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"Role of keratinocyte growth factor receptor (KGFR/FGFR2b) expression and signaling in the control of human keratinocyte differentiation "

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ABSTRACT

The fibroblast growth factor receptors (FGFRs) trigger divergent responses, such as proliferation and differentiation, and the cell type as well as the context-dependent signaling are crucial for the functional outcome. The FGFR2b/KGFR is expressed exclusively on epithelial cells and plays a key role in skin homeostasis. In the first part of this work I analyzed in vitro the role of KGFR in the early differentiation of keratinocytes modulating its expression by KGFR cDNA transient transfection or KGFR siRNA microinjection and inducing a synchronous wave of differentiation in pre-confluent cells. Immunofluorescence, biochemical and molecular approaches demonstrated that KGFR overexpression increased the early differentiation marker keratin 1 at both transcriptional and translational levels, while receptor depletion reduced it. Ligand-dependent receptor activation and signaling were required for this differentiative effect. Overexpression of kinase negative KGFR mutant or Tyr769 KGFR signaling mutant, which is not able to recruit and activate PLCγ showed that the receptor kinase activity, but not its PLCγ-mediated signaling, is required for differentiation. Reduction of K1 expression, obtained by AKT inhibition, demonstrated that the PI3K/Akt signaling pathway is involved in the control of KGFR-mediated keratinocyte differentiation. This in vitro experimental model indicates that KGFR expression represents a key event regulating keratinocyte early differentiation during the switch from undifferentiated to differentiating cells.

Since membrane and actin cytoskeleton dynamics during phagocytosis can be triggered and amplified by the signal transduction of receptor tyrosine kinases and the epidermal keratinocytes appear to use the phagocytic mechanism of uptake to ingest melanosomes released by the melanocytes playing a pivotal role in the transfer process, in the second part of this work I investigate the contribution of KGFR expression, activation and signaling in regulating the phagocytic process. We have previously demonstrated that the keratinocyte growth factor KGF/FGF7 promotes the melanosome uptake through activation of its receptor tyrosine kinase KGFR. Phagocytosis was analyzed in vitro using fluorescent latex beads on human keratinocytes induced to differentiate. KGFR depletion by siRNA microinjection and overexpression by transfection of KGFR

wild type or defective mutants previously described were performed to demonstrate the direct effect of the receptor on phagocytosis. Colocalization of the phagocytosed beads with the internalized receptors in phagolysosomes was analyzed by optical sectioning and 3D reconstruction. KGFR ligands triggered phagocytosis in differentiated keratinocytes and receptor kinase activity and signaling were required for these effects, suggesting that KGFR expression/activity and PLCγ signaling pathway play crucial roles in phagocytosis.

INTRODUCTION

Fibroblast growth factor receptors

The fibroblast growth factors receptors (FGFRs) are expressed on many different tissues and they are involved in the control of different cellular key processes such as cell growth, differentiation, migration and survival (for a recent review see Turner and Grose, 2010). FGFRs are members of a receptor tyrosine kinases (RTKs) family composed by four highly conserved transmembrane tyrosine kinase receptors (FGFR1, FGFR2, FGFR3 and FGFR4) encoded by four different genes (flg, bek, cek-2, frek). Like all receptor tyrosine kinases, the four signalling FGFR1-FGFR4 are composed of an extracellular ligand-binding domain, a single trasmembrane domain and an intracellular tyrosine kinase domain (Figure1) containing the catalytic protein tyrosine kinase core as well as additional regulatory sequences (for a recent review see Haugsten et al., 2010) that transmits the signal to the interior of the cell. The extracellular ligand-binding domain of FGFRs is composed of three immunoglobulin (Ig) like domains (I-III), with an acidic, serin-rich region between domains I and II designated the "acid box" and a conserved positively charged region in domain II that serves as a binding site for heparin (Schlessinger et al., 2000). The first Ig-like domain, together with the acid box, is thought to play a role in receptor autoinhibition (Olsen et al., 2004) while the second and the third Ig-like domains of the receptors constitute the FGF ligand-binding site. An important hallmark of the FGFR family of RTKs is that a variety of FGFR isoforms are generated by alternative splicing of their transcripts. The different isoforms include FGFR with an extracellular domain composed of either two or three Ig-like domains, soluble secreted FGFR forms as well as alternative splicing in the third Ig-like domain that profoundly alters ligand-binding specificity (Miki et al., 1992; Yayon et al., 1992). The alternative splicing in the domain III exists in FGFR1, 2 and 3 but not in FGFR4. In particular, it has been shown that exon 7 of FGFR2 gene encodes for the N-terminal half of the domain III (designated "a"), while exons 8 and 9 alternatively encode for the C-terminal half of the third Ig-like domain (designated as "b" and "c" forms of FGFR, respectively) resulting in either the IIIb or the IIIc isoform of the receptor. The two alternative forms display different ligand-binding specificities: while FGFR2IIIb (FGFR2b/KGFR) specifically binds KGF/FGF7 and FGF10, the FGFR2IIIc isoforms

binds FGF2, but not KGF/FGF7 and FGF10 (Figure3). Furthermore, it has been shown that the FGFR2IIIb isoform is exclusively expressed in epithelial cells, while the FGFR2IIIc is expressed exclusively in mesenchymal cells (Orr-Urtreger et al., 1993). The lineage-specific expression of the IIIb and IIIc isoforms of FGFRs enables interaction between the epithelial and mesenchymal layers during development in response to different FGFs. FGFs also bind to low-affinity the HPSGs (heparin sulfate proteoglycans) present on the plasma membrane of most cells (Freier et al., 2007) and composed by a proteoglycan core that binds two or three negatively charged linear polysaccharides (heparan sulphate chains), necessary for the electrostatic interactions with FGFs. Two FGF-binding sites, a heparin-binding site, and a receptor-receptor interaction site have been identified within the Ig-like domains II and III of the FGFR. Binding of FGFs to the receptors forces the dimerization of a ternary complex consisting of FGF, FGFR and heparin sulphate creating a dimeric 2:2:2 FGF-FGFR-HPSG ternary complex on the cell surface (Schlessinger et al., 2000). The involvement of HPSGs in this complex results important for both protection of the ligands from degradation and also for the complex formation between the FGFs and the FGFRs.

Cell signalling via FGF-receptors

Binding of FGFs to FGFRs induces a ligand-dependent receptor dimerization that in turn leads to a conformational shift in receptor structure and to a break of a hydrogen bonds network, that maintain the kinase in an autoinhibited state, leading to activation and consequently intermolecular transphosphorylation of the intracellular kinase domain (Lew et al., 2009, Chen et al., 2007). Phosphoryated tyrosine residues on the receptor function as docking sites for adaptor protein leading to their recruitment, binding with the activated receptor and phosphorylation of their tyrosines that in turn induces the formation of a complex with additional complement of signalling proteins (Turner and Grose, 2010) (Figure2).

In particular, FGFR substrate 2 (FRS2) is a key adaptor protein largely specific to FGFRs lacking catalytic activity that relay signal transduction from

upstream FGFRs to downstream elements. FRS2 is constitutively associated with the juxtamembrane domain of the FGFRs through the interaction between its phosphotyrosine binding (PTB) domains with an highly conserved sequence on the receptor that serves as a binding site for FRS2α and FRS2β, both members of the FRS2 family (Ong et al., 2000; Dhalluin et al., 2000). In particular, FRS2α functions as a major mediator of signalling via FGFRs and targeted disruption of FRS2α gene causes severe impairment in mouse development resulting in embryonal lethality at embryonic day 7-7.5 (E7-7.5). In contrast, the second member of the family, FRS2β, expressed exclusively in the nervous system of the embryo past E10-10.5, is unable to compensate the loss of FRS2α. In addition to PTB domain, FRS2 proteins also contain a consensus myristylation sequence at the N-terminus for binding to lipids in the plasma membrane constitutively (Schlessinger J 2000; Gotoh et al., 2004, Eswarakumar et al., 2005) and a large region with multiple tyrosine phosphorylation sites at their C-terminus (Kouhara et al., 1997) that becomes phosphorylated on several tyrosine residues upon receptor activation, creating docking sites for additional adaptor protein. In particular, FRS2α contains four binding sites for the adaptor protein growth-factor-receptor-bound protein 2 (Grb2) and two binding sites for the protein tyrosine phosphatase Shp2. FGF-stimulation leads to tyrosine phosphorylation of Shp2 resulting in complex formation with additional Grb2 molecules. Grb2 is thus recruited directly and indirectly via Shp2 upon tyrosine phosphorylation of FRS2α in response to FGF-stimulation. By binding to phosphorylated FRS2α, the adaptor Grb2 recruits the guanine nucleotide exchange factor Sos which activates Ras and the downstream effectors of mitogen-activated protein kinase (MAP kinase) or Grb2-associated-binding protein 1 (Gab1) inducing the recruitment of phosphoinositide 3-kinase (PI3K) and the activation of PI3K/Akt pathway (Figure2). In addition to the central role played by FRS2α in recruitment and activation of the MAP kinase and PI3K, it has been shown that FRS2α plays a role in the recruitment of negative regulators (Wong et al., 2002). In fact, FRS2α phosphorylated forms a complex also with Cbl, a multidomain protein that functions both as an E3 ubiquitin ligase and a platform for recruitment of a variety of signaling proteins (Wong et al., 2002). The complex FRS2α-Cbl results in ubiquitination and degradation of FRS2α and sorting of receptors to the endocytic

lysosomal degradative pathway (Figure2). On the other hand, the binding of Shp protein to phosphorylated FRS2α induces tyrosine phosphorylation of Shp2 followed by strong activation of ERK signalling, a component of MAP kinases family, in response to FGFs (Hadari et al., 1998). It was demonstrated that growth factor-induced activation of ERK might be transient or sustained (Marshall 1995). In a transient phase, the growth factor stimulation induces activation peaks within 5 minutes that returns to basal levels within 1h. In contrast, in a sustained phase activation peak is maintained for several hours with a gradual reduction. The FGF-induced ERK activation fit with a pattern of summation of both transient and sustained phases and FRS2α seems to be a critical mediator. In fact, it is able to induce a strong activation levels of ERK via its Shp2-binding sites, while its Grb2-binding sites activates ERK at moderate levels (Hadari et al., 2001). Nevertheless, the Shp-binding sites and Grb2-binding sites contribute to both phases. It is important noticed that even if the pathway activated is the same, these two different phases can induce divergent response inside cells such as proliferation and differentiation (Gotoh et al., 2008). FGF-stimulation can also induce MAP kinase-dependent phosphorylation of FRS2α on at least eight threonine residues that in turn reduces tyrosine phosphorylation of FRS2α and consequently the recruitment of Grb2, attenuating the MAP kinase response. Thus, FGFRs can utilize FRS2α for the recruitment of positive regulators of FGFR signalling and for the induction of a negative feedback mechanism resulting in signal attenuation and fine-tuning of its own activity (Lax et al., 2002).

Elsewhere on the intracellular portion of the activated receptor, and independently of FRS2 binding, the Src homology 2 (SH2) domain of phospholipase Cγ (PLCγ) binds directly to an autophosphorylated tyrosine in the C-terminal receptor tail, resulting in PLCγ phosphorylation and activation. In FGFR1 the tyrosine 766, well conserved in all four FGFR family members, serves as a binding site for the SH2 domain of PLCγ. Regarding the KGFR, tyrosine 769 is the residue which corresponds to tyrosine 766 in FGFR1 and is the putative PLCγ association site on the receptor. Activated PLCγ hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP2) to produce two second messengers: diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3) that, in turn, trigger the release of intracellular

calcium storages and subsequently activates calcium-dependent members of the proteins kinase C (PCK) family of serine-threonine kinases which partly reinforces the activation of the MAPK pathway by phosphorylating Raf.

Many other signalling molecules have been reported to be activated by FGFRs, including RSK2 (P90 ribosomal protein S6 kinase 2), STATs (signal transducers and activators of transcription) and the non-RTK Src (Zhang et al., 2001; Freier et al., 2007).

Context-dependent signalling of FGFRs

Even if the main FGFR-mediated signaling substrates and pathways are quite similar, numerous studies have demonstrated that FGFR activation can trigger divergent responses such as proliferation and differentiation depending on the cell type as well as the cellular context (Turner and Grose, 2010). In fact, although in most cellular context FGFs induce proliferation and migration (De Moerlooze et al., 2000), in certain cell types physiological FGF signalling induces differentiation and/or cell cycle arrest. As example, it was demonstrated that FGF2-dependent FGFR1 activation is able to induce proliferation in Swiss 3T3 fibroblasts and differentiation in neuronal PC12 cells (Maher et al., 1999). Moreover, it was also demonstrated that during development of endochondral and membranous bone of mouse models, FGFR3 and FGFR2 can negatively regulate proliferation and positively drive differentiation (Yu et al., 2003; Colvin et al., 1996) while their activating human germline mutations cause skeletal dysplasias in embryos (Ornitz and Marie, 2002). These evidences suggest that the cell type and the different cellular context play a crucial role in determining the functional FGFR signaling outcome. In order to explained how the same FGFR signalling pathways can induce different processes, a lot of studies analyzed the MAPK signalling because it seems to be the principal signalling pathway activated downstream of FGFRs. A lot of evidences highlighted the importance of differential effects of MAPK signalling in establishing the cellular response of FGFR activation. In fact, even if the downstream effect of MAPK signalling is mostly cell proliferation, it induces cell cycle arrest and differentiation in other

context, such as the p21-dependent cell cycle arrest in chondrocytes (Raucci et al., 2004) and the differentiation of neuronal PC-12 cells (Maher, 1999). Factors that results important in context specificity include crosstalk with other signalling networks and importantly the kinetics of MAPK signalling: a sustained strong activation of these substrates, in fact, induces differentiation, senescence and apoptosis while their transient activation induces proliferation and survival (Marshall, 1995).

A different MAPK kinetics could be attribuited to the activation of different RTK-induced substrates. For example, after FGFR activation the different kinetics of MAPK activation depends on the substrates recruited by FRS2α: the recruitment of Grb2 induces a transient MAPK activation while the sustained activation of this signalling is attribuited to Shp2 activation (Gotoh et al., 2008).

On the other hand, it has also been demonstrated that the alternative binding of FRS2 or Shc to the same site of another RTK, is able to induce a different MAPK signalling kinetics and consequently a divergent response in PC12 cells: a prolonged activation of MAPK induced by FRS2 activation is associated with differentiation of PC12 cells, while transient activation of this pathway induced by Shc in these cells is associated with proliferation (Gotoh et al., 2008). In addition to the role of MAPK kinetics in the induction of differentiation, other pathways such as PI3K/Akt (Raucci et al., 2004) and PLCγ (Yang et al., 2008) have been also involved in the FGF-mediated induction of this process.

Signalling pathways in keratinocyte differentiation

Among the signalling pathways activated in keratinocytes, PI3K/Akt pathway seems to be involved in the differentiation process of this cell type. In fact, PI3K/Akt pathway is activated early on during differentiation both in cultured keratinocytes and in the epidermis in vivo and its signalling activity is critical for the survival of differentiating cells and the proper execution of the differentiation program (Calautti et al., 2005). Both tyrosine kinases and cadherin adhesion molecules are likely to work cooperatively in the engagement of the

PI3K pathway during keratinocyte differentiation, as this event is abolished either by interference with E-cadherin adhesive functions or by inhibition of the Src-and the EGFR kinases. The EGFR protein may also be involved in the direct recruitment of PI3K, bearing a PI3K docking site at the tyrosine 920 (Stover et al., 1995). Because Src kinases can also phosphorylate the tyrosine 920 of the EGFR, this suggests potential cross-talks between these families of kinases in PI3K activation at this level. Moreover, the physical association of PI3K with cadherin-catenin complexes is increased during differentiation in a tyrosine kinase-dependent manner (Calautti et al., 2005).

More recently, moreover, it has been also demonstrated that the Akt loss-of-function using a stable RNA interference technology (shAKT1) in combination with organotypic culture, a more physiological differentiation system, resulted in a severe defect in stratification with a thin parabasal region but a thickened stratum corneum, or hyperkeratosis, when compared with control. In particular, the presence of a granular layer and lamination of the upper squamous layer as observed in control cultures was absent in shAKT1 cultures, suggesting disruption of aspects of late differentiation (Thrash et al., 2006). Moreover, it was demonstrated in vivo that KGF treatment triggers duct-to-beta cell differentiation by the sequential activation of beta cell specific markers in ductal cells via PI3K/Akt pathway (Uzan et al., 2008). In fact, histological examination of rats pancreatic sections revealed that the number of cells positive for the transcription factor PDX1 and consequently for its target Glut2, a crucial marker for terminal differentiation of the beta cells is increased by KGF treatment compared to control cells and results drastically reduced by treatment with PI3K/Akt pathway inhibitors (Uzan et al., 2008).

Another possible signaling pathway involved in keratinocyte differentiation is that of PLCγ. In fact, PLC-γ is able to hydrolyze PIP2 to IP3 and DAG second messengers and consequently induce a sustained intracellular calcium rise that in turn triggers the calcium-induced differentiation response of human keratinocytes (Xie and Bikle, 1999; Xie et al., 2009). The requirement of intracellular calcium for triggering of keratinocytes differentiation was demonstrated analyzing the inhibitor effect on keratinocytes differentiation

markers of intracellular calcium rise blocking induced by calcium chelators (Li et al., 1995). The induction of PLCγ-mediated keratinocyte differentiation was attribuited to the formation of a p120-catenin-dependent E-cadherin-catenin complex that recruits and activates phosphatidylinositol 3-kinase (PI3K) that in turn produced IP3 in the plasma membrane recruiting and activating PLCγ. Moreover, it has been recently demonstrated that the FGF2-dependent activation of PLCγ is able to induce a sustained activation of MAPKs that in turn triggers transdifferentiation of bone marrow stromal cells in neuronal cells (Yang et al., 2008).

The keratinocytes growth factor receptor (KGFR/FGFR2b): role in keratinocyte differentiation and phagocytosis.

The keratinocyte growth factor receptor (KGFR/FGFR2b) is a splicing transcript variant of the fibroblast growth factor receptor 2 (FGFR2) expressed exclusively on epithelial cells (Miki et al., 1992) and activated by the specific high affinity binding of keratinocyte growth factor (KGF/FGF7) and fibroblast growth factor-10 (FGF10) (Rubin et al., 1989; Igarashi et al., 1998) (Figure3). KGF and FGF10 are two members of the FGF family that are secreted by dermal fibroblast and acts in a paracrine way on epithelial cells. When compared to the other members of the FGF family, both the amino acid sequence and the tissue expression of FGF-10 and KGF show high similarity even if the two growth factors show a different sensitivity to heparin, implying that the extracellular matrix may play a role in regulating the cellular response to FGF10 and KGF (Igarashi et al., 1998). After ligand-induced KGFR activation, its signaling is attenuated and regulated by clathrin-mediated endocytosis similarly to other receptor tyrosine kinases (Belleudi et al., 2007; Marchese et al., 1998; Belleudi et al., 2006). Our previous studies on the endocytic pathways followed by KGFR have demonstrated the opposite endosomal sorting of the receptor triggered by the two ligands: in fact, KGF targets the receptor to the degradative pathway, whereas FGF10 induces KGFR sorting to the indirect recycling route (Belleudi et al., 2007).

Some evidences have suggested an important role of KGFR as a critical mediator of organogenesis and skin development during embryogenesis. In fact, it was demonstrated that KGFR germline knockout results in mice that die at birth from multiple developmental defects (De Moerlooze et al., 2000, Revest et al., 2001) and showing a severe epidermal hypoplasia that demonstrated its crucial role for normal epidermal growth and development as well as for subsequent hair follicle morphogenesis (Petiot et al., 2003). Some reports have also suggested a key role for KGFR expression in the post natal skin homeostasis (Petiot et al., 2003; Grose et al., 2007; Yang et al., 2010), regulating the balance between proliferation and differentiation. In fact, it was demonstrated that KGFR is up-modulated in vitro during differentiation from basal to suprabasal keratinocytes (Marchese et al., 1997; Capone et al., 2000), and it is primarily localized in the spinous suprabasal layer (La Rochelle et al., 1995; Marchese et al., 1995) suggesting that receptor expression may control the proliferative-differentiative cell program. Moreover, ligand-induced KGFR activation promotes the differentiation program of human keratinocytes (Marchese et al., 1990; Marchese et al., 2001). More interestingly, it was identified that mice lacking the KGFR in keratinized epithelia display altered cell proliferation in the basal layer and compromised late differentiation with clear evidence of parakeratosis, although the expression of early differentiation markers, such as K1, does not seem to be profoundly affected (Petiot et al., 2003; Grose et al., 2007; Yang et al., 2010). However, the results obtained in these "in vivo" models appeared frequently discordant and not conclusive, at least concerning the proliferative ability of the keratinocytes when KGFR is knocked out. Consistent with this statement, Yang et al. (Yang et al., 2010) have very recently demonstrated that the hyperproliferative effect induced by the lack of FGFR1b and FGFR2b/KGFR observed "in vivo" in KO mice was not confirmed in the corresponding "in vitro" model of cultured keratinocytes derived from these mice: this finding has been explained by the fact that, in the "in vivo" models, many microenvironmental factors, such as the presence of inflammatory components, may act hiding the specific functions of the receptors in skin homeostasis. This appears to suggest that the role of KGFR expression in the regulation of keratinocyte differentiation cannot be properly investigated "in vivo". On the other hand, the use of "in vitro" models has been particularly

appropriated for the demonstration of the key role of KGFR as a tumour suppressor controlling epithelial cell differentiation: in fact, several studies have demonstrated that the re-expression of KGFR in cultured cells from epithelial tumours in which this receptor is down-regulated was able to inhibit cell growth and to induce differentiation (Feng et al., 1997; Matsubara et al., 1998; Ricol et al., 1999; Zhang et al., 2001; Yasumoto et al., 2004). In fact, the downregulation of KGFR was correlated with the progression of prostate tumors to malignancy *(*Wang et al., 2002*)* and the re-expression of KGFR in human salivary adenocarcinoma and in prostate cancer cells inhibits growth and induces cell differentiation both *in vitro* and *in vivo* (Zhang et al., 2001; Feng et al., 1997). Moreover mice lacking KGFR in keratinized epithelia display spontaneous papillomas and great sensitivity to chemical carcinogenic insult (Grose et al.,2007).

Thus, to evaluate the single contribution of KGFR expression in both the induction of keratinocyte differentiation and in the maintenance of this process in cells already committed to differentiate, we have believed useful to develop an "in vitro" cellular model in which the modulation of the receptor expression, as well as the differentiation process, could be highly controlled and easily monitored. We have thought that a rapid and synchronous modulation of the receptor expression could be efficiently obtained in cultured keratinocytes by transient transfection of KGFR cDNA or by microinjection of KGFR siRNA, while a synchronous wave of differentiation in pre-confluent cells would be generated by treatment with Thapsigargin (TG), an inhibitor of endoplasmic reticulum Ca^{2+}-ATPase pump family that induces a rapid, dose-dependent release of stored calcium increasing its cytosolic concentration (Rosenberger et al., 2007). In addition, this strategy of KGFR modulation and forced cell differentiation would also permit to study the signaling pathways responsible for the differentiative response. Among the possible candidates for the regulation of KGFR-mediated early differentiation in keratinocytes we considered the PI3K/Akt and PLCγ pathways, since these two pathways play a role in keratinocyte differentiation as previously described. In particular, to identify the possible receptor-mediated signaling pathways involved in the earlier induction of differentiation, we used the KGFR signaling mutant Y769F (KGFR Y769F), in which tyrosine 769 was substituited by phenylalanine

and consequently that is not able to recruit and activate PLCγ (Ceridono et al., 2005) and a kinase negative KGFR mutant (KGFR Y656F/Y657F) (Belleudi et al., 2006) as negative control. In fact, our group and others have shown that the KGFR autophosphorylation site Y769 is required for PLCγ binding and activation (Ceridono et al., 2005; Cha et al., 2009). Although the specific mutagenesis of this tyrosine site in phenylalanine (Y769F) has been used to analyze the role of PLCγ in the KGFR-mediated proliferative signaling and response (Ceridono et al., 2005; Moffa et al., 2007), its effect on keratinocyte differentiation remains to be investigated. On the other hand, the possible involvement of the PI3K/Akt signaling pathway was also investigated through the use of a specific Akt inhibitor.

Another process closely controlled by RTK signaling is the phagocytosis. Phagocytosis is a mechanism of endocytosis used for the ingestion either of large particles which can be more than 5 Ãm in diameter or microorganisms. In general the endocytosis mechanism provide for the internalization of macromolecules concentrated in portion of the plasma membrane through the building and closing cup-shaped invaginations that forms vescicles inside cells. Depending on size and origin of the particles ingested it is possible to identify different types of endocytosis: the clathrin-mediated endocytosis is used for internalization of complexes formed by the binding of protein hormones or growth factor with specific plasma membrane receptors, the caveolae-mediated endocytosis is characterized by a plasma membrane invagination of 50-80 nm called “caveolae” present in microdomains rich in cholesterol and sphingolipids involed in the transcytosis process through the capillary endothelium, the pinocytosis is induced by cells for solutes and fluid assumption through the formation of small vescicles, the macropinocytosis is used for the ingestion of extracellular solutes and fluid into 0.2 to >5.0 Ãm diameter vescicles called “macropinosomes” (Swason et al., 2008) and the phagocytosis. The phagocytic process is known to involve reorganization of the cortical actin filaments, required for the formation of actin-enriched membrane protrusions and cups surrounding the particle to be ingested forming intracellular membrane-bounded compartments called “phagosomes”. Phagocytosis have significant roles in animal development, innate immunity, the

initiation of specific immune responses and entry of pathogens into host cells, so the mechanisms of their regulation have broad implications (Stuart and Ezekowitz, 2008; Watts and Amigarena, 2000; Blander and Medzhitov, 2006; Raddien and Harvitz, 2004; Amstutz et al., 2008). Both membrane and actin cytoskeleton dynamics during phagocytosis can be triggered and amplified by the signal transduction of receptor tyrosine kinases and by the modifications of phospholipids at the cell plasma membrane (Swason et al., 2008). In particular, phospholipases contribute significantly to phagocytosis generating phosphoinositides with important roles in the regulation of the movement during this process. The phospholipases in fact hydrolyze PIP2 to PIP3 that is capable of binding and increasing the activity of proteins that modify membrane chemistry and the actin cytoskeleton. In addition to the professional phagocytes, such as macrophages, dendritic cells and neutrophils, many different cell types are able to actively engulf large particles: among them, the epidermal keratinocytes appear to use the phagocytic mechanism of uptake to ingest the melanin pigment released by the neighbouring melanocytes for photoprotection and pigmentation (Boissy et al., 2003). In fact, the most supported model of intercellular melanin transport in the skin implies packaging and storage of the synthesized pigment in large (0.5 μm) lysosomal-related organelles termed melanosomes and their transfer through a regulated double process of exocytosis from the melanocytes and phagocytosis by the surrounding keratinocytes (Van Den Bossche et al., 2003; Wasmeier et al., 2008). We and others have demonstrated that the expression of some receptors, such as the tyrosine kinase keratinocyte growth factor receptor (KGFR/FGFR2b) and the G-protein coupled protease-activated receptor 2 (PAR-2), which are present on the plasma membrane of the keratinocytes but not of the melanocytes, regulate the melanosome uptake (Cardinali et al., 2005; Cardinali et al., 2008; Seiberg et al., 2000a; Seiberg et al., 2000b): these findings clearly indicate that the epidermal keratinocytes play a pivotal role in the transfer process and suggest that melanosome phagocytosis could be the major mechanism involved (Cardinali et al., 2008; Sharlow et al., 2000; Ando et al., 2009), although a deeper knowledge of the molecular mediators affecting skin pigmentation and controlling phagocytosis is still needed. Among the autocrine and paracrine factors which play a role in melanin uptake, we have recently reported that KGF/FGF7 is able to promote the

melanosome transfer from melanocytes to keratinocytes and we have proposed that this function could be exerted by receptor-mediated stimulation of the phagocytic process in the recipient keratinocytes (Cardinali et al., 2005; Cardinali et al., 2008). However, a number of crucial open questions still remain to be solved, mostly related to the receptor signaling pathways involved in such stimulation and the possible role of receptor endocytosis in the melanosome phagocytic process. Thus, considering that phagocytosis is a crucial event for melanosome uptake in keratinocytes and it is reasonable to speculate a role of PLCγ pathway during this process, the second aim of this study was to investigate the contribution of the KGFR expression, ligand-induced activation and in particular its PLCγ-mediated signaling in regulating the phagocytic process.

MATERIALS AND METHODS

Cells and treatments

The human keratinocyte cell line HaCaT was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. HaCaT cells were transiently transfected with the pCI-neo expression vector containing human KGFR WT (HaCaT KGFR WT) or a kinase negative mutant KGFR Y656F/Y657F (HaCaT KGFR Y656F/Y657F) or a signaling mutant KGFR Y769F (HaCaT Y769F) using jetPEI™ DNA Trasfection Reagent (Polyplus-trasfection, New York, NY, USA) according to manufacturer's instructions. For RNA interference and KGFR silencing, HaCaT cells were transfected with Bek small interfering RNA (Bek siRNA) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or with unrelated siRNA as a control, using Lipofectamine 2000 Trasfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.
For growth factor stimulation, cells were serum starved for 12h and then incubated with 20 ng/ml KGF (Upstate Biotechnology, Lake Placid, NY, USA) for 24h at 37°C. Alternatively, to induce KGFR activation and signaling, cells were serum starved for 4h and incubated with 100 ng/ml KGF (Upstate) for 10 minutes at 37°C. To inhibit Akt, cells were incubated with the specific Akt inhibitor 1L-6-hydroxy-methyl-chiro-inositol2-(R)-2-O-methyl-3-O-octadecylcarbonate
(Calbiochem, San Diego, CA) 1μM for 1h at 37°C before treatment with KGF in the presence of the inhibitor. To induce the differentiation program in pre-confluent conditions, HaCaT cells were incubated with different doses of Thapsigargin (TG) (0,1 μM, 0.5 μM, 1 μM and 2,5 μM) for 1h at 37°C and followed by incubation at 37°C for 48h. Since TG stock (1mg/ml) was diluted in solvent dimethyl sulfoxide (DMSO), control cells were treated with an equal amount of DMSO. Alternatively, to induce cell differentiation with high extracellular calcium (Muller et al., 2008), cells were placed in medium containing 2 mM Ca^{2+} .

Primary cultures of normal human keratinocytes (NHKs) were derived from skin biopsies and maintained in Medium 154-CF (Cascade Biologics, Portland, OR, USA) supplemented with Human Keratinocyte Growth Supplement (HKGS, Cascade Biologics) plus antibiotics and Ca^{2+} 0,03 mM (CascadeBiologics Inc.).

To induce cell differentiation, primary human keratinocytes were placed in medium containing Ca^{2+} 1,5 mM. Primary cultures of normal human fibroblasts (HFs) were also obtained from skin biopsies. The sample was cut into small pieces, digested with dispase 0,1mg/ml and collagenase I 0,35% for 45 min at 37°C, pelleted, resuspended and plated in and maintained in DMEM medium containing 10% FBS and antibiotics. Supernatant (SN) obtained from cultured HFs kept in serum-free medium for 48h was collected and frozen at -30°C until use.

To analyze the uptake of beads in keratinocytes, HKs and HaCaT cells were serum starved for 12h and then incubated with fluorescent microspheres 0.5 μm (red) in diameter (FluoSpheres Fluorescent Microspheres, Molecular Probes, Eugene, OR, USA) at the concentration of 72 x 10^7 particles/ml for 4 hours. To evaluate the effects of KGFR activation on the phagocytic ability, the uptake was performed in the presence of KGF 20 ng/ml (Upstate Biotechnology, Lake Placid, NY, USA) or of FGF10 20 ng/ml (PeproTech, London, UK) + heparin 0,3 μg/ml or of SN collected from cultured HFs as above. For inhibition of KGFR activity, cells were pre-incubated with a specific FGFR tyrosine kinase inhibitor SU5402 25 μM (Calbiochem, Nottingham, UK) for 1 hour before treatments with growth factors or SN-HFs. To analyze the co-localization of KGFR and beads in the phagosomal compartments, HaCaT cells were previously incubated with anti-Bek polyclonal antibodies (1:50 in DMEM medium; H-80, Santa Cruz Biotechnology), directed against the extracellular portion of KGFR, for 1h at 4°C and then with the fluorescent microspheres as above. To induce a synchronous wave of KGFR and beads internalization, cells were incubated with anti-Bek polyclonal antibodies (Santa Cruz Biotechnology), fluorescent microspheres and KGF 100 ng/ml for 1h at 4°C and then warmed to 37°C for 30 minutes or 2h.

Microinjection

Microinjection was performed with an Eppendorf microinjector (Eppendorf, Hamburg, Germany) and an inverted microscope (Zeiss, Oberkochen, Germany). Injection pressure was set at 80–100 hPa and the injection time at 0.5 s. For KGFR depletion, a mixture of 100 nM KGFR small interfering RNA (Bek

siRNA) (Santa Cruz Biotechnology) and 3 mg/ml mouse (Cappel Research Products, Durham, NC, USA) or rabbit IgG (Sigma Chemicals, St Louis, Missouri) in distilled water was microinjected in the cytoplasm of HaCaT cells to induce RNA interference and consequent KGFR silencing. Unrelated siRNA was microinjected as negative control. Cells were then treated with TG as above and processed for immunofluorescence.

Alternatively, cells were then serum starved for 12h at 37°C, treated with beads and KGF as above and processed for immunofluorescence. For clathrin heavy chain (CHC) depletion and block of the clathrin mediated endocytosis, a mixture of 100 nM CHC siRNA (Santa Cruz) and 100ng/µl KGFR cDNA in distillate water was microinjected to simultaneously induce CHC silencing and KGFR overexpression. Cells were starved for 12h at 37°C, treated with beads and KGF as above and processed for immunofluorescence. The efficiency of the CHC depletion was assessed by parallel injection of a mixture of 100 nM CHC siRNA and 3 mg/ml rabbit IgG in distillate water.

Immunofluorescence

Cells, grown on coverslips and incubated as above, were fixed with 4% paraformaldehyde in PBS for 30 minutes at 25°C followed by treatment with 0.1 M glycine for 20 minutes at 25°C and with 0.1% Triton X-100 for additional 5 minutes at 25°C to allow permeabilization. Cells were then incubated for 1h at 25°C with the following primary antibodies: rabbit polyclonal anti-Bek (1:50 in PBS; C-17, Santa Cruz Biotechnology), mouse monoclonal anti-Bek (1:20 in PBS; C-8, Santa Cruz Biotechnology) which recognizes the KGFR/FGFR2b and FGFR2c isoforms, rabbit polyclonal anti-Bek (1:50 in PBS; H-80, Santa Cruz Biotechnology), goat polyclonal anti-tyrosinase (1:50 in PBS; C-19, Santa Cruz Biotechnology), mouse monoclonal anti-cytokeratins (1:100 in PBS; clone MNF116, DAKO, Carpinteria, CA, USA), rabbit polyclonal anti-K1 (1:50 in PBS; Covance, Emeryville, CA, USA), mouse monoclonal anti-clathrin (1:20 in PBS; Santa Cruz Biotechnology) and mouse monoclonal anti-Ki67 (1:50 in PBS; Novocastra, Newcastle-upon-Tyne, U.K.).

The primary antibodies were visualized, after appropriate washing with PBS, using goat anti-rabbit IgG-FITC (1:400 in PBS; Cappel Research Products, Durham, NC, USA), goat anti-mouse IgG-FITC (1:20 in PBS; Cappel), goat anti-rabbit IgG-Texas Red (1:200 in PBS; Jackson Immunoresearch Laboratories, West Grove, PA, USA), chicken anti-goat IgG-Alexa Fluor 488 (1:1000 in PBS; Molecular Probes, Eugene, OR; USA) and donkey anti-rabbit IgG-Alexa Fluor 594 (1:1000 in PBS; Molecular Probes) for 30 minutes at 25°C. Nuclei were stained with DAPI (1:1000 in PBS; Sigma). Coverslips were finally mounted with mowiol for observation. Fluorescence signals were analyzed by recording and merging stained images using a CCD device SPOT-2 camera (Diagnostic Instruments Inc., Sterling Heights, MI) and IAS2000/H1 software (Delta Sistemi, Roma, Italy) or by scanning cells in a series of 0.5mm sequential sections with an ApoTome System (Zeiss) connected with an Axiovert 200 inverted microscope (Zeiss); image analysis was then performed by the Axiovision software (Zeiss). Images were obtained by 3D reconstruction of a selection of three out of the total number of the serial optical sections. The fluorescence intensity of the signals was performed by the analysis of 50 cells for each sample in five different microscopic fields from three different experiments and the cut-off of the signal intensity was selected for both TG-treated and control samples in order to discriminate between K1 positive and negative cells using the KS300 3.0 Image Processing System (Zeiss, Oberkochen, Germany). Quantitative analysis of the percentage of Ki67 and/or K1-positive cells was assessed counting for each sample a total of 50 cells, randomly observed in 10 microscopic fields from three different experiments, except for the single experiment on HaCaT cells treated at low and high Ca^{2+} concentrations. Alternatively, quantitative analysis of the beads uptake was performed by counting the number of internalized beads in 100 cells for each condition, randomly taken from 10 microscopic fields in three different experiments, and values are expressed as the mean value ± standard errors (SE). The cell margins were defined by parallel observation in phase contrast microscopy. Quantitative analysis of the extent of colocalization of beads with KGFR and of the tyrosinase fluorescence intensity for cytoplasmic area was performed by the analysis of 100 HaCaT cells for each sample in five different fields randomly taken from three independent experiments and using the KS300 3.0 Image Processing System

(Zeiss); results are shown as mean values +/- standard error (SE). Statistical analysis was performed and significance level has been defined using Student's t test.

Western blot analysis

HaCaT and HaCaT KGFR cells were lysed in a buffer containing 50 mM HEPES pH 7.5, 150 mM NaCl, 1% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 5 mM EGTA, supplemented with protease inhibitors (10 μg/ml aprotinin, 1mM PMSF, 10μg/ml leupeptin), and phosphatase inhibitors (1mM sodium orthovanadate, 20 mM sodium pyrophosphate, 0.5 M NaF); 50 μg of total protein was resolved under reducing conditions by 8% SDS-PAGE and transferred to reinforced nitrocellulose (BA-S 83, Schleider and Schuell, Keene, NH, USA). The membranes were blocked with 5% non fat dry milk in PBS 0.1% Tween 20, and incubated with anti-Bek (C-17, Santa Cruz) polyclonal antibodies, anti-K1 (Covance) polyclonal antibodies, anti-phospho-Akt (Ser 473, Cell Signaling Technology, Beverly, MA) polyclonal antibodies (followed by enhanced chemiluminescence detection (ECL; Amersham, Alington Heights, IL). The membranes were rehydrated by being washed in PBS-Tween 20, stripped with 100 mM mercaptoethanol and 2% SDS for 30 min at 55°C, and probed again with anti-Akt 1/2 (H-136, Santa Cruz Biotechnology) polyclonal antibodies or anti-actin (Sigma) monoclonal antibody, to estimate the protein equal loading. Densitometric analysis was performed using Quantity One Program (Bio-Rad Laboratoires, Hercules, CA). Briefly, the signal intensity for each band was calculated and the background subtracted from experimental values. The resulting values from three different experiments were then normalized and expressed as fold increase respect to the control value.

Primers

Oligonucleotide primers for target genes and for the housekeeping gene were chosen with the assistance of the Oligo 5.0 computer program (National Biosciences, Plymouth, MN) and purchased from Invitrogen. The following primers were used: for *FGFR2b/KGFR* target gene: 5'-

CAGGGGTCTCCGAGTATGAA-3 (sense), 5′-TCTAAAGGCAACCTCCGAGA-3′ (anti-sense); for *K1* target gene 5′-AGCACAAGCCACACCACCATC-3′ (sense), 5′-CGCCACCTCCAGAACCATAGC-3′ (antisense); for the GAPDH housekeeping gene: 5′-CATCAGCAATGCCTCCTGCAC-3′ (sense), 5′-GTCATGAGTCCTTCCACGATACCAA-3′ (antisense). For each primer pair, we performed no-template control and no-reverse-transcriptase control (RT negative) assays, which produced negligible signals.

RNA extraction and cDNA synthesis

RNA was extracted using the TRIzol method (Invitrogen, Carlsbad, CA) according to manufacturer's instructions and eluted with 0,1% diethylpyrocarbonate (DEPC)-treated water. Total RNA concentration was quantitated by spectrophotometry and the quality was assessed by measuring the optical density ratio at 260/280 nm. RNA samples were stored at -80°C. After denaturation in DEPC-treated water at 70°C for 10 minutes, 1 μg of total RNA was used to reverse transcription using iScript™ cDNA synthesis kit (Bio-Rad) according to manufacturer's instructions.

PCR amplification and real-time quantitation

Real-time PCR was performed using the iCycler Real-Time Detection System (iQ5 Bio-Rad) with optimized PCR conditions. The reaction was carried out in 96-well plate using iQ SYBR Green Supermix (Bio-Rad) adding forward and reverse primers for each gene and 1μl of diluted template cDNA to a final reaction volume of 15 μl. All assays included a negative control and were replicated three times. The thermal cycling programme was performed as follows: an initial denaturation step at 95°C for 3 minutes, followed by 45 cycles at 95°C for 10 seconds and 60°C for 30 seconds. Real-time quantitation was performed with the help of the iCycler IQ optical system software version 3.0a (BioRad), according to the manufacturer's manual. The relative expression of the housekeeping gene was used for standardizing the reaction. The comparative threshold cycle (C_t) method was applied to calculate the fold changes of expression compared to control cells

Results are reported as mean ± standard deviation (SD) from three different experiments in triplicate.

RESULTS

The expression of KGFR regulates early differentiation

The crucial role of keratinocyte growth factor receptor (KGFR/FGFR2b) and of its ligands in the control of the epithelial cell homeostasis has been widely demonstrated (Haugsten et al., 2010). In addition, previous results from our group have demonstrated that KGFR is up-modulated during cell differentiation (Marchese et al., 1997; Capone et al., 2000; Visco et al., 2004). To analyze in detail the contribution of KGFR expression in the induction of keratinocyte early differentiation, we used the human keratinocyte HaCaT cell line, spontaneously immortalized from a primary culture of keratinocytes and widely used as a model of keratinocyte differentiation and stratification (Boukamp et al., 1988; Capone et al., 2000). Pre-confluent HaCaT cells expressing very low levels of KGFR were transiently transfected with KGFR (HaCaT KGFR) and it was assessed the effect of its forced expression on the early differentiation marker keratin 1 (K1). Quantitative real-time RT-PCR showed a considerable increase in both K1 mRNA (Figure 4 A, right panel) and KGFR mRNA (Figure 4A, left panel) expression at 24 hours from transfection in HaCaT KGFR cells compared to control cells. The enhanced expression of K1 induced by KGFR overexpression was also validated at the protein level by Western blot analysis using anti-K1 polyclonal antibodies, which showed that the band at the molecular weight corresponding to K1 protein was increased in HaCaT KGFR cells compared to control cells (Figure 4B). Immunoblot analysis using anti-Bek polyclonal antibodies, which recognize the intracellular portion of the two splicing variants KGFR/FGFR2b and FGFR2c, showed that the 140 KDa specific band corresponding to the receptor molecular weight was well visible only upon KGFR transfection (Figure 4B). The equal loading was assessed with anti-actin antibody and densitometric analysis was performed as described in materials and methods. These results indicated that, in pre-confluent HaCaT cells expressing very low levels of endogenous KGFR, the receptor overexpression up-regulates K1 at both transcriptional and translational levels, suggesting that increased expression of this receptor might be able to trigger early differentiation in undifferentiated keratinocytes.

Then, we wondered if the KGFR overexpression could enhance early differentiation also in differentiating HaCaT cells expressing increased levels of endogenous KGFR. Since cell pre-confluence is required for transient transfection and, consequently, transient KGFR overexpression can not be maintained for the time necessary to permit HaCaT cell stratification (Capone et al., 2000), the differentiation program was induced in pre-confluent conditions using Thapsigargin (TG), an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase that it is known to trigger HaCaT differentiation (Rosenberger et al., 2007) inducing a dose-dependent release of stored Ca^{2+} in the cytoplasm (Thastrup et al., 1990, Lytton et al., 1991). Pre-confluent HaCaT cells were treated with different doses of TG as reported in materials and methods and the number of living metabolically active cells was evaluated by MTT test measuring the absorbance at 570nm by a spectrofotometer (data not shown). Based on this preliminary set-up, we found that, taking in account both cell differentiation and cell viability, the best condition was obtained at the dose of 0.5 μM TG followed by incubation at 37°C for 48h (data not shown). Control cells were treated with equal amount of the solvent dimethyl sulfoxide (DMSO). The differentiative effect of the selected dose of TG was first analyzed by phase contrast microscopy: while cells treated with DMSO alone appeared non differentiated, closely packed and polygonal (Figure 5A, left panels), the TG-treated cells showed morphological changes characteristic of keratinocyte differentiation (Schwartz et al., 1992): in fact, the cells appeared detached each other and more elongated if compared to control cells (Figure 5A, left panels). The proliferation rate and the early differentiation were assessed by immunofluorescence using anti-Ki67 monoclonal antibody and anti-K1 polyclonal antibodies respectively. Quantitative immunofluorescence analysis showed that, consistent with the differentiative stimulus, TG treatment significantly decreased the percentage of cells expressing the proliferative marker Ki67 and increased that of cells positive for K1 (Figure 5A, right panels), as expected (Rosenberger et al., 2007; Hong et al., 2008). The enhanced expression of K1 induced by TG was also demonstrated at both transcriptional and translational levels by real-time RT-PCR (Figure 5B) and Western blot analysis (Figure 5C), confirming the ability of TG to trigger HaCaT cell differentiation. In addition, immunoblot using anti-Bek antibodies showed that the band corresponding to KGFR, hardly detectable in

control cells, was evident upon TG treatment despite the pre-confluent condition (Figure 5C) and correlated with a clear increase of KGFR mRNA levels (Figure 5B). Thus, TG treatment allows to obtain a synchronous wave of differentiation and is able to generate a homogenous population of HaCaT differentiating cells expressing increasing amount of KGFR.

Therefore, having the appropriate tool to induce differentiation in our model system, in order to assess if KGFR overexpression could enhance early differentiation in HaCaT cells undergoing differentiation, we analyzed the effect of KGFR transfection on K1 expression in cells forced to synchronously differentiate by TG treatment. To this aim, pre-confluent HaCaT cells were transiently transfected with KGFR and then treated with TG, while the control cells were kept in DMSO alone: samples were then processed for biochemical or immunofluorescence analysis. Western blot performed as above revealed that the increased expression of KGFR in transfected cells corresponded to a parallel enhancement of the K1 protein levels also upon TG treatment (Figure 6A), indicating that the effect mediated by receptor expression is well detectable in cells induced to differentiate. Double immunofluorescence using anti-Bek monoclonal antibody and anti-K1 polyclonal antibodies showed that the signal of the transfected receptor appeared localized both on the cell surface and in intracellular compartments, as expected (Belleudi et al., 2006). The quantitative analysis showed that the overexpression of KGFR was able to significantly increase the percentage of cells expressing K1 in both TG-treated and control cells (Figure 6B). Thus, KGFR plays a crucial role in the control of early differentiation, since its expression triggers early differentiation in undifferentiated cells as well as enhances this process in cells already committed to differentiation. To confirm the physiological role of KGFR in this process in a well established cell model of keratinocyte differentiation, the experiments described above were repeated using high extracellular calcium addition as an alternative stimulator of differentiation in both HaCaT cells and primary cultures of normal human keratinocytes (HKs). Cells were transiently transfected with KGFR and induced to differentiate in response to increased Ca^{2+} concentration (2 mM for HaCaT cells (M□ller et al., 2008), 1.5 mM for HKs) in the medium or kept in low Ca^{2+} medium (0.1 mM for

HaCaT cells, 0.03 mM for HKs). Quantitative immunofluorescence analysis as above showed that, also upon extracellular Ca^{2+} treatment and in primary keratinocytes, the KGFR expression induces an increase of K1 positive cells (Figure 6C), indicating that the KGFR role in the regulation of the early differentiation represents a physiological general phenomenon in epidermal cells.

To unequivocally demonstrate the specific direct function of KGFR in regulating the early differentiation, we analyzed the effect of the receptor depletion on K1 expression. To this aim, it was performed co-injection of small interfering RNA for FGFR2/Bek (KGFR siRNA) to obtain receptor silencing and mouse IgG to identify the microinjected cells. Microinjection with an unrelated siRNA was performed as a control. After injection, cells were treated with TG or kept in DMSO and then warmed to 37°C as above. Quantitative immunofluorescence analysis showed that in TG-treated cells expressing increased levels of KGFR protein, as stated above (Figure 5C), the KGFR siRNA injection and consequent receptor depletion induced a significant decrease in the percentage of K1 positive cells compared to the uninjected ones surrounding them in the same microscopic fields or to cells injected with control siRNA (Figure 7A). In contrast, in cells kept with DMSO alone expressing very low levels of endogenous KGFR protein (Figure 5C), the low percentage of K1 positive cells appeared unaffected by KGFR siRNA injection (Figure 7A). The efficiency of KGFR depletion was evaluated by transfecting HaCaT cells with KGFR siRNA or with a control siRNA and then treating them with TG as above. Western blot analysis using anti-Bek polyclonal antibodies showed that KGFR protein expression appeared down-regulated in KGFR siRNA-transfected cells (Figure 7B). The equal loading was assessed with anti-actin antibody. Taken together, these results demonstrate that the modulation of the receptor expression is crucial in regulating cell differentiation.

KGFR signaling through PI3K/Akt pathway, but not through PLCγ activation, is involved in the induction of early differentiation.

To first analyze if the ligand-dependent activation of KGFR is required for the induction of early differentiation, HaCaT KGFR cells treated with TG or kept in DMSO alone as above were serum starved and stimulated the last 24h at 37°C with KGF 20ng/ml. Quantitative immunofluorescence analysis using anti-Bek and anti-K1 antibodies showed that KGF stimulation induced an increase in the percentage of K1 positive cells as expected (Marchese et al., 1997; Capone et al., 2000) and this KGF-induced increase was higher in cells overexpressing KGFR compared to cells expressing endogenous receptor levels (Figure 8). This enhancement was particularly evident in undifferentiated cells, because the TG-treated cells were almost all K1 positive independently on KGF addition. Interestingly, in KGF untreated cells, no significant increase in the percentage of K1 positive cells was induced by KGFR overexpression (see Figure 8 compared to Figure 6B) as a consequence of serum deprivation performed in these set of experiments, further confirming the requirement of KGFR activation and signaling in this process.

Since it has been demonstrated that PLCγ activation controls keratinocyte differentiation through PI(4,5)P2 hydrolysis and generation of the second messengers IP3 and DAG (Xie and Bikle, 1999; Xie et al., 2009), we wondered if KGFR-mediated activation of PLCγ might be involved in the induction of HaCaT cell early differentiation. To address this point, cells were transiently transfected alternatively with KGFR WT or with a KGFR signaling mutant in which the tyrosine 769, required for PLCγ binding and activation (Ceridono et al., 2005; Cha et al., 2009) has been substituted by phenylalanine (Y769F) (Ceridono et al., 2005). In addition, cells were transiently transfected with a Y656F/Y657F KGFR kinase dead mutant (Belleudi et al., 2006) as a control. After transfection, cells were treated with TG or kept in DMSO alone as above. Quantitative immunofluorescence analysis using anti-Bek and anti-K1 antibodies showed that, similarly to KGFR WT, KGFR Y769F overexpression was able to significantly increase the percentage of cells expressing K1 either upon treatment with TG or in the presence of DMSO alone (Figure 9A). In contrast, the overexpression of the

kinase negative mutant KGFR Y656F/Y657F did not affect the early differentiation, confirming the role of the receptor signaling (Figure 9A). Quantitative real-time RT-PCR performed in HaCaT cells transiently transfected with KGFR WT or KGFR mutants, and then treated with TG and KGF as above, showed a significant ligand-dependent increase in K1 mRNA expression in HaCaT KGFR WT and HaCaT KGFR Y769F, but not in HaCaT KGFR Y656F/Y657F cells (Figure 9B, right panel). Moreover, we observed a ligand-dependent decrease in KGFR mRNA expression in both HaCaT KGFR WT and HaCaT KGFR Y769F, but not in HaCaT KGFR Y656F/Y657F, due to the receptor internalization and consequent down-modulation induced by KGF (Figure 9B, left panel). These results indicate that, although the KGFR kinase activity is required for the keratinocyte differentiation, recruitment and consequent activation of the receptor substrate PLCγ do not appear to play a role in this process.

It has been previously reported that the PI3K/Akt signaling is important in the regulation of keratinocyte differentiation (Calautti et al., 2005) and it might provide an essential survival signal required for keratinocyte stratification and differentiation (Thrash et al., 2006). In addition, it has been very recently demonstrated a possible role of the PI3K/Akt pathway in the KGFR-mediated differentiation process of pancreatic ductal cells to beta cells (Uzan et al., 2008). To clarify if the PI3K/Akt could be the main differentiative pathway induced by KGFR expression and signaling, first HaCaT cells were transfected with KGFR and then stimulated with KGF (100 ng/ml for 10' at 37°C) to induce receptor activation as previously reported (Belleudi et al., 2006; Belleudi et al., 2007) and the amount of Akt phosphorylation was assessed by Western blot analysis using anti-pAkt antibody. As shown in Figure 10A, in HaCaT control cells, Akt protein phosphorylation was evident following KGF treatment and was specifically blocked by the Akt inhibitor. In KGFR transfected cells, the Akt phosphorylation appeared more intense and clearly detectable also in serum-free untreated cells, possibly as a result of the short time starvation performed in these experiments. In fact, trans-phosphorylation of overexpressed KGFRs can be excluded, based on previous reported evidences (Belleudi et al., 2006; Bernard-Pierrot et al., 2004).

To demonstrate if the PI3K/Akt pathway activated by KGFR could be responsible for the induction of the early differentiative program, HaCaT KGFR cells were serum starved and treated for the last 24h at 37°C with KGF as above to induce K1 expression in presence or not of the specific Akt inhibitor. Quantitative immunofluorescence analysis showed that the Akt inhibitor drastically blocked the increase of K1 positive cells observed upon KGFR overexpression and KGF stimulation (Figure 10B). Moreover, the Akt inhibitor was able to significantly decrease the KGF-mediated differentiation also in cells expressing endogenous levels of the receptor (Figure 9B). Thus, these results indicate that the early differentiation induced by KGFR expression occurs through the Akt signalling pathway.

KGFR ligands promote phagocytosis in differentiated keratinocytes.

In previous studies from our group, we have demonstrated that KGF is able to promote the phagocytic process in human keratinocytes through activation of its receptor KGFR (Cardinali et al., 2005; Cardinali et al., 2008). Here, to analyze the KGF effect on cells expressing different levels of the receptor during differentiation, we used the human keratinocyte HaCaT cell line grown at different densities, which are known to induce up-modulation of KGFR following cell confluence (Capone et al., 2000). Cells were then incubated with inert latex fluorescent (red) beads 0.5 μm in diameter, widely used to study the phagocytic capacity of epidermal keratinocytes (Wolff et al., 1972; Virador et al., 2002; Desjardins and Griffiths, 2003). To induce phagocytosis, HaCaT cells were serum starved for 12h and incubated with the beads for 4h at 37°C in presence of KGF (20 ng/ml) or in absence of the growth factor. The experimental procedure was comparable to that performed in our previous studies (Cardinali et al., 2005; Cardinali et al., 2008) and the KGF or beads concentrations as well as the times of treatment were selected based on our published results. Nuclei were stained with DAPI. The immunofluorescence analysis showed that the internalized beads were located in the perinuclear area of the keratinocytes and that, as expected (Cardinali et al., 2005; Cardinali et al., 2008), the KGF treatment (20 ng/ml)

strongly increased the uptake of the beads (Fig 11A). Interestingly, while in cell cultures treated with the beads in absence of the growth factor the uptake was visible only in cells located at the periphery of the islets (Figure 11A, arrows), the addition of KGF triggered phagocytosis in the confluent differentiating cells at the center of the colonies (Figure 11A, arrowheads), which are known to display an augmented response to the proliferative and differentiative effects of KGF as a consequence of the receptor up-modulation (Capone et al., 2000). The differentiating phenotype of the confluent central keratinocytes was demonstrating by the positivity for the early differentiation marker K1 (Figure 11A). The quantitative analysis of the number of intracellular fluorescent beads, performed as described in Materials and Methods, showed that KGF treatment significantly increased ($p<0,001$) the beads uptake only in differentiated cells, but not in the undifferentiated keratinocytes (Fig 11D).

Both KGF and FGF10 bind and activate specifically the KGFR. However, FGF10 appears less motogenic than KGF in migration assays of human keratinocytes and it exerts an effect on the reorganization of the cortical actin which is delayed compared to KGF (Ceccarelli et al., 2007). Therefore, we wondered if FGF10 could act differently from KGF in inducing phagocytosis. The phagocytosis assay performed above was then applied to cells treated with FGF10, at the concentration of 20 ng/ml and in the presence of heparin (0,3 μg/ml) as required to induce KGFR up-modulation and actin cytoskeleton reorganization (Ceccarelli et al., 2007). Quantitative immunofluorescence analysis showed that, similar to KGF, FGF10 was able to increase the beads uptake compared to control, although less effective than KGF (Figure 11B, D), and this effect was evident again not only in peripherally (Figure 11B, arrows) but also in centrally (Figure 11B, arrowheads) located cells inside the colonies.

To analyze the possible functional role of the paracrine KGFR ligands, secreted by the dermal fibroblasts in the skin microenviroment, in inducing phagocytosis on the epidermal keratinocytes, HaCaT cells were treated with a culture supernatant (SN) collected from primary skin derived human fibroblasts (HFs) and we performed the phagocytic assay as above. The fibroblast supernatant was also added to the cells in the presence of the specific FGFR

tyrosine kinase inhibitor SU5402. The amount of the internalized fluorescent beads was increased by incubation with the supernatant alone, but strongly reduced by the addition of the KGFR inhibitor (Fig 11B, D), indicating that the phagocytic effect of the SN was dependent on specific KGFR ligands released in the fibroblast culture medium.

To ascertain if the promoting action of KGF on the differentiated cells could represent a general phenomenon occurring in skin, a parallel phagocytic test was performed on primary cultures of human keratinocytes (HKs) induced to differentiate in response to increased Ca^{++} concentration (1.5 mM) in the medium. The beads internalization was much higher when the keratinocytes were induced to differentiate before treatment with the growth factor (Figure 11C, D), further suggesting the role of KGFR expression in phagocytosis.

KGFR/FGFR2b expression regulates the phagocytic process.

To unequivocally demonstrate the specific direct function of KGFR/FGFR2b in regulating the phagocytic process, we analyzed the effect of the receptor depletion on the uptake of the fluorescent beads. To this aim, we performed co-injection of small interfering RNA (siRNA) for FGFR2/Bek to obtain KGFR silencing and rabbit IgG to identify the microinjected cells. Microinjection with an unrelated siRNA was performed as a control. After injection, cells were serum starved for 12h and incubated with the beads and KGF at 37°C for 4h as above. The quantitative immunofluorescence analysis showed a decreased number of internalized beads in the IgG-positive cells injected with Bek siRNA, compared either to the uninjected cells surrounding them in the same microscopic fields or to cells injected with control siRNA (Fig 12A, C). The efficiency of KGFR depletion was evaluated by transfecting the HaCaT cells with Bek siRNA or with a control siRNA, followed by Western blot analysis using anti-Bek polyclonal antibodies: as shown in Figure 12B, KGFR protein expression appeared down-regulated in siRNA-transfected cells. The equal loading was assessed with anti-actin antibody. Thus, KGFR silencing is able to inhibit the beads uptake induced by KGF.

We have previously demonstrated that KGF induces KGFR internalization by clathrin-mediated endocytosis (Marchese et al., 1998; Belleudi et al., 2006) and receptor trafficking toward the degradative endocytic pathway (Belleudi et al., 2007). Since we have also reported that KGFR colocalized with the marker dextran in the intracellular phagosomal compartment (Cardinali et al., 2008), we analyzed here the extent of colocalization of the receptor with the internalized beads in the perinuclear phagocytic structures. To follow the receptor in the phagocytic compartment, HaCaT keratinocytes were transiently trasfected with KGFR WT, serum starved for 12h and treated 1h in vivo at 4°C with an anti-Bek polyclonal antibodies, directed against the extracellular portion of KGFR, before the incubation with the beads for 4h at 37°C as above. The colocalization of the KGFR (green) and beads (red) signals was assessed on series of 0.5 μm sequential optical sections obtained with a microscope scanning system as described in Materials and Methods. In KGF-untreated cells, the KGFR signal remained mainly associated to the cell plasma membrane and no colocalization was observed with the perinuclear internalized beads (Figure 13A, left panel). In contrast, frequent colocalization of the phagocytosed beads with the internalized receptors (yellow dots in Figure 13A, right panels) was observed by the addition of KGF. The small yellow dots in proximity or surrounding the beads were suggestive of possible fusion events of the phagosomes containing the beads with late-endosomes or lysosomes containing the internalized receptors (Figure 13A, arrows).

To analyze in detail early and late steps of the KGF-induced uptake and the intracellular transport of the fluorescent beads as well as to evaluate the extent of colocalization with the receptor during phagocytosis, we induced a synchronous wave of KGFR and beads internalization. To this purpose, HaCaT KGFR WT transfected cells were serum starved as above, incubated with the anti-KGFR Ab, KGF and the fluorescent beads for 1h at 4°C followed by warming to 37°C for 30′ or 2h before fixation. Although less frequent respect to the 4h incubation, colocalization of the two signals was visible also in some peripheral structures beneath the cell plasma membranes (Figure 13B, left panels, circles) and in early endophagosomes (Figure 13B, left panels, arrows) after 30′ of warming, when the endocytosed receptor is known to reach the MVBs/late endosomes (Belleudi et al.,

2007). In contrast, after 2h of warming, colocalization was clearly evident in late perinuclear structures probably corresponding to phagolysosomes (Figure 13B, right panels, arrows). Quantification of the extent of colocalization, performed as described in Materials and Methods showed that the increase in the percentage of the colocalization of the beads with the KGFR signal was significant only at the later step (after 2h of warming) and not significant at the earlier time of internalization (after 30′ of warming), suggesting that KGF treatment does not induce receptor concentration either in the ingestion cup responsible for the uptake of the beads or in structures involved in their transport to early endophagosomes.

KGF-induced phagocytosis is dependent on KGFR kinase activity and on PLCγ binding and signaling.

Our previous published results (Cardinali et al., 2005; Cardinali et al., 2008) and the present above observations indicated that KGFR expression and activation are able to enhance phagocytosis. Since KGFR signaling is involved in cortical actin assembly through the tyrosine phosphorylation of the actin-binding protein cortactin (Ceccarelli et al., 2007), we wondered if an abolished receptor kinase activity would influence the phagocytic process. To elucidate this point, HaCaT cells were transiently transfected alternatively with KGFR WT or with a KGFR kinase negative mutant Y656F/Y657F (Belleudi et al., 2006) and analyzed by the phagocytic assay (4h at 37°C) described above. However, differently from the experiments shown in figure 13, the immunofluorescence KGFR staining was performed after beads uptake and fixation and permeabilization, in order to visualize both surface and intracellular receptors. Quantitative analysis of the number of beads internalized in response to KGF revealed an higher amount in cells overexpressing KGFR WT ($p<0,05$ compared to untransfected cells) (Figure 14 A, B), either located at the periphery (Figure 14A, arrow) or in the central part (Figure 14A, arrowhead) of the colony, further indicating that KGFR expression plays a key role in the process. The KGF promoting effect was totally abolished by expression of the kinase negative mutant KGFR Y656F/Y657F (Figure 14A, B),

demonstrating that receptor kinase activity and signal transduction are required for the KGFR-mediated phagocytosis.

Among the molecules which regulate the actin cytoskeleton and the phagocytic process, phospholipases recruited and activated by receptor tyrosine kinases as well as plasma membrane phospholipids modified by the receptor signal transduction are known to contribute significantly to the particle ingestion (for a review see Swanson et al., 2008). In particular, it has been proposed that the presence of PIP2 and active PLCγ at the phagocytic cup is essential for phagocytosis (Botelho et al., 2000). To assess the possible role of PLCγ activation induced by KGFR signaling on the regulation of the beads uptake, HaCaT cells were transiently transfected with a mutant KGFR in which the tyrosine 769 residue, required for PLCγ binding and activation (Ceridono et al., 2005; Cha et al., 2009), has been substituted by phenylalanine (Y769F) (Ceridono et al., 2005). The beads phagocytic assay performed as above revealed that, although the receptor was internalized by KGF-dependent endocytosis as expected (Belleudi et al., 2006; Ceridono et al., 2005), the growth factor failed to promote the beads uptake (Figure 14A, B), suggesting a crucial role of tyrosine 769 and PLCγ in the KGF-induced ingestion.

KGF-induced phagocytosis is independent on KGFR endocytosis

To rule out the possible effect of KGFR endocytosis and traffic in controlling the phagocytic process, the receptor internalization were blocked by siRNA interference to selectively inhibit the clathrin-mediated pathway through silencing of clathrin heavy chain (CHC): in fact, in a previous paper from our group we have shown that this approach is able to block KGFR endocytosis (Belleudi et al., 2007). The efficiency of CHC depletion was assessed by co-injection of CHC siRNA and rabbit IgG to identify the microinjected cells. Microinjection with an unrelated siRNA was performed as a control. The immunofluorescence analysis with anti-clathrin antobodies revealed that the punctate signal corresponding to clathrin-positive structures was drastically reduced in cells injected with CHC siRNA compared to the surrounding

uninjected cells (Figure 15A) or to cells injected with the control siRNA (not shown). Then, to confirm the effect of CHC depletion on the receptor endocytosis and to evaluate in parallel the beads uptake, we performed co-injection of KGFR cDNA and CHC siRNA to simultaneously obtain KGFR overexpression and CHC depletion. Co-injection of KGFR cDNA with an unrelated siRNA was performed as a control. After injection, cells were serum starved for 12h and incubated with the anti-KGFR Ab, KGF and the fluorescent beads for 1h at 4°C followed by warming to 37°C for 2h before fixation as above. The immunofluorescence analysis showed that the receptor endocytosis induced by KGF treatment was blocked in clathrin-depleted cells, while KGFR internalization appeared unaffected in cells injected with the control siRNA (Figure 15B). Despite the drastic effect on the receptor endocytosis, the phagocytic uptake of the beads in response to KGF was not reduced by clathrin-depletion (Figure 15B). The quantitative analysis of the number of beads internalized in response to KGF (Figure 15C) indicates that the phagocytosis of the beads in response to KGFR activation occurs independently on endocytosis and suggests that the receptor signaling triggers the phagocytic process from the plasma membrane.

DISCUSSION

The possible key role of FGFR2b/KGFR in controlling cell differentiation stems mostly on the fact that this receptor is down-modulated in several epithelial tumors (for a recent review see Haugsten et al., 2010) and that its re-expression alone is able to induce cell differentiation (Feng et al., 1997; Zhang et al., 2001; Yasumoto et al., 2004). However, only a few reports have suggested that the expression of the receptor might be crucial in the normal cell physiology of the epithelial differentiation (Petiot et al., 2003; Grose et al., 2007; Yang et al., 2010) although this possibility is sustained by evidences that the expression of other FGFRs, such as FGFR1 and FGFR3 (Yang et al., 2008; Maher et al., 1999; Dailey et al., 2005) or other receptor tyrosine kinases, such as TrkA (Raucci et al., 2004), can be directly involved in the regulation of cell growth arrest and differentiation. With the aim to elucidate the role of KGFR in the differentiative process, we used a controlled system of human keratinocytes led to express K1 and to change their morphology in a synchronized manner through the treatment with thapsigargin. In addition, in our model system the treatment with thapsigargin was also able to generate a homogeneous population of differentiating cells expressing increasing amounts of KGFR, allowing us to analyze the effects of a synchronized enhancement in the expression levels of endogenous receptor.

Among the well known different stages which characterized the sequential differentiation of the epidermal keratinocytes, we focused our attention on the earlier differentiation step, because it has been previously demonstrated by our group and by others that KGFR and K1 expression are increased during the transition from basal to suprabasal cells (Marchese et al., 1997; Capone et al., 2000; La Rochelle et al., 1995) and that this step is critical for the response to the ligand KGF (Hines et al., 1996). We modulated here KGFR expression by cDNA transient transfection or by siRNA microinjection in the HaCaT human keratinocyte cell line, which are immortalized and warrant a constant and reproducible behaviour in vitro, and we evaluated its effects on early differentiation monitoring K1 expression by immunofluorescence and molecular approaches. In addition, through the rapid increase of intracellular Ca^{2+} released in response to thapsigargin, as well as through the increase in extracellular Ca^{2+} by its addition to the medium, we could compare on pre-confluent cells the effects of KGFR modulation in undifferentiated versus differentiating keratinocytes. Our results

demonstrated that the forced expression of KGFR alone is able to increase K1 expression at both transcriptional and translational levels, which suggests that the receptor triggers and controls early differentiation in undifferentiated as well as in differentiating keratinocytes, in agreement with the previous reports on transformed epithelial cells (Feng et al., 1997; Zhang et al., 2001; Yasumoto et al., 2004). Moreover, differently from what reported by Petiot et al. (Petiot et al., 2003) Grose et al. Grose et al., 2007) and Yang et al. (Yang et al., 2010) in KGFR KO mouse models, we found that the receptor depletion by KGFR siRNA microinjection reduced K1 expression. This contrasting result may be explained considering that the "in vivo" and "in vitro" models represent quite different contexts. In fact, growth factors and cytokines, such as those released by an inflammatory or wound healing microenvironment, may act simultaneously and may interfere with the activity of KGFR, possibly hiding its single contribution. In agreement with these hypothesis, Yang et al. (Yang et al., 2010) have very recently demonstrated that the hyperproliferative effect of FGFR1b and FGFR2b/KGFR depletion observed in the basal layer of the KO mice skin was not found in cultured keratinocytes isolated from these mice tissues, since hyperproliferation could be an indirect effect of the inflammation derived from the reduced epidermal barrier function. Thus, we believed that our "in vitro" experimental model is suitable to demonstrate that the modulation of KGFR expression is a specific key event regulating K1 expression and consequently the keratinocyte early differentiation during the switching from undifferentiated to differentiating cells.

To further elucidate the receptor role, we wondered whether the differentiative role of KGFR implies receptor activation and signaling. Since it has been shown that FGFR2b/KGFR overexpression in epithelial cells does not induce receptor trans-phosphorylation (Belleudi et al., 2006; Bernard-Pierrot et al., 2004), the results obtained here by serum-starving HaCaT KGFR cells and then treating them with KGF have unequivocally demonstrated that ligand-dependent KGFR activation is required for the receptor function in the induction of early differentiation. These results are also in agreement with those obtained by Zhang et al. (Zhang et al., 2001) and Feng et al. (Feng et al., 1997) in nude mice tumors derived from implants of epithelial tumor cells, in which the down modulated

KGFR was exogenously re-expressed leading to slow down the tumor growth and to enhance cellular differentiation: in fact, the importance of stroma cells, producing KGF, in the induction of epithelial tumor cell differentiation was clearly underlined in these papers. Moreover, comparing the effects of the overexpression of the receptor kinase dead mutant KGFR Y656F/Y657F with those obtained overexpressing KGFR WT, it was further demonstrated that the intrinsic receptor tyrosine kinase activity is required for the differentiative function.

Since our present results pointed out the importance of the ligand-dependent KGFR signaling in the control of keratinocyte differentiation, we then focused our attention on the possible signaling pathway that might be involved in the induction of KGFR-mediated differentiation. Current knowledge points to the MAPK pathway as the downstream FGFR signaling involved in the differentiative outcome, suggesting that differentiation might be induced by a FGFR-mediated sustained MAPK signaling (Turner and Grose, 2010; Marshall 1995; Gotoh, 2008). Because it has been also proposed that the PLCγ signaling pathway is required for the FGFR-mediated sustained activation of the MAPKs (Yang et al., 2008) and that this substrate is able to trigger the calcium-induced differentiation response in human keratinocytes (Turner and Grose, 2010; Xie et al., 2009), in this work we investigated if PLCγ signaling could be the main KGFR-mediated differentiative pathway in keratinocytes. To this aim, we overexpressed a KGFR Y769F signaling mutant, which is not able to recruit and activate PLCγ (Ceridono et al., 2005) and we found that this mutant receptor acts similarly to the WT receptor in triggering the expression of K1, implying that this signaling pathway is not involved in the KGFR-induced early differentiation.

Because it has been reported that the Akt phosphorylation plays an important role in keratinocyte differentiation (Calautti et al., 2005) and that the PI3K/Akt signaling might induce a survival outcome crucial for keratinocyte stratification and differentiation (Thrash et al., 2006), it was reasonable to investigate if the KGFR-mediated activation of PI3K/Akt pathway could be required for the induction of keratinocyte differentiation. The results obtained in this work showed that the KGF-dependent Akt phosphorylation/activation, which is inhibited by the specific Akt inhibitor, was more intense in cells overexpressing KGFR; moreover, the Akt inhibitor significantly reduced the early differentiation induced

by KGF in both non transfected HaCaT cells and in HaCaT KGFR transfected cells, suggesting that this pathway is involved in the KGFR-mediated keratinocyte differentiation. These results are in agreement with those obtained by Uzan et al. (Uzan et al., 2008) showing that the KGF-mediated differentiation of pancreatic cells is controlled by the PI3K/Akt signaling pathway. Similarly, the possible involvement of Akt signaling in the cell growth arrest and differentiation induced by other members of the FGFR family has been proposed (Arnaud-Dabernat et al., 2008), even if the topic is still debated (Raucci et al., 2004; Priore et al., 2006).

In FGFRs, the PI3K/Akt signaling starts from the phosphorylation of the docking adaptor platform FRS2□ (for a recent review, see Gotoh, 2008), in which six tyrosine residues are critical in determining the cellular context-specific signaling outcome. In a previous study from our group, we have observed that, during keratinocyte differentiation, the expression of FRS2□ and Akt, as well as the Akt phosphorylation, was increased parallel to that of KGFR and K1, (Lotti et al., 2007), already suggesting that this pathway would be more active in differentiated cells. Thus, we can conclude that KGFR plays a key role in the regulation of early stages of the keratinocyte differentiation program and that the up-modulation of this receptor in differentiating suprabasal keratinocytes induces the K1 expression probably through a profile-specific phosphorylation of FRS2□.

In the second part of this work I analyzed the role of KGFR-mediated PLCγ pathway in the induction of phagocytic process

Assembly and remodeling of the cortical actin cytoskeleton represents the driving force for phagocytosis and cell movement. The regulatory molecular mechanisms involved in actin reorganization are only partially clarified, but the central roles of small GTPases of the Ras superfamily, such as Rho, Rac and Cdc42, have been fully established (Hall, 1994; Insall and Macheskyet, 2009). In accordance with this key point, we have previously demonstrated that KGF treatment of cultured keratinocytes triggers the formation of membrane protrusions surrounding the beads to be ingested and we have shown, by the use of specific inhibitors of the pathways mediated by activation of Rho or Rac/Cdc42, that this KGF-induced phagocytic process is dependent on both mechanisms (Cardinali et al., 2005).

Among the molecular mediators more recently included in the list, the actin-binding protein cortactin has been proposed as a key regulator of actin assembly (Olazabal and Machesky, 2001; Weed and Parsons, 2001). Its crucial role in the modeling of the cortical actin network leading to the extension of membrane protrusions such as lamellipodia and ruffles has been demonstrated in response to several growth factors and during cell motility (Weed and Parsons, 2001). Interestingly, it has been also suggested that tyrosine phosphorylation and recruitment of cortactin at the site of bacteria entry could be responsible for the pathogenic cell invasion (Dehio et al., 1995, Lambotin et al., 2005). Both KGF and FGF10 induce migration of keratinocytes through stimulation of actin assembly in lamellipodia and ruffles: expression and Src-dependent tyrosine phosphorylation of cortactin as well as its recruitment to the plasma membrane are required for such motility, although FGF10 appears less efficient than KGF in exerting this effect (Ceccarelli et al., 2007). We have proposed that the functional differences might be ascribed to the reduced, more transient and delayed phosphorylation of cortactin induced by FGF10 compared to KGF, possibly as a consequence of the opposite behaviour in heparin requirement for the binding of the two growth factors to their common receptor. These findings have suggested that the composition of HSPGs on the extracellular matrix might differently modulate the biological responses to KGF or FGF10 (Ceccarelli et al., 2007). Similarly, in the present study it was observed a less efficient effect of FGF10 compared to KGF in the phagocytic assay, which might indicate that the extracellular matrix concentration in the skin microenvironment can affect also the melanosome uptake by the keratinocytes.

Using our model system, which permits to dissect the contribution of the recipient keratinocytes, we found that treatment with either KGF or FGF10 was able to trigger both phagocytosis and melanosome entry in differentiated keratinocytes, which are known to express an increased amount of receptors (Capone et al., 2000), suggesting that KGFR activation could be a crucial event for the melanosome transfer from melanocytes located on the epidermal basal layer to suprabasal keratinocytes of the spinous layer. Since these K1-positive keratinocytes are also more exposed to damages by UVB radiation, our proposed

scenario is also consistent with the fact that the exposure to UVB, which are well recognized stimulators of melanosome transfer (Imokawa et al., 2004), are able to trigger ligand-independent activation of KGFR (Belleudi et al., 2006).

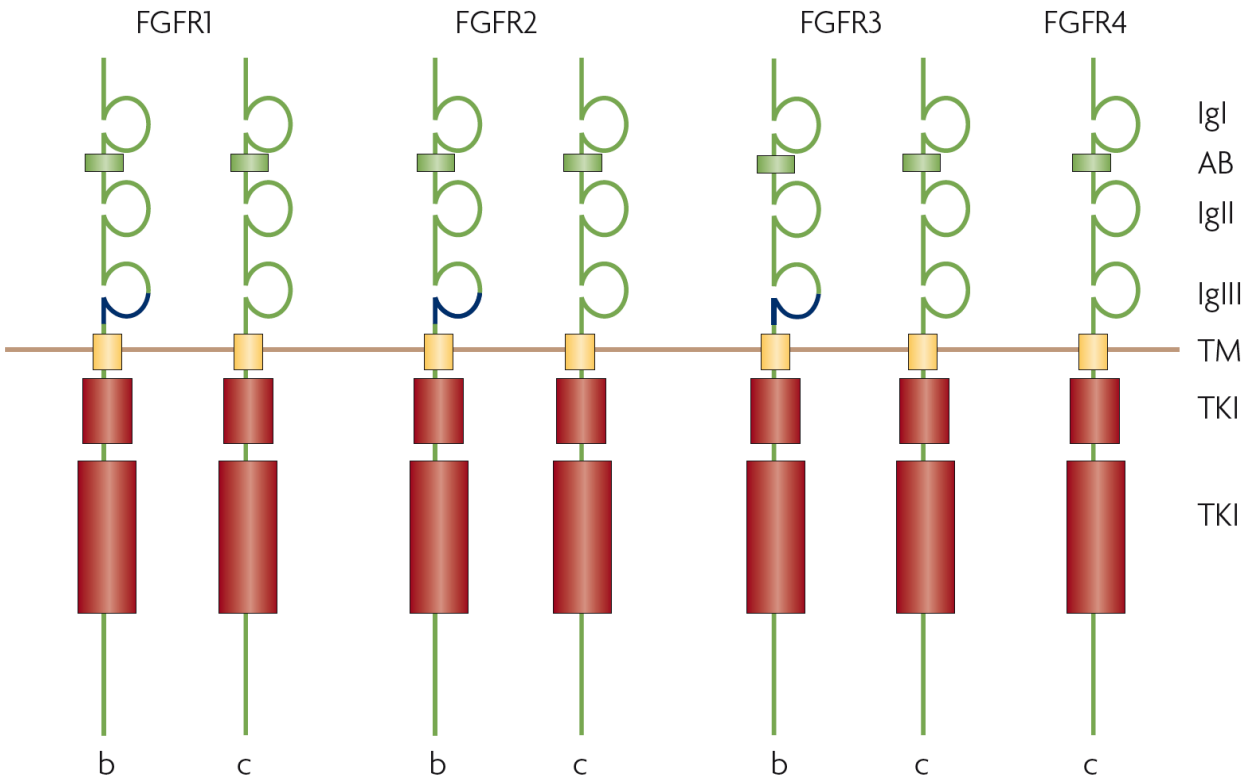
KGFR depletion or overexpression of wild type or defective mutants in human cultured keratinocytes demonstrates the direct promoting effect exerted by the activated receptor on the phagocytic ingestion of inert latex beads. In addition, the role of the receptor and of its signaling on the regulation of melanosome transfer was highlighted using co-cultures models of keratinocytes and melanocytes and through receptor depletion or overexpression as above, providing novel findings on the molecular mechanisms controlling the complex process leading to skin pigmentation.

The actin cytoskeleton and phagocytic process are also regulated by phospholipases recruited and activated by receptor tyrosine kinases as well as by plasma membrane phospholipids modified by the receptor signal transduction (Swanson, 2008). In particular, it has been reported that the spatial concentration of PIP2 and active PLCγ at the phagocytic cup is essential for phagocytosis (Botelho et al., 2000). These present results indicate the crucial role of the KGFR tyrosine 769 residue responsible for the activation of PLCγ signaling pathway in the receptor-mediated mechanism of uptake. Since the most recent models of molecular regulation of the phagocytic ingestion propose that lipids generated by receptor activation might regulate proteins spatially distant from the ligand-receptor complexes (Swanson, 2008), It is possible speculate that KGFR would participate to the phagosome cup formation and closure either as part of the membrane domain involved or acting at distance through diffusion of signals. However, the colocalization of internalized KGFR with the phagocytosed beads observed in our experiments mostly at late steps of the process, which demonstrate the presence of receptor molecules in intracellular phagosomes, appear to imply fusion of KGFR-carrying endosomes with late phagocytic structures more than involvement of activated receptors in the ingestion cup from the beginning. In accordance with this observation, the inhibition by RNA interference of the clathrin-mediated receptor endocytosis does not affect KGF activity in promoting the phagocytosis, unequivocally demonstrating the key role

of receptor signaling and activation of the PLCγ downstream pathway from the plasma membrane.

In conclusion I propose that phosphorylation of the Y769 site, followed by activation and recruitment to the receptor of PLCγ, controls diacylglycerol (DAG) formation by PIP_2 hydrolysis and then cortical actin reorganization and phagocytosis (Figure 16A). Point mutation of Tyr769 to phenylalanine (Y769F) impairs PLCγ activation and phagocytosis, but not receptor endocytosis, as expected (Ceridono et al., 2005; Cha et al., 2009) (Figure 16B). Block of the receptor kinase activity and signaling by double tyrosine mutation (Y656F/Y657F) inhibits both phagocytosis and receptor endocytosis (Figure 16C). Block of the receptor clathrin-mediated endocytosis by RNA interference does not impair phagocytosis (Figure 16D).

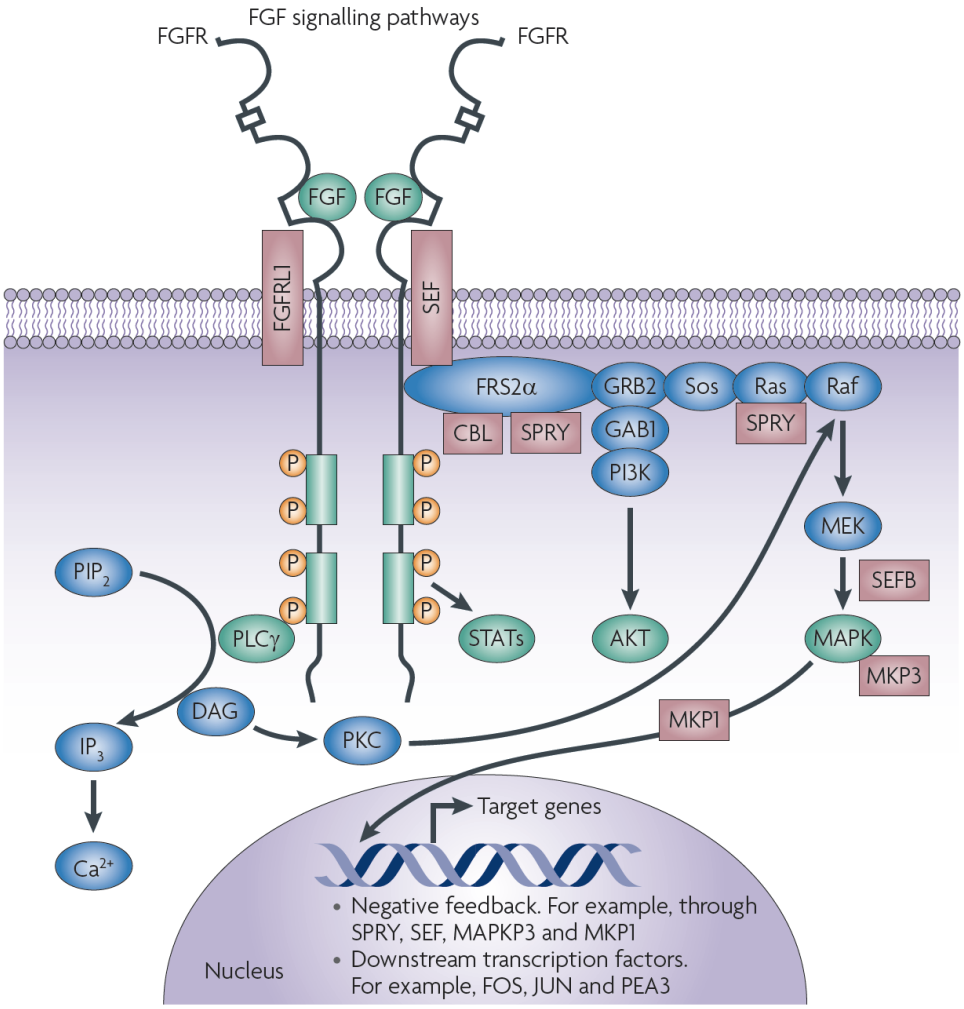
FIGURES



(Mason, 2007)

Fig.1

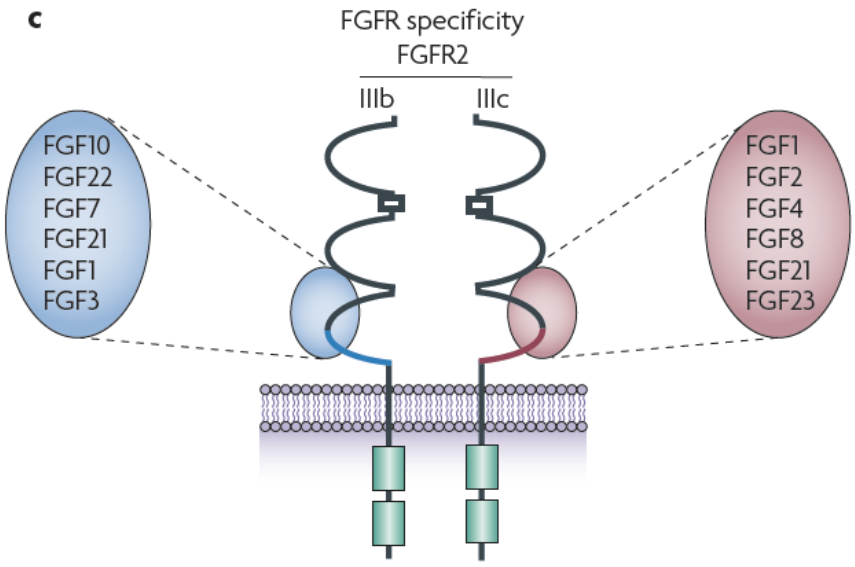
Figure 1. The 3-immunoglobulin (Ig) isoforms of the four vertebrate FGFRs. The extracellular domain contains the three Ig loops (IgI, IgII and IgIII), and the acid box (AB), which is located between IgI and IgII. The AB is removed in certain splice variants. The alternatively spliced sequences in IgIII, which distinguish the 'b' and 'c' isoforms of FGFR1–3 are coloured blue for the 'b' variants. There is a transmembrane domain (TM), and intracellular sequences include a split tyrosine kinase enzyme domain (TKI and TKII). The binding site for FGF ligands comprises the C-terminal part of IgII and the N-terminal portion of IgIII, whereas the binding site for CAM ligands includes the acid box. CAM, cell adhesion molecule.



(Turner and Grose, 2010)

Fig.2

Figure 2. FGFR signalling network. The signal transduction network downstream of fibroblast growth factor (FGF) receptors (FGFRs), along with negative regulators. Following ligand binding and receptor dimerization, the kinase domains transphosphorylate each other, leading to the docking of adaptor proteins and the activation of four key downstream pathways: RAS–RAF–MAPK, PI3K–AKT, signal transducer and activator of transcription (STAT) and phospholipase Cγ (PLCγ) (green). Signalling can be negatively regulated at several levels by receptor internalization or the induction of negative regulators, including FGFR-like 1 (FGFRL1), SEF, Sprouty (SPRY), CBL, MAPK phosphatase 1 (MKP1) and MKP3 (brown). These regulators may modulate ligand binding (FGFRL1 and SEF) or interfere with intracellular signalling, principally through modulation of the MAPK pathway. DAG, diacylglycerol; FRS2α, FGFR substrate 2α; GRB2, growth factor receptor-bound 2; IP3, inositol triphosphate; P, phosphorylation; PIP2, phosphatidylinositol-4,5-biphosphate; PKC, protein kinase C; Sos, son of sevenless.



(Turner and Grose, 2010)

Fig.3

Figure 3. FGFR2IIIb and FGFR2IIIb isoforms structure. Examples of the extent to which ligand specificity can differ between FGFRIIIb and FGFRIIIc isoforms, illustrated with the differing ligand specificty of FGFR2 isoforms. The FGFR2IIIb ligands are shown in blue and the FGFR2-IIIc ligands are shown in brown. For example, FGF7 and FGF10 bind specifically to FGFR2-IIIb and have essentially no binding to FGFR2IIIc.

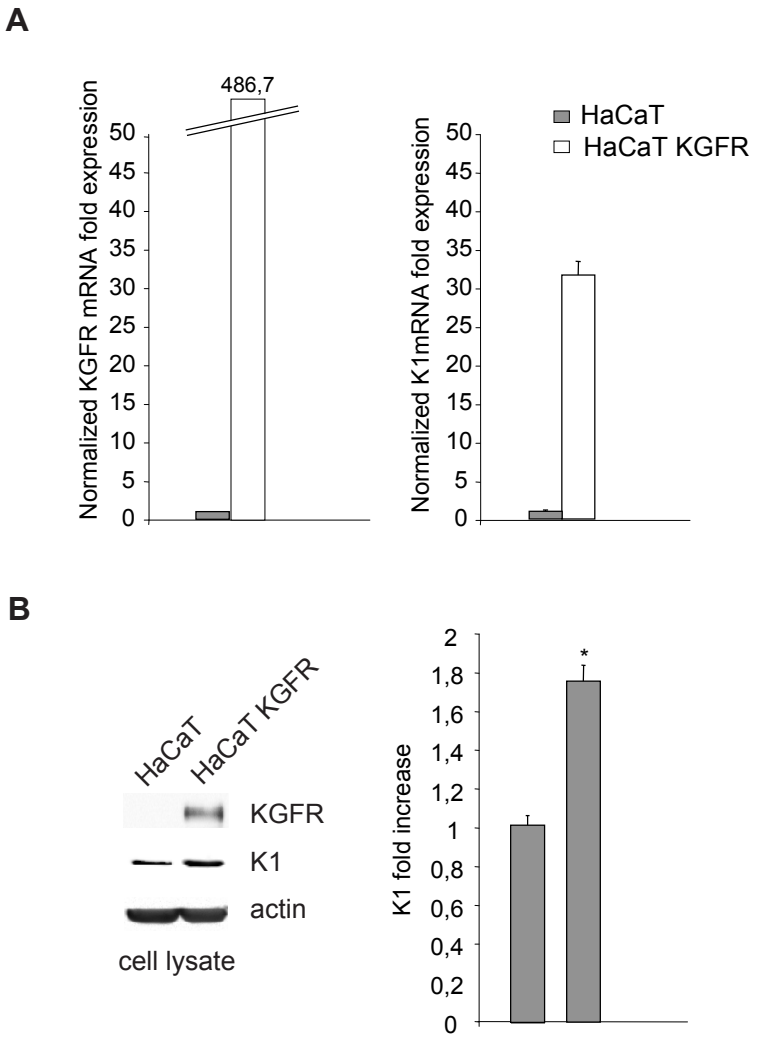

A
486,7
Normalized KGFR mRNA fold expression
50
45
40
35
30
25
20
15
10
5
0
HaCaT
HaCaT KGFR
Normalized K1mRNA fold expression
B
HaCaT
HaCaT KGFR
KGFR
K1
actin
cell lysate
K1 fold increase
2
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
*

Fig.4

Figure 4. KGFR expression triggers the early differentiation in undifferentiated keratinocytes. **(**A) HaCaT cells were transfected with the pCI-neo expression vector containing human KGFR cDNA (HaCaT KGFR). After 24h from transfection, the KGFR and K1 mRNA transcript levels were quantitated by real-time RT-PCR: a clear fold increase in both K1 mRNA (right panel) and KGFR mRNA (left panel) expression is observed in HaCaT KGFR cells compared to control ones. **(**B) Western blot analysis on HaCaT KGFR and HaCaT cells using anti-K1 monoclonal antibody and with anti-Bek polyclonal antibodies, which recognize the KGFR/FGFR2b protein, shows that the band of K1 is increased by KGFR overexpression in HaCaT KGFR compared to control cells. The specific band corresponding to KGFR protein is well visible only upon KGFR transfection. The equal loading was assessed with anti-actin antibody. For densitometric analysis of the band corresponding to K1 protein the values from three independent experiments were normalized, expressed as fold increase and reported in graph as mean values +/- standard deviation (SD).Student' t test was performed and significance level has been defined as *p< 0.005 vs the corresponding untransfected cells.

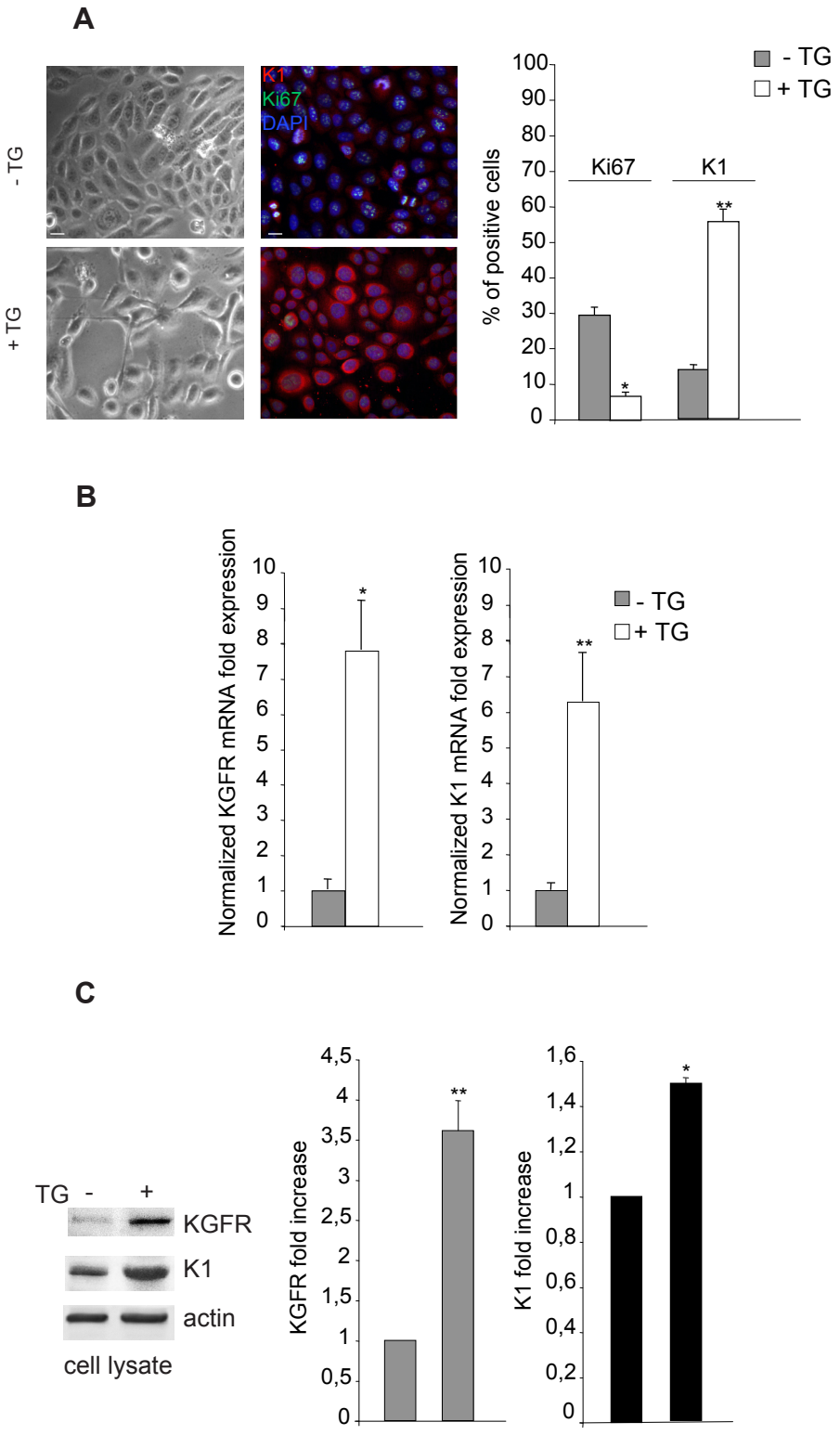

A
- TG
+ TG
K1
Ki67
DAPI
100
90
80
70
60
50
40
30
20
10
0
% of positive cells
Ki67
K1
- TG
+ TG
*
**
B
Normalized KGFR mRNA fold expression
Normalized K1 mRNA fold expression
10
9
8
7
6
5
4
3
2
1
0
*
**
- TG
+ TG
C
TG - +
KGFR
K1
actin
cell lysate
KGFR fold increase
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
**
K1 fold increase
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
*

Fig.5

Figure 5. Thapsigargin-induced differentiation is associated with increased KGFR expression. **(**A) HaCaT cells were treated with TG 0.5 μM for 1h at 37°C following by incubation at 37°C for 48h. Cells treated with equal amount of DMSO solvent were used as a control. Phase contrast microscopy analysis shows that the TG-treated cells appear detached each other and elongated, while control cells are closely packed and polygonal (left panels). Quantitative immunofluorescence analysis using anti-Ki67 and anti-K1 antibodies shows that TG treatment significantly decreases the percentage of cells expressing the proliferative marker Ki67 and increases that of cells positive for the early differentiation marker (right panels). Cell nuclei were visualized by DAPI. The quantitative analysis was assessed by counting for each sample a total of 50 cells, randomly observed in 10 microscopic fields from three different experiments. Cut-off of the K1 signal intensity was determined for TG-treated and control samples as described in materials and methods. Results are expressed as mean values ± standard errors (SE). Student's t test was performed and significance level has been defined as *p< 0.001 and **p< 0.05 vs the corresponding untreated cells. **(**B) Quantitative real-time RT-PCR of KGFR and K1 mRNA transcript levels on TG-treated and control HaCaT cells shows an evident increase in both KGFR mRNA (left panel) and K1 mRNA (right panel) expression upon TG-treatment. Student's t test was performed and significance level has been defined as *p< 0.005 and **p< 0.001 vs the corresponding untreated cells. **(**C) Western blot analysis shows that the KGFR band, which is hardly detectable in control cells, becomes well visible in TG-treated cells. Enhancement of K1 protein expression is also evident upon TG treatment. The equal loading was assessed with anti-actin antibody. For densitometric analysis of the bands, the values from three independent experiments were normalized, expressed as fold increase and reported in graphs as mean values +/- SD. Student's t test was performed and significance level has been defined as *p< 0.001 and **p< 0.001 vs the corresponding untreated cells.

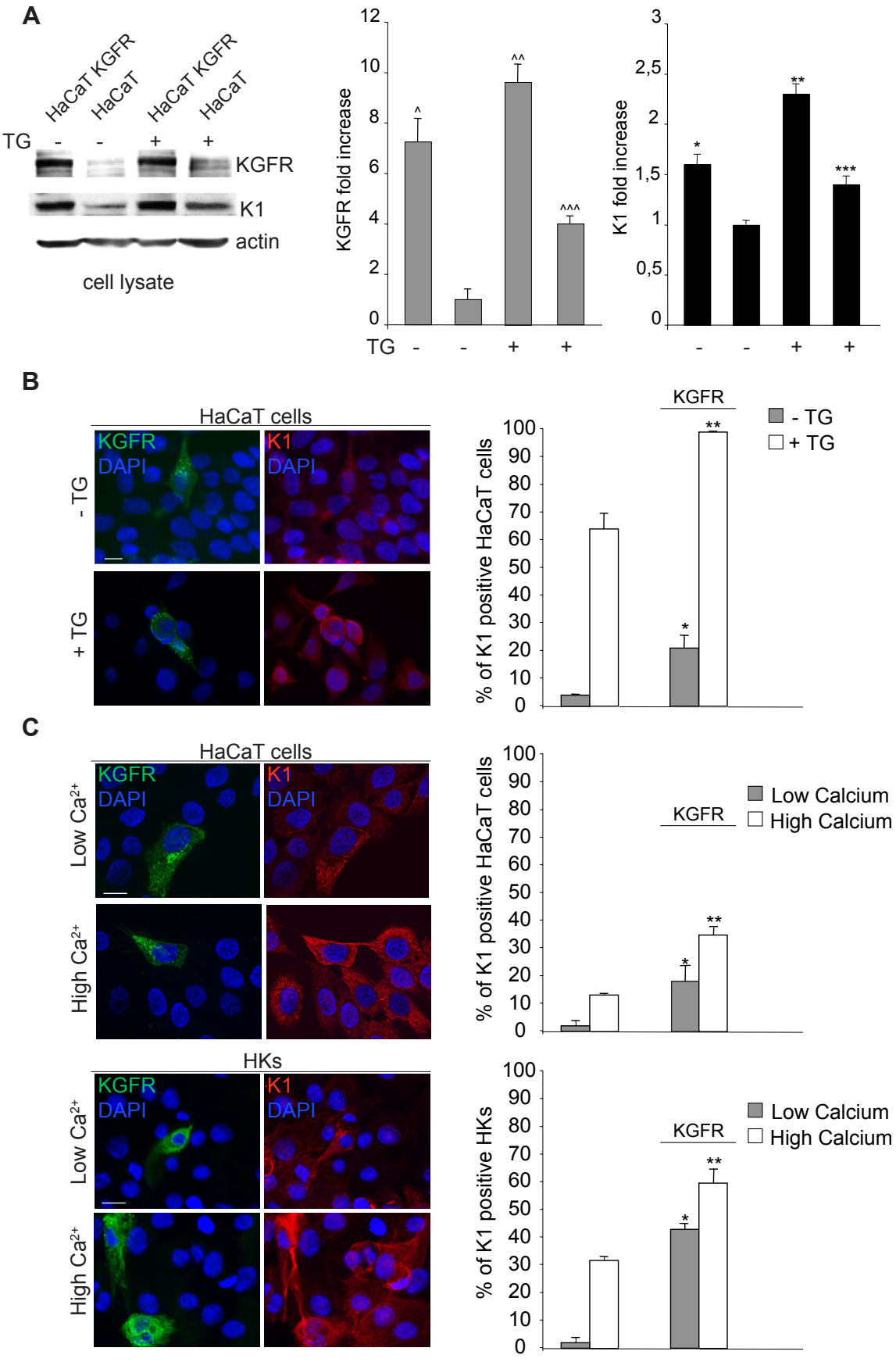

A
HaCaT KGFR
HaCaT
HaCaT KGFR
HaCaT
TG - - + +
KGFR
K1
actin
cell lysate
KGFR fold increase
K1 fold increase
TG - - + +
B
HaCaT cells
KGFR
DAPI
K1
DAPI
- TG
+ TG
KGFR
- TG
+ TG
% of K1 positive HaCaT cells
C
HaCaT cells
Low Ca2+
High Ca2+
Low Calcium
High Calcium
% of K1 positive HaCaT cells
HKs
% of K1 positive HKs

Fig.6

Figure 6. KGFR expression enhances early differentiation in differentiating keratinocytes. HaCaT KGFR transfected cells and HaCaT control cells were treated with TG as above, while the control cells were kept in DMSO alone. (A) Western blot followed by densitometric analysis, as described in Figure 5C, shows that the strong increase of the KGFR band in transfected cells corresponds to a parallel enhancement of the K1 protein levels in both TG-treated or untreated samples. Student's t test was performed and significance level has been defined as ^p<0.001 Vs the corresponding untranfected cells, ^^p<0,05 and ^^^p<0,005 vs the corresponding TG-untreated cells, *p<0,005 vs the corresponding untransfected cells, **p< 0.05 and ***p< 0.005 vs the corresponding TG-untreated cells **(**B) Double immunofluorescence staining with anti-Bek and anti-K1 antibodies shows that the signal of the transfected receptor appears localized either on the plasma membrane and in intracellular compartments. Quantitative analysis shows that KGFR overexpression significantly increases the percentage of cells expressing K1 in both TG-treated and control cells. (C) HaCaT cells and primary cultured HKs were transiently transfected with KGFR and induced to differentiate by incubation with high Ca^{2+} concentration (2 mM for HaCaT cells and 1.5 mM for HKs) in the medium or kept in low Ca^{2+} (0.1 mM for HaCaT cells and 0.03mM for HKs). Double immunofluorescence analysis performed as above and its quantitation confirms the effect of KGFR overexpression in increasing the percentage of K1 positive cells in either high Ca^{2+} or low Ca^{2+} conditions. The quantitative analysis in B and C was assessed counting for each sample a total of 50 cells overexpressing KGFR randomly observed in 10 microscopic fields from three different experiments and comparing them with the surrounding cells that do not display receptor overexpression. Cut-off of K1 signal intensity was determined as above. Results are expressed as mean values ± SE. Student's t test was performed and significance level has been defined as *p< 0.001 and **p< 0.01 vs the corresponding surrounding cells that do not show KGFR overexpression (B), *p< 0.05 and **p< 0.05 (C: HaCaT cells) and *p< 0.005 and **p< 0.05 (C: HKs) vs the corresponding surrounding cells that do not show KGFR overexpression . Bars, 10 μm

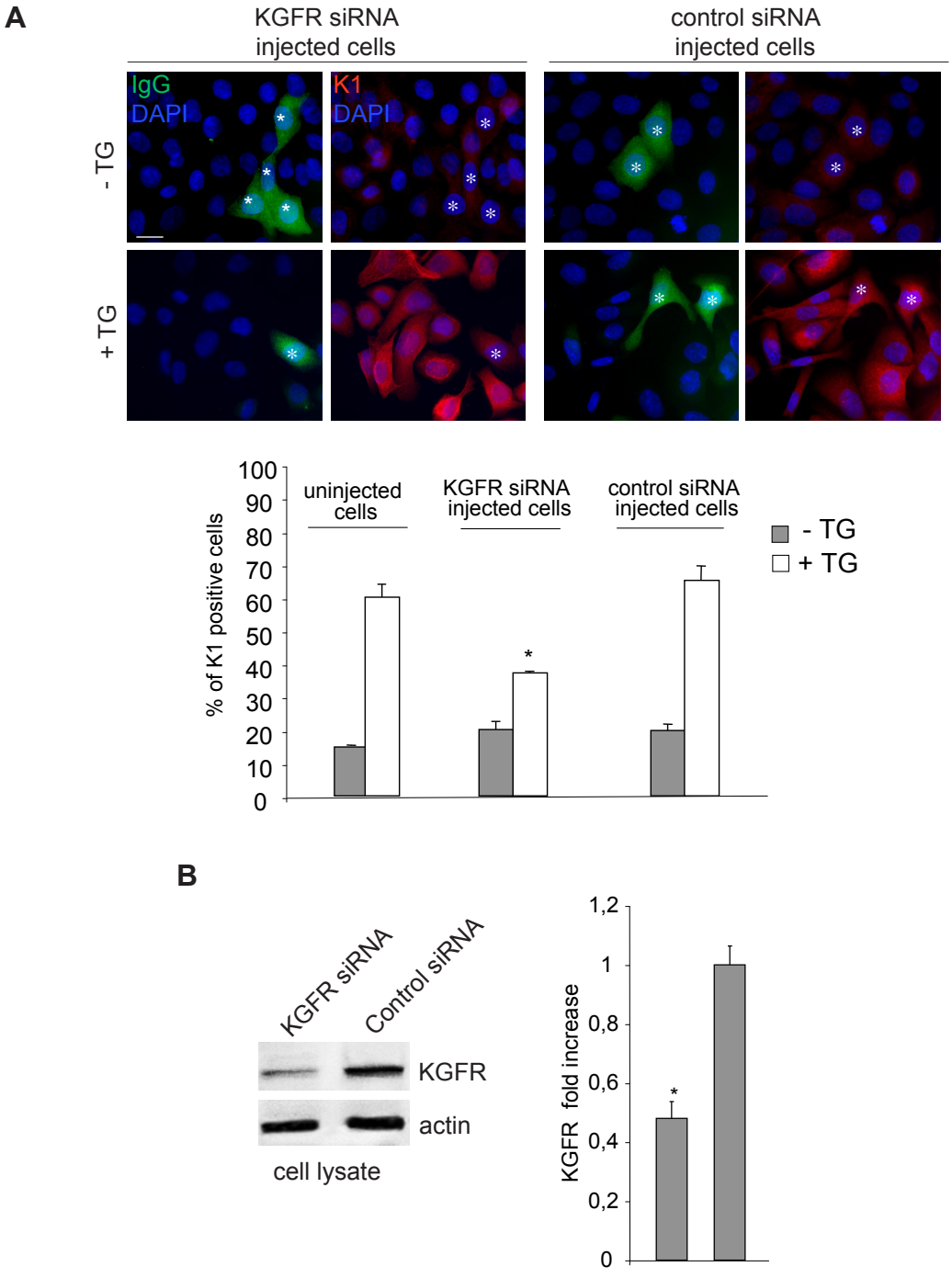

A
KGFR siRNA injected cells
control siRNA injected cells
IgG
DAPI
K1
DAPI
- TG
+ TG
100
90
80
70
60
50
40
30
20
10
0
% of K1 positive cells
uninjected cells
KGFR siRNA injected cells
control siRNA injected cells
- TG
+ TG
*
B
KGFR siRNA
Control siRNA
KGFR
actin
cell lysate
1,2
1
0,8
0,6
0,4
0,2
0
KGFR fold increase
*

Fig.7

Figure 7. KGFR/FGFR2b depletion inhibits early differentiation in keratinocytes. **(**A) HaCaT cells were coinjected with KGFR siRNA to obtain KGFR silencing and mouse IgG to identify the microinjected cells. Control cells were injected with an unrelated siRNA. After injection, cells were treated with TG as above. Quantitative immunofluorescence analysis using anti-K1 polyclonal antibodies shows that the percentage of K1 positive cells in KGFR-depleted and TG-treated cells is significantly decreased if compared either to uninjected cells in the same microscopic field or to cells injected with control siRNA. The quantitative analysis was assessed as described in figure 6B. Results are expressed as mean values ± SE. Student's t test was performed and significance level has been defined as *p< 0.001 vs the corresponding uninjected cells. Bar, 10 μm. **(**B) Western blot analysis with anti-Bek polyclonal antibodies in HaCaT cells transfected with KGFR siRNA or with the control unrelated siRNA and treated with TG as above. The KGFR protein expression is down-regulated in KGFR siRNA-transfected cells. The equal loading was assessed with anti-actin antibody. For densitometric analysis of the band corresponding to KGFR protein, the values from three independent experiments were normalized, expressed as fold increase and reported in graph as mean values +/- SE. Student's t test was performed and significance level has been defined as *p< 0.01 vs the corresponding uninjected cells

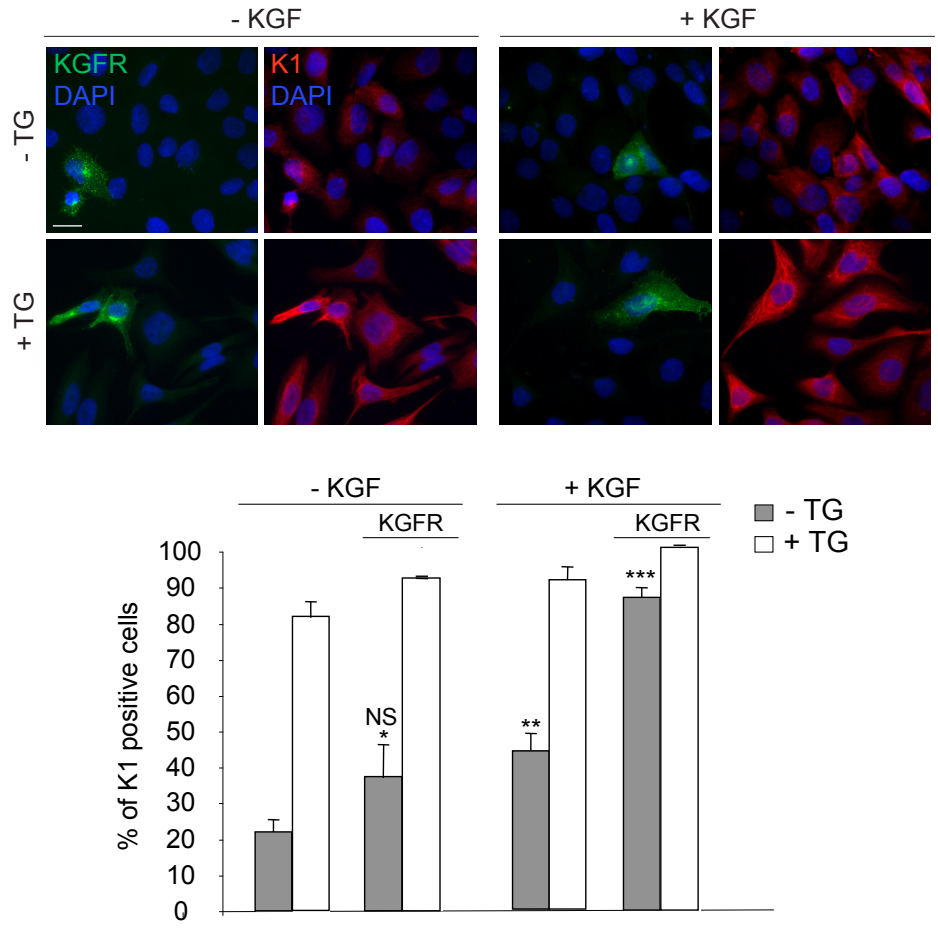

- KGF
+ KGF
KGFR
DAPI
K1
DAPI
- TG
+ TG
- KGF
+ KGF
KGFR
KGFR
- TG
+ TG
% of K1 positive cells
100
90
80
70
60
50
40
30
20
10
0
NS
*
**
***

Fig.8

Figure 8. KGF-induced KGFR activation is required for the induction of early differentiation. HaCaT KGFR cells were treated with TG as above, serum starved for 12h and stimulated the last 24h at 37°C with 20ng/ml KGF. Quantitative immunofluorescence analysis performed as described in figure 6B shows that KGF stimulation increases the percentage of K1 positive cells. This KGF-induced increase is higher in cells overexpressing KGFR compared to cells expressing endogenous receptor levels and is particularly evident in undifferentiated cells, while the TG-treated cells are almost all K1 positive independently on KGF addition. No significant increase in the percentage of K1 positive cells is induced by KGFR overexpression in KGF untreated cells as a consequence of serum deprivation. Results are expressed as mean values ± SE. Student's t test was performed and significance level has been defined as *: NS vs the corresponding surrounding cells that do not display KGFR overexpression, **p< 0.01 and ***p< 0.001 vs the corresponding unstimulated cells. Bar, 10 μm

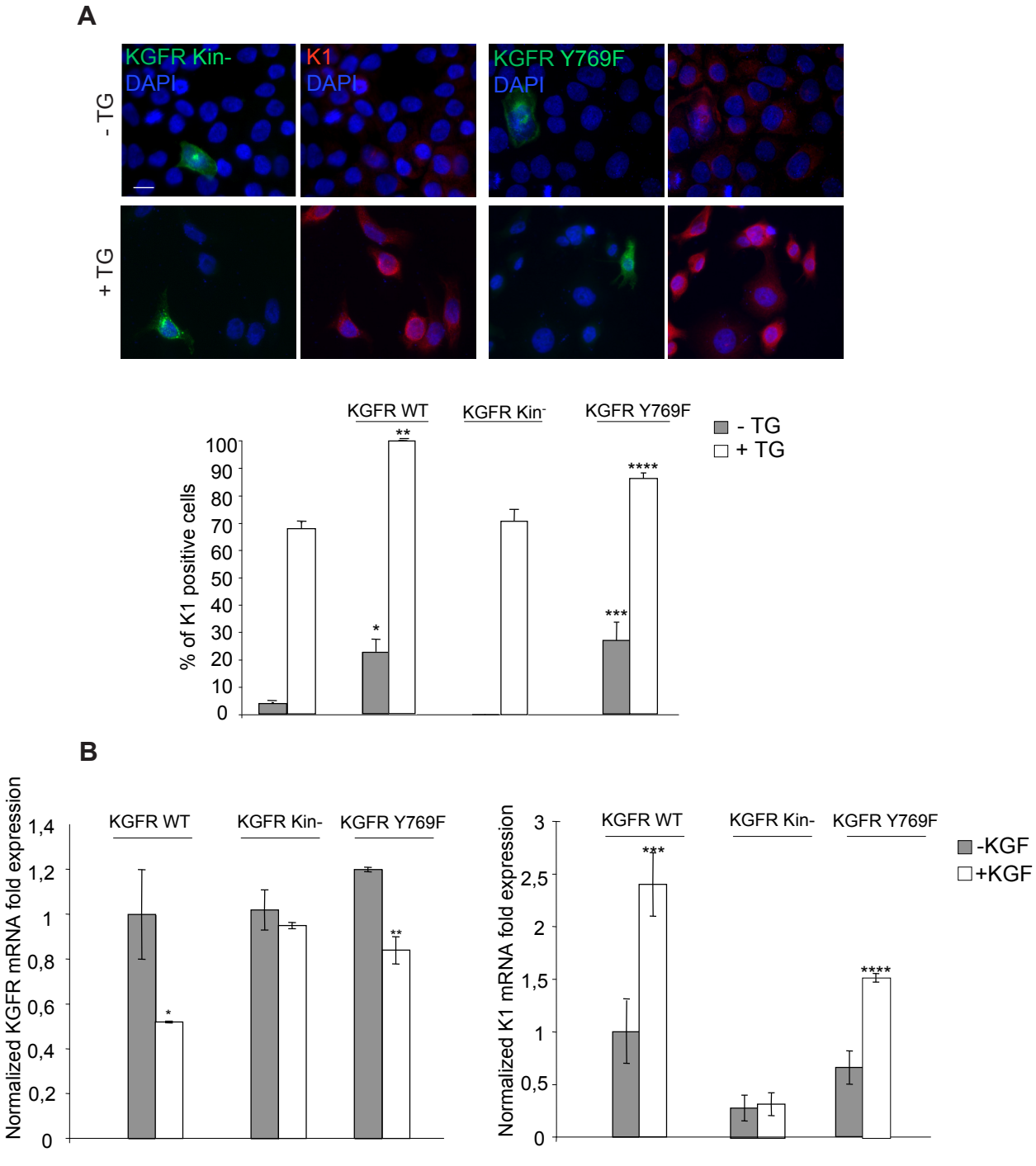

A
KGFR Kin-
DAPI
K1
DAPI
KGFR Y769F
DAPI
- TG
+ TG
KGFR WT
KGFR Kin-
KGFR Y769F
- TG
+ TG
% of K1 positive cells
100
90
80
70
60
50
40
30
20
10
0
*
**
***
****
B
KGFR WT
KGFR Kin-
KGFR Y769F
Normalized KGFR mRNA fold expression
1,4
1,2
1
0,8
0,6
0,4
0,2
0
*
**
KGFR WT
KGFR Kin-
KGFR Y769F
Normalized K1 mRNA fold expression
3
2,5
2
1,5
1
0,5
0
***
****
-KGF
+KGF

Fig.9

Figure 9. KGFR-mediated early differentiation is independent on PLCγ recruitment and activation. (A) HaCaT cells transiently transfected with KGFR WT, with KGFR Y769F signaling mutant or with KGFR Y656F/Y657F kinase dead mutant were treated with TG as above. Quantitative immunofluorescence analysis performed as described in figure 6B shows that, similarly to KGFR WT, KGFR Y769F overexpression significantly increases the percentage of cells expressing K1 either in TG-treated and in the control cells, while the KGFR Y656F/Y657F overexpression does not affect it. Results are expressed as mean values ± SE. Student's t test was performed and significance level has been defined as *p< 0.001, **p< 0.01, ***p< 0.001 and ****p< 0.05 vs the corresponding surrounding cells that do not display KGFR overexpression. Bar, 10 μm. (B) HaCaT cells were transfected and treated with TG and KGF as above. KGFR mRNA (left panel) and K1 mRNA (right panel) transcript levels were quantitated by real-time RT-PCR: a ligand-dependent increase in K1 mRNA expression is observed in cells transfected with KGFR WT and with KGFR Y769F but not in cells transfected with KGFR Y656F/Y657F. A ligand-dependent down-modulation in KGFR mRNA expression is observed in HaCaT KGFR WT cells and in HaCaT KGFR Y769F cells but not in KGFR Y656F/Y657F cells. Student's t test was performed and significance level has been defined as *p< 0.001, **p< 0.05, ***p< 0.005 and ****p< 0.001 vs the corresponding unstimulated cells.

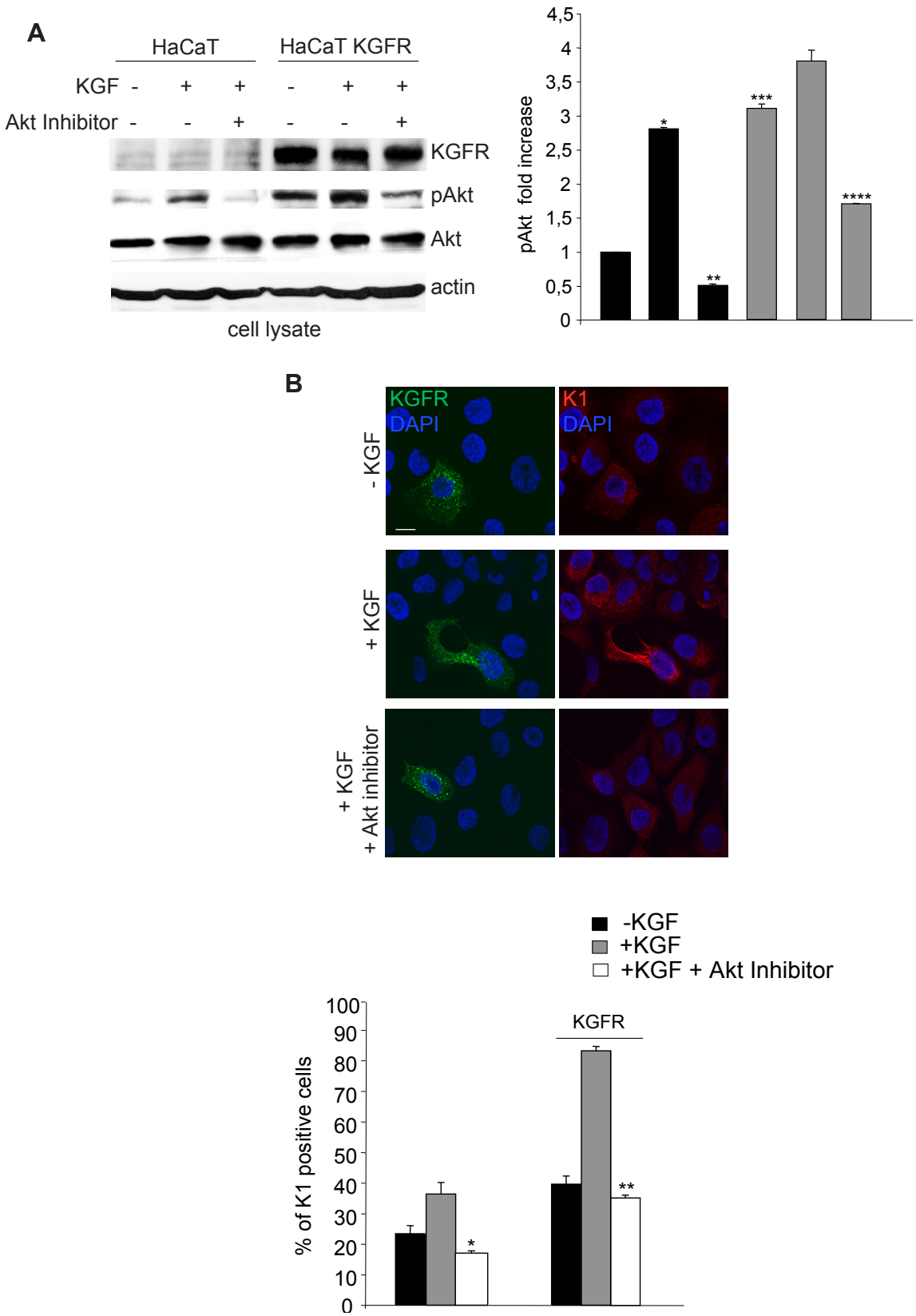

A
HaCaT
HaCaT KGFR
KGF - + + - + +
Akt Inhibitor - - + - - +
KGFR
pAkt
Akt
actin
cell lysate
pAkt fold increase
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
*
**
***
****
B
KGFR
DAPI
K1
DAPI
- KGF
+ KGF
+ KGF
+ Akt inhibitor
-KGF
+KGF
+KGF + Akt Inhibitor
KGFR
% of K1 positive cells
100
90
80
70
60
50
40
30
20
10
0
*
**

Fig.10

Figure 10. The PI3K/Akt signaling pathway is involved in KGFR-mediated early differentiation. (A) HaCaT KGFR and HaCaT cells were serum starved for 4h and treated with 100ng/ml KGF for 10' at 37°C. Western blot analysis using anti-Bek and anti-phospho-Akt polyclonal antibodies shows that Akt protein phosphorylation is evident following KGF treatment and it is specifically blocked by the Akt inhibitor in HaCaT control cells. In KGFR transfected cells, the Akt phosphorylation is more intense and clearly detectable also in serum-free untreated cells. The specific band corresponding to the receptor is well visible only in KGFR transfected cells. The equal loading was assessed with anti-actin antibody. For the densitometric analysis of band corresponding to phospho-Akt protein band the values from three independent experiments were normalized, expressed as fold increase and reported in graph as mean values +/- SE. Student's t test was performed and significance level has been defined as *p< 0.001 vs the corresponding KGF-untreated cells, **p< 0.005 vs the corresponding Akt inhibitor-untreated cells, ***p< 0.001 vs the corresponding untransfected cells and ****p< 0.005 vs the corresponding Akt inhibitor-untreated cells. (B) HaCaT KGFR and HaCaT cells were serum starved for 12h and then stimulated with 20ng/ml KGF for the last 24h at 37°C in presence or not of the specific Akt inhibitor. Quantitative immunofluorescence analysis shows that the Akt inhibitor drastically blocks the increase of K1 positive cells observed upon KGFR overexpression and KGF stimulation. The Akt inhibitor decreases the KGF-mediated differentiation also in cells expressing endogenous levels of the receptor. Results are expressed as mean values ± SE. Student's t test was performed and significance level has been defined as *p< 0.001 and **p< 0.001 vs the corresponding, KGF-treated cells. Bar, 10 μm.

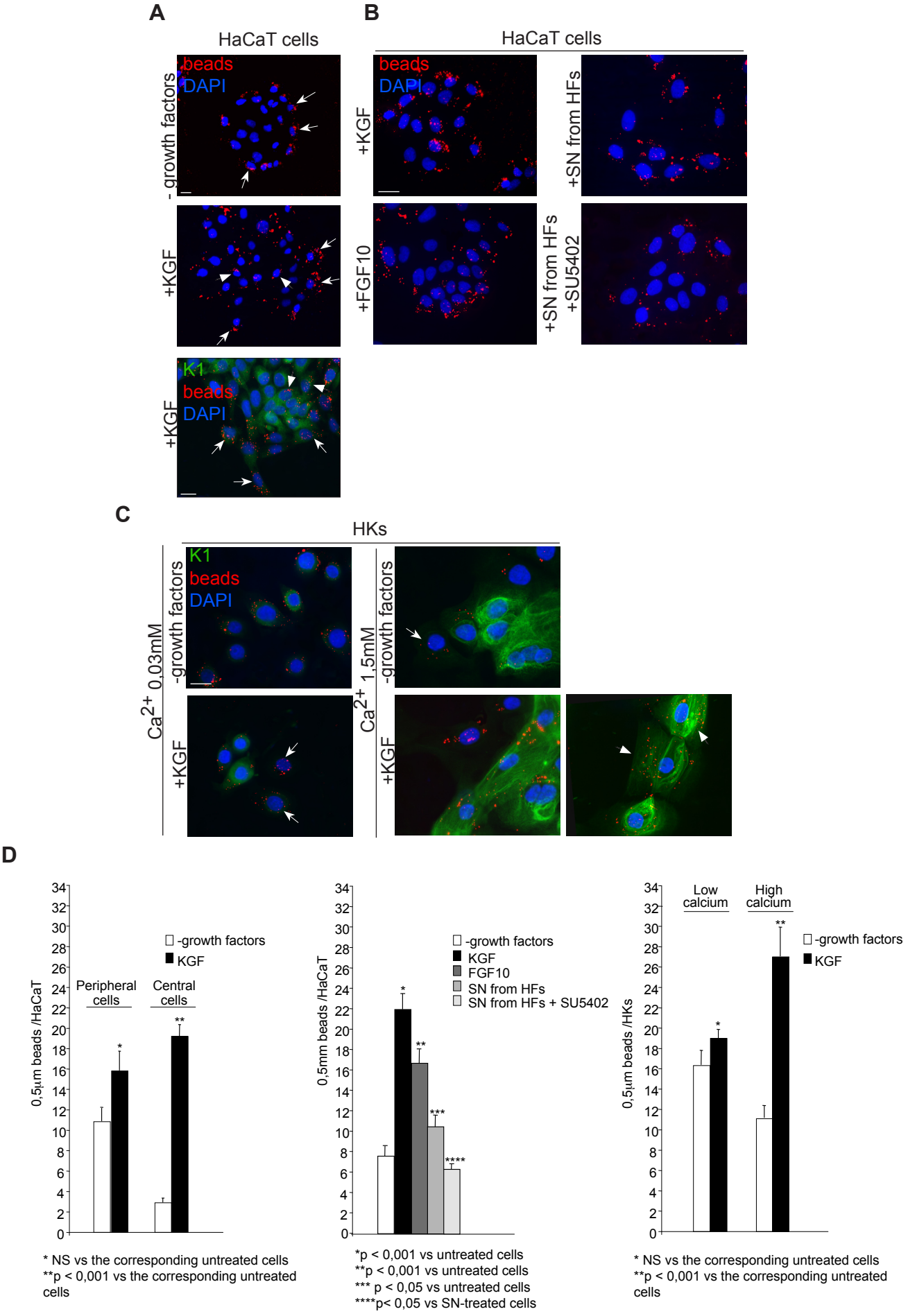

A
HaCaT cells
beads
DAPI
- growth factors
+KGF
K1
beads
DAPI
+KGF
B
HaCaT cells
beads
DAPI
+KGF
+SN from HFs
+FGF10
+SN from HFs
+SU5402
C
HKs
K1
beads
DAPI
-growth factors
Ca2+ 0,03mM
+KGF
Ca2+ 1,5mM
-growth factors
+KGF
D
0,5µm beads /HaCaT
-growth factors
KGF
Peripheral cells
Central cells
* NS vs the corresponding untreated cells
**p < 0,001 vs the corresponding untreated cells
0,5mm beads /HaCaT
-growth factors
KGF
FGF10
SN from HFs
SN from HFs + SU5402
*p < 0,001 vs untreated cells
**p < 0,001 vs untreated cells
*** p < 0,05 vs untreated cells
****p< 0,05 vs SN-treated cells
0,5µm beads /HKs
Low calcium
High calcium
-growth factors
KGF
* NS vs the corresponding untreated cells
**p < 0,001 vs the corresponding untreated cells

Fig.11

Figure 11. Phagocytosis of fluorescent latex beads promoted by KGFR ligands in differentiated keratinocytes. A) HaCaT cells were grown up to semi-confluence, serum starved and incubated with latex fluorescent (red) beads 0.5 μm in diameter for 4h at 37°C with or without the addition of KGF (20 ng/ml). After fixation and permeabilization, cell nuclei were visualized by DAPI. In parallel experiments, cells were also double-stained with anti-K1 antibody. Immunofluorescence analysis shows that the uptake of the beads in the absence of growth factors occurs mostly in cells located at the periphery of the colonies (arrows) and that the internalized beads are mostly distributed on the perinuclear cytoplasmic area. Addition of KGF induces an increased phagocytosis of the beads in confluent differentiated cells (arrowheads), expressing the K1 early differentiation marker. Bar: 10μm**.** B) Treatment with the alternative KGFR ligand FGF10 or with a culture supernatant (SN) collected from primary skin derived human fibroblasts (HFs) enhances the phagocytosis of the beads also in confluent HaCaT keratinocytes. The KGFR tyrosine kinase inhibitor SU5402 inhibits the internalization induced by the SN, indicating that the promoting effect is dependent on the release of KGFR ligands in the HF culture supernatant. Bar: 10μm**.** C) Primary human keratinocytes (HKs) were induced to differentiate by incubation with high Ca^{++} concentration (1.5 mM in the culture medium or kept in low Ca^{++} (0.03 mM) before the above phagocytic assay: similarly to the results on HaCaT cells, the beads internalization is enhanced by KGF treatment and differentiation. Arrowheads point to K1-positive differentiated cells, while arrows to less differentiated keratinocytes. Bar: 10μm**.** D) Quantitative analysis of the number of internalized beads was performed as described in Materials and Methods. Results are expressed as mean values +/- SE (standard errors). Student's t test was performed and significance levels have been defined.

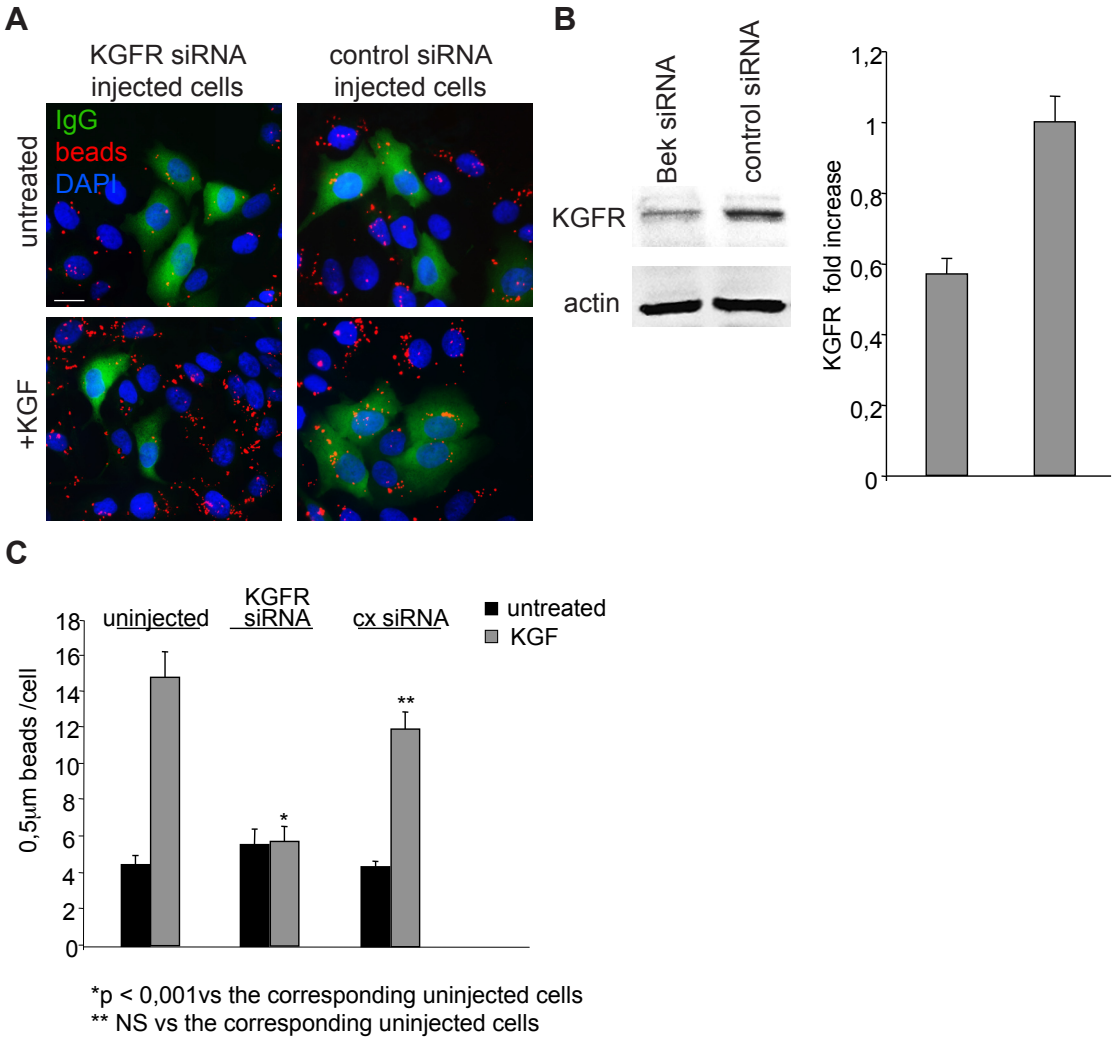
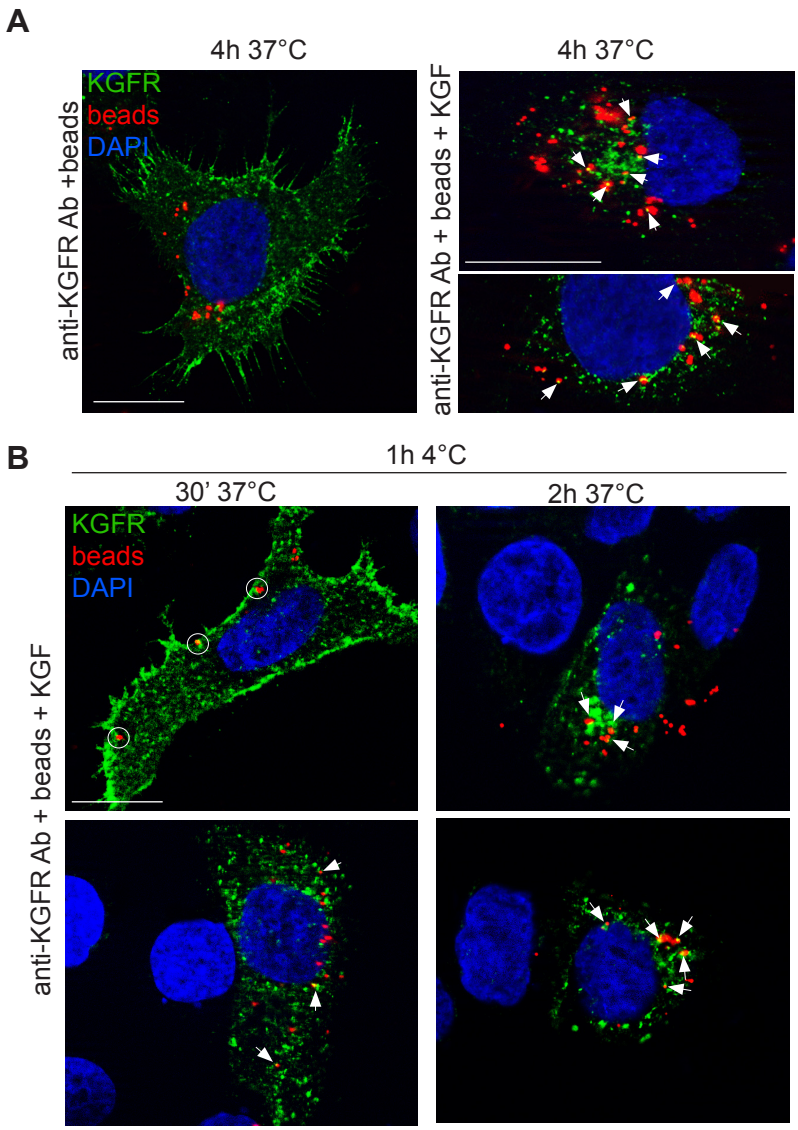

A
KGFR siRNA injected cells
control siRNA injected cells
IgG
beads
DAPI
untreated
+KGF
B
Bek siRNA
control siRNA
KGFR
actin
KGFR fold increase
1,2
1
0,8
0,6
0,4
0,2
0
C
uninjected
KGFR siRNA
cx siRNA
untreated
KGF
0,5µm beads /cell
18
16
14
12
10
8
6
4
2
0
*
**
*p < 0,001vs the corresponding uninjected cells
** NS vs the corresponding uninjected cells

Fig.12

Figure 12. Depletion of KGFR/FGFR2b inhibits the phagocytic process in keratinocytes. A, C) HaCaT cells were coinjected with siRNA Bek to obtain KGFR silencing and with rabbit IgG to identify the microinjected cells. Control cells were injected with an unrelated siRNA. After injection, cells were serum starved and incubated with the beads and KGF as above. The number of internalized beads in the IgG-positive KGFR-depleted cells is drastically decreased, compared either to the uninjected cells in the same microscopic field or to cells injected with control siRNA. Bar: 10μm**.** B) Western blot analysis with anti-Bek polyclonal antibodies, recognizing the FGFR2b/KGFR protein, in HaCaT cells transfected with Bek siRNA or with the control unrelated siRNA. The receptor expression, shown by the specific 140 kDa band corresponding to the molecular weight of KGFR, is down-regulated in siRNA-transfected cells. The equal loading was assessed with anti-actin antibody. Densitometric analysis of the bands: the values from three independent experiments were normalized, expressed as fold increase and reported in graph as mean values +/- SE.



* p NS vs the corresponding untreated cells
** p < 0,0001 vs the corresponding untreated cells
*** p < 0,005 vs cells treated for 30' at 37°C

Fig.13

Figure 13. KGFR colocalize with the internalized beads at early and late steps of the phagocytic pathway. A) HaCaT keratinocytes were transiently trasfected with KGFR WT, serum starved for 12h and treated 1h in vivo at 4°C with an anti-Bek polyclonal antibodies, directed against the extracellular portion of KGFR, before the incubation with the beads and KGF for 4h at 37°C as above. Colocalization of the signals of KGFR (green) and beads (red) was assessed on series of 0.5 μm sequential optical sections obtained with the microscope scanning system described in Materials and Methods. In KGF-untreated cells, the KGFR signal appears associated to the cell plasma membrane and does not colocalize with the perinuclear internalized beads (left panel). In contrast, colocalization of the receptors with the beads is evident after endocytosis of the KGF-receptor complexes (yellow dots in right panels) in treated cells. The small yellow dots in proximity or surrounding the beads remind fusion events among the phagosomes containing the beads and late-endosomes or lysosomes containing the internalized receptors (arrows). B) HaCaT KGFR WT transfected cells were serum starved as above, but incubated with the anti-KGFR Ab, KGF and the fluorescent beads for 1h at 4°C, followed by warming to 37°C for 30' or 2h before fixation, to induce a synchronous wave of KGFR and beads internalization. At 30' of warming, colocalization of the two signals is evident in peripheral structures beneath the cell plasma membranes (left panels, circles) and in early endo-phagosomes (left panels, arrows), whereas after 2h of warming, colocalization is visible in late perinuclear structures probably corresponding to phago-lysosomes (right panels, arrows). Quantitative analysis of the percentage of colocalization of the internalized beads with the receptor was performed as described in Materials and Methods. Results are expressed as mean values +/- SE. Student's t test was performed and significance levels have been defined. Bar: 10μm

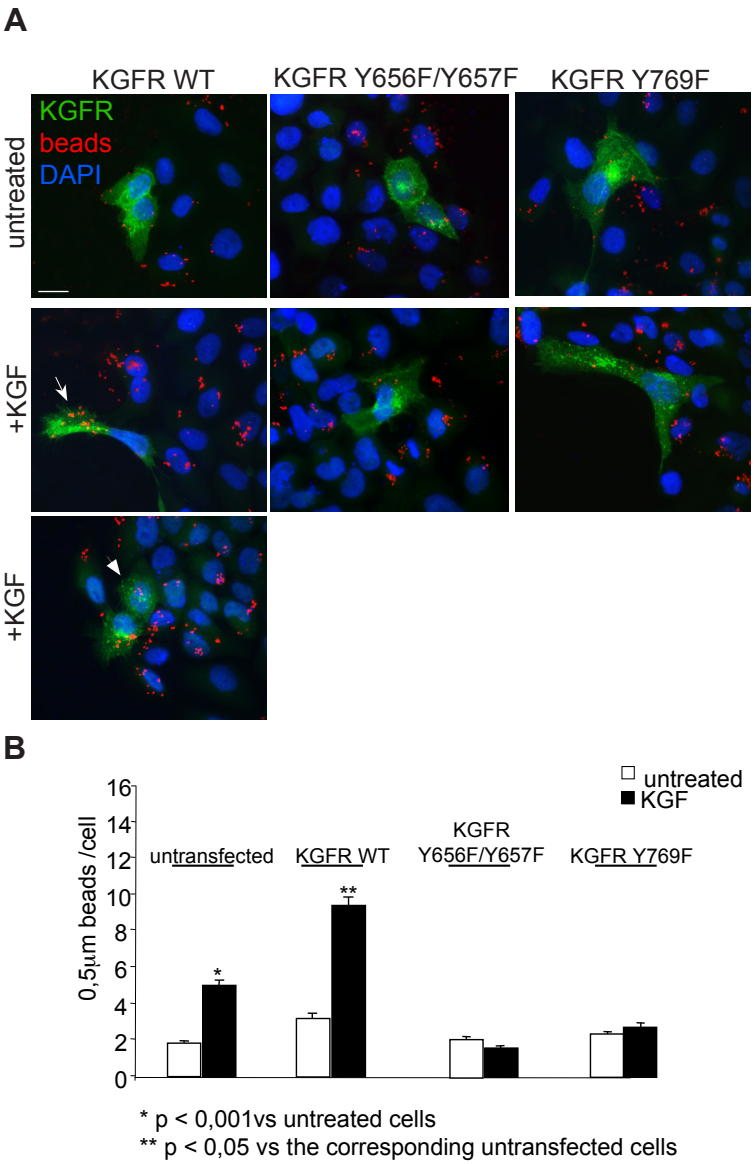

A
KGFR WT
KGFR Y656F/Y657F
KGFR Y769F
KGFR
beads
DAPI
untreated
+KGF
+KGF
B
untreated
KGF
0,5µm beads /cell
16
14
12
10
8
6
4
2
0
untransfected
KGFR WT
KGFR Y656F/Y657F
KGFR Y769F
*
**
* p < 0,001vs untreated cells
** p < 0,05 vs the corresponding untransfected cells

Fig.14

Figure 14. KGF-promoted phagocytosis is dependent on receptor kinase activity and on PLCγ recruitment and signaling. A, B) HaCaT cells were transiently transfected alternatively with KGFR WT (left panels), with a kinase negative mutant KGFR Y656F/Y657F (central panels) or with a KGFR Y769F signaling mutant (right panels), serum starved and incubated with the beads as above. Cells were then fixed and permeabilized before immunofluorescence staining with anti-KGFR antibodies. The signal of the transfected receptors appears localized both on the cell surface and in intracellular compartments (A). Quantitative analysis of the number of beads internalized in response to KGF (A, B) shows an increased amount in cells overexpressing KGFR WT, either at the periphery (A, arrow) or in the central part (A, arrowhead) of the colony. The KGF promoting effect is abolished by expression of the kinase negative mutant KGFR Y656F/Y657F (A, B), demonstrating that receptor kinase activity and signal transduction are required for the KGF-mediated phagocytosis. The KGF-stimulated uptake of the beads is also drastic reduced in cells expressing the KGFR Y769F mutant, indicating that PLCγ activation and recruitment to the receptor is critical for the phagocytic promotion. Bar: 10μm

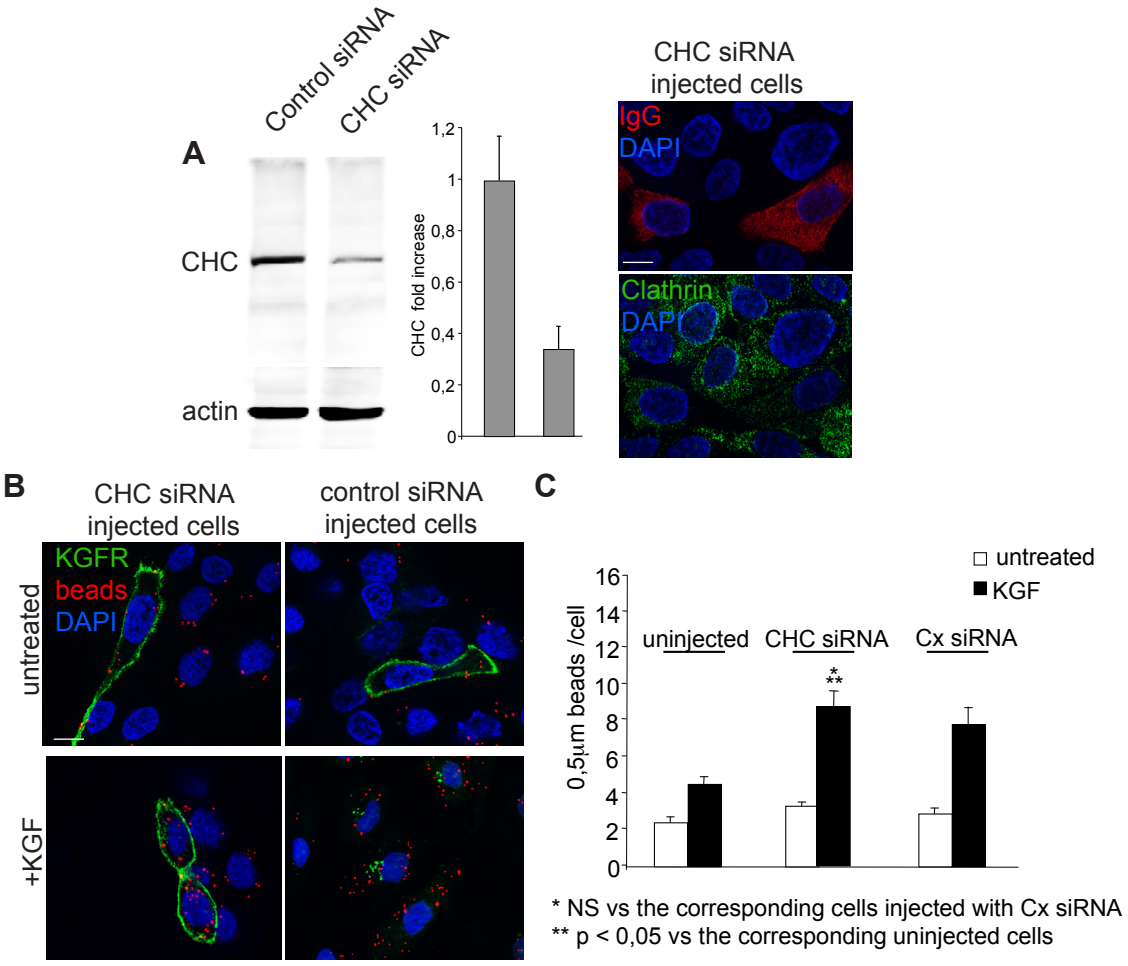
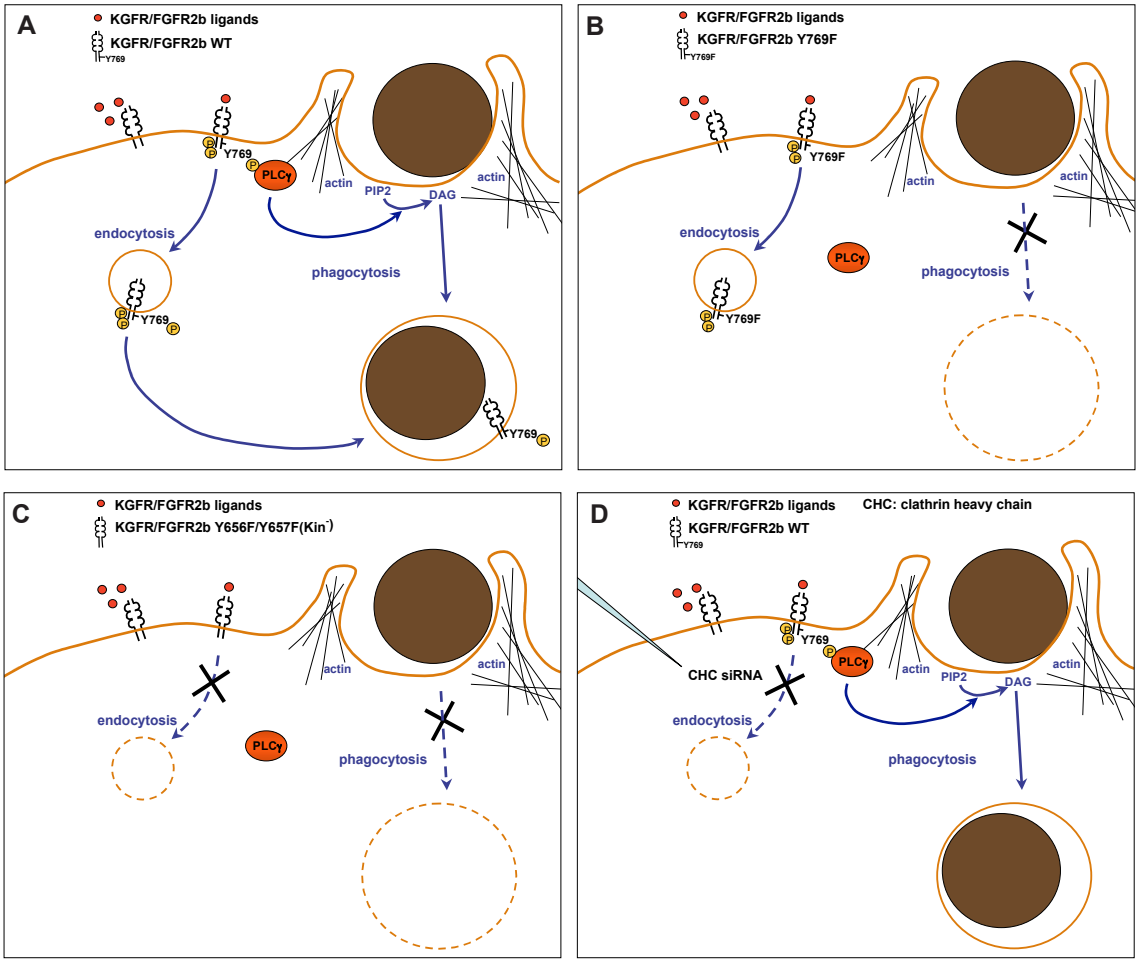

A
Control siRNA
CHC siRNA
CHC
actin
CHC fold increase
1,2
1
0,8
0,6
0,4
0,2
0
CHC siRNA
injected cells
IgG
DAPI
Clathrin
DAPI
B
CHC siRNA
injected cells
control siRNA
injected cells
untreated
+KGF
KGFR
beads
DAPI
C
□ untreated
■ KGF
0,5µm beads /cell
16
14
12
10
8
6
4
2
0
uninjected
CHC siRNA
Cx siRNA
*
**
* NS vs the corresponding cells injected with Cx siRNA
** p < 0,05 vs the corresponding uninjected cells

Fig.15

Figure 15. KGF-induced phagocytosis is independent on receptor endocytosis. A) HaCaT cells were co-injected with siRNA CHC to obtain clathrin silencing and with rabbit IgG to identify the microinjected cells. Control cells were co-injected with an unrelated siRNA. Immunofluorescence analysis with anti-clathrin antobodies shows a drastic reduction of the punctate signal in cells injected with CHC siRNA compared to the surrounding uninjected cells. B, C) HaCaT cells were co-injected with KGFR cDNA and CHC siRNA to simultaneusly obtain KGFR overexpression and clathrin depletion. Co-injection of KGFR cDNA with an unrelated siRNA was performed as a control. After injection, cells were serum starved for 12h and incubated the anti-KGFR Ab, KGF and the fluorescent beads for 1h at 4°C, followed by warming to 37°C for 2h before fixation. Receptor endocytosis induced by KGF treatment is blocked in clathrin-depleted cells, while appears unaffected in cells injected with the control siRNA. Quantitative analysis of the number of beads shows that the phagocytic uptake in response to KGF is increased by receptor expression but not affected by clathrin-depletion. Bar: 10μm


A
KGFR/FGFR2b ligands
KGFR/FGFR2b WT
Y769
PLCγ
actin
PIP2
DAG
endocytosis
phagocytosis
B
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KGFR/FGFR2b Y769F
Y769F
C
KGFR/FGFR2b ligands
KGFR/FGFR2b Y656F/Y657F(Kin-)
D
KGFR/FGFR2b ligands
KGFR/FGFR2b WT
CHC: clathrin heavy chain
CHC siRNA

Fig.16

Figure 16. Schematic drawing of the proposed role of KGFR/FGFR2b and of its Tyr769 in the promotion of phagocytosis: the ligands KGF/FGF7 and FGF10, secreted by dermal fibroblasts, bind and activate the receptor expressed on epidermal keratinocytes. In A, the tyrosine kinase activity induces phosphorylation of the Y769 residue, which is required for activation and recruitment of PLCγ to the receptor. In turn, through diacylglycerol (DAG) formation by PIP_2 hydrolysis, PLCγ controls cortical actin reorganization and phagocytosis. In B, the point mutation of Tyr769 to phenylalanine (Y769F) impairs PLCγ activation and phagocytosis, but not receptor endocytosis. In C, the block of the kinase activity and receptor signaling by double tyrosine mutation (Y656F/Y657F) inhibits both phagocytosis and receptor endocytosis. In D, the block of the receptor clathrin-mediated endocytosis by RNA interference does not impair phagocytosis.

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Cardinali, G., Bolasco, G., Aspite, N., Lucania, G., Lotti, L.V., Torrisi, M.R. and Picardo, M., (2008) Melanosome transfer promoted by keratinocyte growth factor in light and dark skin derived keratinocytes. *J. Invest. Dermatol*. **128**, 558-557.

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