lamellipodia. Such a phenotypical aspect was evidenced in FRT cultures when cells were treated with tamoxifen to activate the transfected exogenous Rac1 protein. It is known that Rac1 can direcly or indirectly regulate actin and actin-associated proteins in microfilaments and in the plasma membrane-related cortical meshwork. Changes in actin organization have been in fact described in MDCK cells expressing Rac1V12, in which F-actin no longer appeared organized in stress fibers and formed sharp cortical bundles, lined along cell-cell contacts. Altough changes in cytoskeloton were not investigated in deep in support to the other findings presented in the thesis, it should be said that this aspect is under investigation, as a part of a different experimental project developed in the laboratory.

5.5 INHIBITION OF RAC1 ACTIVITY DISSOCIATES RAC FROM PLASMA MEMBRANE

As mentioned in the Materials and metods section diferent fixation protocols for immunofluorescence staining have been experimented and we are now convinced that in studying Rac1 subcellular distribution in cytoplasmic compartments one should carefully consider wich is the best procedure to adopt. This is expecially true since Rac1 molecules dynamically exchange with different membranes in different locations and following different types of stimuli.

We show that in growing cells FRT cells Rac1 can be detected at the plasma membrane level, in cytosolic clusters, around the nuclear envelope and in lamellipodia. In this thesis we examined the dynamics of Rac-1 localization during the time that follows the inhibition of Rac by the treatment with the inhibitor NSC23766. We show that if cells are treated with the inhibitor of Rac1 activation a great reduction in the plasma membrane-associated quota and apperance large Rac1-positive granules could be

evidenced. As mentioned Rac1 seems to be required for the accumulation of actin filaments at cell-cell contact sites and for the establishment of cadherin/β-catenin-mediated cell adhesion. It has been shown that overespressing dominant negative Rac1 (RacN17) in MDCKII cells reduces Rac1, actin and cadherin accumulation at the plasma membrane (Takaishi et al. 1997). Our observations in FRT cells are in good agreement also with the data reported in the paper of Noritake and coll. They showed that Rac1 suppression by siRNA in MDCKII epithelial cells reduces actin accumulation at sites of cell- cell contact. Interestingly immunofluorescence analysis showed that Rac1 was reduced at sites of cell-cell contacts, in the cytoplasm and in the nuclei (Noritake et al. 2004). In the same experimental conditions the authors demonstrated that the intensity of E-cadherin and β-catenin staining on lateral membranes was reduced. ZO-1 intensity was slightly reduced. However the expression levels of actin, E-cadherin and β-catenin, as checked by immunoblot analysis, did not alter upon knock-down of Rac1. Again the observations we made in the FRT system in which the E-cadherin staining fades away at the plasmamembrane after NSC23766 treatment, but the total amount of the protein remain almost unchanged, are consistent with the data by Noritake and coll.

5.6 WHY RAC1 DISSOCIATES FROM THE PLASMA MEMBRANE ?

Why is Rac1 detaching from tha plasma membrane and why apparently does it take so much time? Rac1 cycles between the membrane and the cytosol. Solubility in the cytosol is conferred by the binding of Rac to GDIs. According to the experimental conditions settled by Moissoglu et al.. conversion of GTP- to GDP-Rac is the major pathway for dissociation of Rac from the membrane (Moissoglu et al.,2006). To what extent GTP-Rac on the membrane must interact with a GAP and convert to GDP-Rac before dissociation from the membrane is currently unclear.

Moissoglu and coll.. did a very careful analysis of the in vivo dynamics of Rac-membrane interactions. The dissociation rate costant of membrane-bound Rac was measured on protrusive areas of cells spreading on fibronectin developing a photobleaching method, and reported to be 0,048 s-1 for wild type Rac1. These dissociation rates are much faster however than persistent times for migrating cells indicating that the pathways that determine local Rac activation and deactivation during migration must operate on slower time scales.

Equivalent experiments focusing on the dynamics of Rac1 localized at the lateral plasma membrane in regions of cell-cell contacts have not been attempted to our knowledge. Association of Rac molecules to proteins in the junctional complexes and/or associated to the actin meshwork could probably modify this parameter to a great extent. If Rac1 molecules cycle from a cytoplasmic pool to membranes and viceversa the actual amount of the protein, in a given compartment, will depend on the concentration range of the classes of molecules that regulate activation and/or localization of Rho GTPases, and on the type of interactions that these molecules have with other factors that partecipate to the process. GEFs promote membrane localization and it has been shown that Tiam1 overespression in NIH 3T3 cells may do so in part by slowing the dissociation of Rac1 from the membrane. The proposed mechanism to explain this effect is that Tiam1 competes wiyh the GAP-RhoGDI pathway by converting the GDP-Rac formed at the membrane into GTP-Rac before it gets dissociated. (Moissoglu et al.,2006). In vitro data support this reasoning showing that Rho GTPases can be extracted from the membranes more efficiently in the GDP-bound form. It was suggested that GTP-Rac binds less well to GDI and/or it interacts with effector proteins that limit the access to GDI. The role of GDIs in establishing Rac1 amount in the plasma membrane is complex: as mentioned Rho GDI can extract Rho GTPases from membranes, confer solubility in the cytoplasm and inhibit activation by GEFs. GDI molecules may be involved in the regulation of membrane association. It has been shown that RhoGDI downregulation increases Rac1 membrane targeting with only slight effects on the dissociation of Rac! from the membrane (Moissoglou et al. 2006), suggesting that the controll of GDI activity and/or availability should partecipate in regulating the rate of association to the membrane. As an example GDFs (GDI displacement factors) have been described that initiates the activation of the Rho protein displacing Rho from Rho-GDI at a step before membrane targeting (Faure and al. 1999; Takahashi and al. 1997; Yamashita and al. 2003). Furthermore the Rac1 effector PAK1 phosphorilates RhoGDI to reduce its binding to Rac1 and increase Rac targeting to the membrane. (DerMardirossian and al. 2004). Therefore a positive loop can be imagined were high Tiam1 concentrations can sustain Rac1 localization in the membrane by keeping high levels of activated PAK1. On the other hand an excess of cellular GDI could generate opposite results.

We do not have investigated all these aspects in our system and so we do not think to have at the moment a sufficient amount of information to interpret the progressive

release of Rac1 from the membrane observed in FRT cells treated with the GEF inhibitor. One possibility is that by increasing the amount of inactive Rac1 molecules the amount of active PAK1 is also reduced, leading to a reduced GDI phosphorilation. This in turn should increase the affinity of GDI for Rac and promote Rac1 dissociation from the membrane. Our observations in FRT cells suggest that Rac dissociation is slow. One possibile interpretation is that in the beginning Rac1 molecules, that have been already activated, are bound to their effectors and therefore can not be approached by GDI, since effector interaction sites overlap with GDI-binding sites. Later on GAP-dependent conversion to GDP-Rac increases the number of molecules that can be dissociated from the membrane by GDI. Another possibility is that the Tiam1 concentration in FRT cells is high and Tiam1 competes with Rac-GDI, converting Rac-GDP in Rac-GTP, so that the effccts promoted by Rac- GDI (and by the inhibitor NSC23766.) are not so rapid as expected.

5.7 FATE OF RAC1 MOLECULES IN RAC1-INHIBITED CELLS

An interesting feature of prolonged incubation of FRT cell with the inhibitor is the appearance of large Rac-positive granules in the cytosol. It is not clear at the moment by which mechanism they are generated, so that we can not establish if they contain Rac1 molecules released from the plasma membrane or from different intracellular pools.

Intracellular Rac granules have been described in MDCK cells espressing the dominant negative RacN17 proteins and stained with anti-myc antibodies to reveal only the exogenous protein.They have been shown to colocalize with cadherin and actin but have not been further characterized (Jou and Nelson, 2005).

An interesting paper by Lynch and coll. discuss the possibility that the proteasome activity has a role in the documented down-regulation of Rac1-GTP and total Rac1 observed during cell scattering that follows loss of adhaerent jnctions in MDCKII cells stimulated with HGF (Lynch etal., 2006). They report that a very low level of ubiquitinated Rac-1 was also detected in polarized cells before scattering. It could be interesting in the next future to investigate in our FRT cell system wether a proteasome-based mechanism of Rac1 degradation is operative in NSC23766-treated cells.

5.8 A RAC1-CADHERIN LOOP

Rac1 seems to be required for the accumulation of actin filaments at cell-cell contacts sites and for the establishment of cadherin-mediated cell-cell adhesion. During the very early phases of polarity generation Cdc42 and Rac-1 are activated by the initiation of cell contacts formed by trans-interactions of nectins or E-cadherin (Fukuhara A, and al. 2003; Kukuhara T. and al. 2004). As mentioned, overespression of constitutively active Rac1 induces a greater accumulation of E-cadherin, beta catenin and actin at sites of cell-cell contacts in MDCK cells. However how the localization and activation of Rac1 are regulated at sites of cell-cell contacts is not fully elucidated. It has been reported that Rac1 translocates to the cytosol during disruption of E-cadherin mediated cell-cell adhesion by calcium chelation (Nakagawa et al.,2001) suggesting that E–cadherin mislocalization affects Rac distibution in the cells.

In this thesis changes in E-cadherin localization were examined in parallel to the analysis the dynamics of Rac-1 localization during the period that follows the inhibition of Rac1 by the treatment with NSC23766. Whe demonstrated that GFP-Rac1 colocalized with E-cadherin at sites of cell-cell contacts. As indicated in the Results section a significative reduction of plasma membrane staining is visibile at regions of cell-cell contacts for both proteins. Furthermore analyzing the distribution of GFP-Rac1 and E-cadherin in treated cells it was evident that where Rac1 is dissociated from the membrane also E-cadherin is absent, and where Rac1 is still present E-cadherin is also present, suggesting that both are translocated simultaneously to the cytosol during the disuption of cell-cell adhesion. Therefore Rac1 mislocalization can affect E-cadherin distribution in the cells.

5.9 RAC1 INHIBITION AND POLARIZED TRAFFIC OF CADHERIN

The observations presented in this project concerning the accumulation of E-cadherin in large multiple intracellular vescicles (see Results section) suggest that Rac1 can influence cadherin dynamics in different ways. The dynamic traffic of E-cadherin to and from the lateral surface of epithelial cells is crucial to delivery newly synthesized E-cadherin to the adhaerens junctions and thereafter to balance and modulate cadherin-based adhesions.

It is now widely recognized that surface cadherins can be internalized costitutively and recycled in confluent monolayers or endocytosed via different endocytic carriers and pathways in response to growth factors (Lee et al. 1999; Bryant et Stow 2004).

The exocytosis of newly synhesized E-cadherin requires the sorting and polarized transport to the basolateral membrane in epithelial cells. A dileucine motif in the tail domain mediates this traffic of E-cadherin (Miranda et al. 2001).

There is a strong evidence linking Rho GTPases to E-cadherin at the cell surface. Rac1 has been shown to regulate the endocytosis of E-cadherin, making non nonadhesive E-cadherin molecules available for internalization. (Izumi et al. 2004). At the cell surface RhoA, Rac1, and Cdc42 act to directly regulate components of the cadherin-catenin complex to modulate cadherin-based adhesion and signaling (Fukata and Kaibuchi 2001).

Wang and collaborators (Wang et al. 2005) showed that both Rac1 and Cdc42 are involved in the polarized trafficking of E-cadherin in MDCK kidney cells, determining efficient post-Golgi sorting of E-cadherin and its delivery to the lateral cell surface.

Data presented in this thesis show that in FRT cells intracellular cadherin colocalizes with markers of the Golgi membranes and that cadherin colocalizes with Rac1 at the plasma membrane. When cells are treated with the Rac1-GEF inhibitor a reduced staining for both Rac1 and E-cadherin is observed at the plasma membrane level. Concomitantly intracellular E-cadherin appeared in large vescicular structures and E-cadherin staining at the Golgi disappeared. How does Rac1 inhibition correlates with changes in E-cadherin localization? Intracellular accumulation may be related to destabilization of plasma membrane-associated protein, with increased endocytosis, or disregulation of the intracellular traffic and accumulation in a compartment associated with the post-Golgi transport of the protein. Reduced surfaced staining of E-cadherin have been described in polarized MDCK cells after the knock down of Rac-1 using small interfering RNA (Noritake et al. 2004) and intracellular vescicles heve been observed in MDCK cells expressing Rac 1 mutants (Jou and Nelson 1998). The data obtained in the FRT system of polarized cells and presented in this report are consistent with these observations.

In the mentioned work of Wang and col. (2005) the authors, on the basis of absence of colocalizaion with the LBPA marker for late endosomes, suggested that E-cadherin accumulated in the exocytic rather than an endocyic pathway. They suggest that Rac1 accumulates E-cadherin at o adjacent to the TGN, after the dileucine mediated sorting. Overespression of dominant negative Rac accumulated E-cadherin-GFP in large vescicular structures that overlapped significantly wit the staining of GM130 and also with the TGN marker p230 golgin. The structure of the Golgi complex or the TGN were not significantly affected by the overespression of mutated GTPases. These last mentioned observations do not match what we report for the FRT cells treated with the Rac1-GEF inhibitor. In fact as

indicated in the Results section we evidenced a vesciculation and dispersal of the Golgi stacks and the absence of colocalization of the cadherin-containing large vescicles with the GM130 Golgi marker in cultured cells treated with NSC23766.

We do not know at the present the reason for these discrepancies. One possibility is that the analysis have been conducted with a different timing. Our immunofluorescence-based morphological investigations were initiated at 24 hours following the treatment with the Rac1-GEF inhibitor, when fading of plasma membrane-associated E-cadherin appears to be noticeable. It should be recognized that cadherin-containing cytosolic structures await to be chracterized in our system. However from data not presented in this report we can exclude that the large vescicles correspond to the early endosomal compartment (Santoriello M. unpublished).

Furthermore the data presented in this report outlines the involvement of Rac1 activity in the control of the Golgi apparatus assembly and suggest that an additional pathway by wich the molecule can control the polarized phenotype in epithelial cells may exist and is linked to the control the polarized sorting of glycoproteins to different domains of the cells via the post-Golgi compartment.

5.10 MIGRATION AND TER ACQUISITION IN FRT CELLS TREATED WITH THE RAC1 INHIBITOR

The observations made on TER (transepithelial resistance) acquisition indicated that when the inhibitor is added at an a early time (few hours after plating on filters) FRT cultures manifest significantly lower values of TER compared to the control. Loss of plasma membrane E-cadherin and consequent impairment in tight junction assembly could represent the molecular basis of the defect in cell polarization, evidenced monitoring the TER. When cells were plated on filters at high density and that have already reached plateau values inhibition of Rac1 in this culture condition is very much less effective in eliciting changes in TER. This results are consistent with he observation that also morphological changes are less evident when FRT monolayers are very confluent, and are in agreement to data reported for MDCK cells expressing the dominant negative form of Rac1 in which more dramatic changes where observed at low cell density (Jou and Nelson,1998). Recent studies indicate that Rho GTPases collaborate with polarity proteins in fibroblasts an epithelial cells to control directional cell migration. Cell migration is

achieved through the balance of Cdc42 and Rac1 function in the front of the cell and RhoA function in the rear (Iden and Collard, 2008). Components of the PAR,Scribble an Crumbs complexes crosstalk with Rho GTPases and regulate front-rear polarization and wound healing of cultured epithelial cells. FRT cell, as other types of epithelial cells, move as groups of adhaerent cells in a way epithelials cells are used to do during tissues morphognesis. One intriguing aspect that has to do with the migratory activity of inhibitor-treated cells in monolayer is the observation that, although cells have lost intercellular contacts, they do not tend to separate from each other and scatter. One speculation could be that disassembly of adhaerens junctions contribute to the acquisition of the migratory potential but molecular signalling from activated Rac1 is mandatory for the achievement of this type of motile activity. It is also true that we evidenced changes in the aspect of the cell colonies that suggest their tendency to fuse by an active process of cell locomotion. Also the data from the wound healing test imply that level of Rac1 inhibition attained with the inhibitor concentrations we used is not sufficient to completely abolish migration in FRT cells.

5.11 RAC1 AND THE CONTROL OF MORPHOGENETIC EVENTS IN FRT CELLS

Cell polarization is achieved by the concerted actions of polarity proteins. By assembling multiprotein complexes they induce downstream signaling to trigger the establishment of cellular asimmetry. Crosstalk between small GTPases and polarity proteins is crucial in cell polarization (an ecellent review is: Iden and Collard, 2008).

There is strong evidence for a predominant function of Rac1 in the formation of epithelial apico–basal polarization (Takaishi et al 1997; Chen and Macara 2005; Mertens et al. 2005). The interaction between Tiam1 and Par3 couples Rac1 activation to the activation of aPKC, and loss of Tiam1 impairs the establishment of functional tight junctions in keratinocytes. Furthermore, Rac1 controls extracellular matrix-induced reorientation of apico–basal polarity in three-dimensional cultures of epithelial cysts in a PI3K- and aPKC-dependent manner (O'Brien et al. 2001; Liu et al. 2007). Functional integrity of the junctional complexes is a pre-requisite for cel-cell adhesion between epithelial cells (Miyoshi and Takai 2005). The adhaerens junctions (AJ) are considered to be the archetypes oj jnctional complexes, and AJ formation may precede tight junctions (TJ) formation in epithelial cells. According to working models adapted to cells grown on monolayers, assembly of the apical junctional complex is triggered by the initiation of cell-cell adhesion by nectins and E-cadherin. The nectin based cell-cell

adhesions are associated with the E-cadherin-based AJs through their respective adaptor proteins, afadin and alfa catenin. Nectin and E-cadherin are thought to form AJs cooperatively. When migrating cells recognize their neighbors cells primordial spot-like junctions are first formed at the tips of cell protrusions (Adams et al. 1998; Yonemura et al. 1995; Vasioukhin et al 2000). These primordial junctions fuse with each other to form short line-like planes wich develop into matured AJs. The contact between two cells generate nectin-based microclusters that then recruit E-cadherin and JAMS to the apical side of the AJs. Recruitment to this primordial junctions of others components of TJs follow, which eventually leads to the establishment of the claudin-based TJs (see on this topic also the Introduction section). The model according to wich interactions of nectins facilitate the formation of E-cadherin based AJs and claudin based TJs has been validated in the MDCK system. Cdc42 and Rac-1 are activated by the initiation of cell contacts formed by trans-interactions of nectins or E-cadherin (Fukuhara A. et al. 2003; Fukuhara T. et al. 2004). We already demonstrated the ability of FRT thyroid epithelial cells to form polarized structures when they are cultured in suspension on agarose-coated dishes. In this culture condition they generate compact cell aggregates that undergo compaction, possibly due to junctions formation. A compact aggregate can be regarded as an unstable structure whose final configuration will depend upon the type of enviroment: compact aggregates will be converted into polarized cysts (three-dimensional structures filled with fluid with inside-out polarity) if they are cultured in suspension culture, or will form follicular structures with lumina if they are embedded in dilute collagen gel or gelatin (Garbi et al. 1987). The whole sequence of events leading to the described polarized structures may be regarded as a morphogenetic sequence that resemble that occurring in epithelia in vivo. Its tempting to speculate that suspended cells although in a completely different enviroment try to form primordial junctions in the moment they come in close contact one the the other. Morphological observations of the aggrgates show that in a initial stage individual cell components can be easily distinguished, probably due to the fact that the surface area involved in establishing contacts is limited. Later on aggregates show a smoother surface and single cells can not be traced anymore. This step corresponds to a stage defined as compaction, and may indicate that more solid and extended junctions, ie mature AJs, have been assembled. If this model holds true, than one can speculate that Rac1 activity is promoted by contacts and necessary for compaction and to prepare the ground to TJs assembly. At this point an asimmetric apico-basal polarity will be

established and depending on external and intrinsic cues a polarized permanent structure will form. With thyroid epithelial cells it has been shown that embedding cell aggregates in a collagen gel a follicular structure is generated with the outside-in configuration, that closely riproduce the follicular orientation in the gland. If aggregates are kept in suspension in the medium cysts with inverted polarity will form (inside–out configuration). A major issue on this subject is: does Rac1 controls the fate of aggregates also in the this final step that follows compaction?

In this thesis the the partecipation of Rac1 to the process of cell aggregation and polarization of aggregated cells in suspension culture have been investigated. In one set of experiments the role of Rac1 in the morphogenetic process leading to formation of follicles was investigated in FRT parental cell grown in suspension culture and treated with the Rac1 inhibitor. The inhibitor was added alternatively at the onset of culture, or few hours laters at the moment when the initial aggregation of cell have already taken place. In a second set of experiments FRT-β1B cells expressing the RacER(QL) protein were used. The FRT-β1B cells have been shown to form smaller aggreggates and only a reduced number of cysts. It was expected tha upon tamoxifen treatment and induction of the exogenous Rac1 activity they could espress the normal phenotype back again.

The results obtained with FRT aggregates tell us that Rac1 activity is necessary to promote aggregation and formation of polarized structures. The treatment with the inhibitor in fact inhibits both the processes. The tamoxifen-based experiments indicate that iperstimulation of Rac1 is able to impair cyst formation, independently from the type of cells that is used. Identical results were obtained with both FRT ER-Rac(QL) cells and FRT-β1B cells espressing ER-Rac(QL). From the data collected, although the issue deserves additional verification, it can be proposed that: if the Rac1 activity is kept low then aggregates evolve into inverted follicles, if the Rac1 activity is inhibited aggregates stay as they are and do not form polarized structures, if Rac1 activity is greatly stimulated the cell aggregation process is promoted further on, originating large clusters of aggregated cells. It also possibile that TJ junctions is perturbed in this condition. In this respect many studies suggest that activities of the Rho family must be carefully balanced at levels optimal for TJ and AJ integrity. In epithelial and endothelial cells constitutively activated forms of RhoA and Rac-1 perturbs TJ function (Braga et al. 1997; Wójciak-Stothard et al. 2001). Wang and coll. reported that also MDCK cells form cysts with inverted polarity with the apical pole facing the free cellular surface of the cyst periphery when grown in suspension culture (Wang et al. 1990). Using the

same cellular system O'Brien and coll. investigated the role of Rac1 in the process of cell polarization. They observed that when cysts are embedded in a collagen gel they form a lumen and establish the apical pole in the interior, as indicated by the localization of the TJ protein ZO-1 (O'Brien et al. 2001). They showed that expression of costitutively active Rac1 did not signifcantly alter MDCK cyst morphogenesis, while expression of dominant-negative Rac1 (N17Rac1) caused a selective inversion of the apical pole to the cyst periphery and N17Rac1 cysts did not form lumina.

Rac1 activity controls laminin assembly in apical pole polarization. N17 Rac1 cysts exhibited a marked decrease in alfa3 integrin levels that could relate with the alterations of laminin assembly that they have documented. Interistingly exogenous laminin restored proper apical orientation in N17Rac1 cysts. An interesting comment in that paper is in correlation to the role of Rac1 in the polarization process. Both monolayer and suspension culture are indicated as inherently anisotropic providing cells with a free and adhesive cellular surfaces. On the contrary cells in collagen are confronted with an isotropic enviroment, in which all cell surfaces are adhesive,either to collagen or to other cells.These cells must break the symmetry of their enviroment and polarize without external cues by forming a free luminal surface. Intracellular Rac1 activity should have a special role in guiding the cells throughout this process.

5.12 INTEGRIN SIGNALING AND RAC1. THE FRT-β CELLS AS A MODEL TO INVESTIGATE THE ROLE OF RAC1 ACTIVITY IN POLARIZED CELLS

Cell adhesion to extracellular matrix (ECM) is necessary to achieve a sustained activation of Rac1 and other signaling molecules. The integrino, (ECM receptors), engagement is crucial in this respect. How integrins signal Rac-1 activation following cell adhesion is not completely clear. It has been shown that adhesion to ECM regulates the coupling of the Rac1 to its effetor Pak (del Pozo et al.,2000), that integrin induces GTP-Rac translocation to the plasmamembrane (Grande-Garcia et al.,2005), and that this process is a phospholipase D-dependent effect (Chae et al.,2008)

The role of integrin signaling in the acquisition/maintenance of cell polarity has been studied to some extent in polarized epithelial cells in culture. It has been shown, in particular, that beta1 integrins plays a critical role in orienting the polarity of FRT and MDCK cells. In suspension culture, both cell type form hollow cysts where a monolayer of cells has the apical surface facing the outside. If the cysts are placed in a

collagen matrix, reversal of polarity occurs (Garbi etal., 87) and this process depends on β1 integrin (Ojakian et al.,2001) Furthermore, it has been shown that β1 integrin lies upstream of Rac1 in a pathway controlling orientation of polarity (Yu et al.,2005). Activation of integrin causes activation of Rac1, which in turn orients the polarity of the cells in a process that, in MDCK cells, also requires laminin-dependent assembly of a basement membrane. Recent evidences indicate that phosphoinositides may lye upstream of these signaling cascades and may be directly involved in the generation of an apical (Martin-Belmonte F, et al,2007 a and b) or a basolateral (Gassama-Diagne A, et al., 2006) membrane domain. To directly demonstrate that β1 integrins play a role in the acquisition of polarity, FRT cells have been transfected with the dominant-negative β1B integrin that inactivates the endogenous β1A integrin (Calì et al.,1998). In these cells a great reduction of fibronectin fibrils associated the basal membrane was observed (Calì et al.,1998). RhoA is required to promote fibronectin matrix assembly in FRT cells and the activation of the signal transduction pathway downstream Rho can overcame the inhibitory effect of β1B (Calì et al.,1999). Moreover formation of of polarized cysts in suspension culture and cell migration of cells embedded in a collagen gel were impaired. Both are are very likely Rac-dependent process. In fact the phenotical changes that we observed in FRT- β1B cells are remarkably similar to those manifested by FRT parental cell in which Rac1 activity have been inhibited. Furthermore β1B integrin differs from the canonic β1A the aminoacid sequence in its cytoplasmic tail and the integrin β1 tail has been shown to be required and sufficient to regulate adhesion signaling to Rac1 (Berrier et al.,2002). Since b1 integrin lies upstream of Rac1 in a pathway controlling orientation of polarità we speculated that the integrin signaling converging on Rac1 is impaired in FRT- β1B cells. We tested in this work the hypothesis that could be possibile to rescue the normal phenotype in these ells implementing Rac1 activity. Starting from the 8i clone of the FRT-β cells FRT-β 8i cells were obtained that stably expressed an inducible constitutively active Rac1 protein, ER-Rac1(QL). As indicated in the Materials and methods section the inducibility was obtained by fusing the Rac1(QL) downstream of a 4-OH-Tamoxifen-sensitive mutant of the estrogen receptor ligand binding domain.

Polarized activities such as migration, transepithelial resistance development and polarized cyst formation were analyzed in the absence and in the presence of tamoxifen.

5.13 RAC1 OVERESPRESSION INDUCED BY TAMOXIFEN REDUCES THE GROWTH RATE OF THE CELLS

Owing to the impossibility to directly measure Rac1 activity two in direct evidences suggested that the activity of the exogenous Rac molecule was effectively stimulated. The first was the demonstration by immunofluorescence that upon tamoxifen treatment a noticeable amount of ER-Rac1(QL) moved from the cytosol to the plasma membrane at sites of cell-contacts. As indicated in the Materials and methods this effect is very rapid. Once stimulated cells keep the ER-Rac1(QL) protein stably associated to the membrane. The second was inferred from the observation that prolonged incubation with tamoxifen led to appearance of multinucleated cells in the monolayer. A similar observation has been reported following the overespression of the constitutively activated RacV12 (Yoshizaki et al,2004). In our FRT cellular system constitutive activation of Rac1 induces an arrest of cell proliferation. This result is in agreement with data reporting that Rac1 downregulation is essential at the end of the mitosis during cytokinesis, while RhoA activity is upregulated (Yoshizaki et al, 2004, Glotzer, 2005, Wolf et al., 2007)). Rac1 is sequestrated into the nucleus immediately before mitosis and released into the cytoplasm during cytokinesis (Michaelson et al., 2008).

5.14 A CONSTITUTIVELY ACTIVE RAC1 IMPAIRS THE ACQUISITION OF EPITHELIAL CELL POLARITY

We use the FRT-β1B cells as a model to investigate the role of Rac1 in epithelial cell polarity with an approach wich is different from that carried with the Rac1 inhibitor in FRT parental cells. As a general comment on the results obtained expressing the inducible ER-Rac1(QL) construct in 8i cells (the clone of the FRT-β1B cells selected for transfection) is that they all give the indication that a prolonged and sustained Rac1 activity in our system is not a guarantee that the proper polarization of the cells is achievable. The FRT-β cells polarity defect not only can not be rescued by the iperespression of active Rac1 molecules but also a general impairment of polarized activities is promoted. We tend to esclude that these observations are linked to special features of the clones generated by the transfection. First all three clones analyzed behave in the same way. Furthermore also the FRT parental cells that have been transfected with the same construct show reduced wound healing efficiency, impaired formation of polarized cysts in suspension culture and manifest only negligible levels of TER when treated with tamoxifen. Finally tamoxifen alone did not induce any modification in the

parental FRT cells used as control. The most obvious conclusion is that a constitutively active Rac1 do not promote. or implement, but instead impair the acquisition of cell polarity. Altogether all the data reported in this thesis emphasize the concept that Rac1 activity must be carefully controlled both spatially and temporally.

5.14 RAC1 IN FRT CELLS GROWN IN MONOLAYER: A MODEL

We believe that major biological phenomena that occur in "in vitro" culture, such as colony migration, colonies fusion, proliferation, acquisition of the polarized phenotype and generation of polarized structures (domes in monolayer culture, or follicular structures in three-dimensional collagen gels), are reminescent of morphogenetic events that take place during differentiation and organogenesis. FRT cells synthesize extracellular matrix (ECM) components such as laminin and fibronectin and organize them in the form of a complex, mostly fibrillar, extracellular deposits (Calì et al. 1998). In non-confluent cultures fibronectin (FN) is mainly organized into extracellular fibrils and larger fibers of different size and orientation. During their functional differentiation in culture FRT cells, initially plated as single cellular entities, form small colonies that progressively fuse generating a monolayer. The fibrillar basal FN progressively disappeares with time in culture and is substituted, in confluent cultures, by FN deposited at sites of cell-cell contacts in the form of granular deposits (Nezi et al. 2002). Domes only form in confluent monolayers and only after the cells have gained the polarized phenotype. Data presented in this thesis clearly show that Rac1 localizes at the plasma membrane both in small colonies and in confluent coltures. Therefore a fraction of activated Rac1 should be constantly present in the cells. Although very speculative in nature a model can be proposed, that apply to the FRT system. In the first period in culture RhoA activity is prevalent on Rac1 activity and this assures microfilament assembly, fibronectin fibrillar matrix deposition and colonies migration and fusion. Previous work from the laboratory showed that the $\alpha 5\beta 1$ dimer is the main integrin involved, and evidenced the critical role of the Rho A small GTPase in the process of FN fibril organization (Calì et al. 1999). With time the balance could change in favor of a more sustained Rac1 activity. A sustained activation of Rac by Tiam1 could downregulate Rho activity as it was shown in the NIH3T3 cell model (Sander et al.,1999). This could implement cell growth, initiate the process of fibronectin degradation and enforce junctional complexes assembly. In agreement with this is the observation made in the study presented here that the treatment of FRT cultures with the Rac1 inhibitor reduced

the growth rate of the cells. Furthermore it has been shown that Rac1 is a mediator of collagen-stimulated MMP2 (metalloprotease) activity increase (Zughe and Xu 2001), and that in endotelial cells MMP2 production increases in response to actin depolymerization in a Cdc42/Rac1 dependent process. (Ispanovic and Haas 2004). Previous work from the laboratory showed that in FRT cells basal fibrillar FN is susceptible to degradation by proteolitic activities. FRT cells synthesize MMP2 and express MT1-MMP, necessary for cell membrane-mediated MMP2 activation (Nezi et al. 2002). In addition, a transition from stress fibers-containing cells to cells with depolymerized filaments is clearly detectable in FRT cultures at the stage when colonies fuse, forming extended areas of the monolayer. Finally FRT cells synthesize and deposit a laminin-enriched extracellular matrix and, according to what have been described in MDCK cells, this should be a Rac1-dependent process. A fully polarized monolayer is only formed after stable and solid junctions have been established. The balance between different Rho GTPases might be a crucial determinant of polarization. We expect that also in this final step the relative amount of activated Rho and Rac should be carefully controlled. In this respect it has been shown that Rho activity decreases with maturation of cell-cell contacts (Noren et al. 2001) and Rac1 has been shown to suppress RhoA activity at adhaerens junctions, by stimulating the association of p190 RhoGAP with cadherin-bound p120-catenin during cell-cell contact formation (Wildenberg et al. 2006).

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