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SPERIMENTALE

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"***EMT/autophagy crosstalk: role of the aberrant expression of the mesenchymal FGFR2c isoform in epithelial tumorigenesis***"

DOTTORANDO — DOCENTE GUIDA

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Abstract

In epithelial context the aberrant expression of the mesenchymal FGFR2c isoform in epithelial cells induces impaired differentiation inhibition of autophagy as well as the induction of the epithelial-mesenchymal transition (EMT). Considering the widely proposed negative loop linking autophagy and EMT in the early steps of carcinogenesis, in this thesis work we investigated the possible involvement of FGFR2c aberrant expression and signalling in orchestrating this crosstalk. In human keratinocytes context, biochemical, molecular, quantitative immunofluorescence analysis and in vitro invasion assays, coupled to the use of specific substrate inhibitors and transient or stable silencing approaches, showed that AKT/MTOR and PKCε are the two hub signalling pathways, downstream FGFR2c, intersecting with each other in the control of both the inhibition of autophagy and the induction of EMT and invasive behavior. These results indicate that the out of context expression of FGFR2c, could represent a key upstream event responsible for the establishment of a negative interplay between autophagy and EMT.

FGFR2 isoform switch is recognized as one of the oncogenic events occurring during pancreatic carcinogenesis, whose contribution in EMT induction and cell invasion, as well as in the dysregulation of autophagy still appears controversial.

In fact, pancreatic ductal adenocarcinoma (PDAC) is a treatment-resistant malignancy characterized by a high malignant phenotype including acquired EMT signature and deregulated autophagy. Since we have previously described that the aberrant expression of the mesenchymal FGFR2c and the triggering of the downstream PKC signaling are involved in epidermal carcinogenesis, the aim of this work has been to assess the contribution of these oncogenic events also in the pancreatic context. Biochemical, molecular and immunofluorescence approaches showed that FGFR2c expression impacts on PDAC cell responsiveness to FGF2 in terms of intracellular signaling activation, upregulation of EMT-related transcription factors and modulation of epithelial and mesenchymal markers compatible with the pathological EMT. Moreover, shut-off via specific

protein depletion of PKC signaling, activated by high expression of FGFR2c resulted in a reversion of EMT profile, as well as in a recovery of the autophagic process. The detailed biochemical analysis of the intracellular signaling indicated that PKCε, bypassing AKT and directly converging on ERK1/2, could be a signaling molecule downstream FGFR2c whose inhibition could be considered as possible effective therapeutic approach in counteracting aggressive phenotype in cancer.

Introduction

The fibroblast growth factor receptor family

The fibroblast growth factor receptors (FGFRs) are receptor tyrosine kinases (RTKs) belonging to a family composed by four highly conserved transmembrane tyrosine kinase receptors (FGFR1, FGFR2, FGFR3 and FGFR4) encoded by four different genes. These are involved in the management of physiological key processes such as cell proliferation, differentiation, migration and survival and are expressed on several tissues (Turner and Grose, 2010; Goetz and Mohammadi, 2013). FGFRs are stimulated by fibroblast growth factor (FGF) family members, which are 18 members grouped in five paracrine subfamilies and one endocrine subfamily. Paracrine FGFs are secreted glycoproteins immobilized by HPSGs in the extracellular matrix near the site of their secretion. These FGFs are released from the extracellular matrix by enzymes or specific FGF binding proteins in cooperation with HPSGs, that stabilize the FGF-FGFR interaction, forming a ternary complex. FGFRs structure consist of an extracellular domain, a singlepass transmembrane domain and an intracellular tyrosine kinase domain (Figure 1).

The extracellular domain is made by three immunoglobulin (Ig)-like domains (I-III), an acid serine-rich box region linking I and II domains and a conserved charged binding site for heparin (Schlessinger et al., 2000). The first Ig-like domain, together with the acid box, seems to be involved in receptor autoinhibition (Olsen et al., 2004), while the second and third Ig-like domains form the attaching site for FGF ligands (Mohammadi et al., 2005). The ligand specificity of FGFR1, FGFR2 and FGFR3 is defined by the alternative splicing of the third Ig-like domain of these receptors, which generates FGFRb and FGFRc isoforms (Figure 1). For FGFR2 the N-terminal portion of the Ig-III domain is encoded by the exon 7 (exon IIIa), while the C-terminal portion is encoded alternatively by exon 8 (exon IIIb), and 9 (exon IIIc), which determines respectively FGFR2IIIb and FGFR2IIIc isoforms with different ligand-binding specificities.

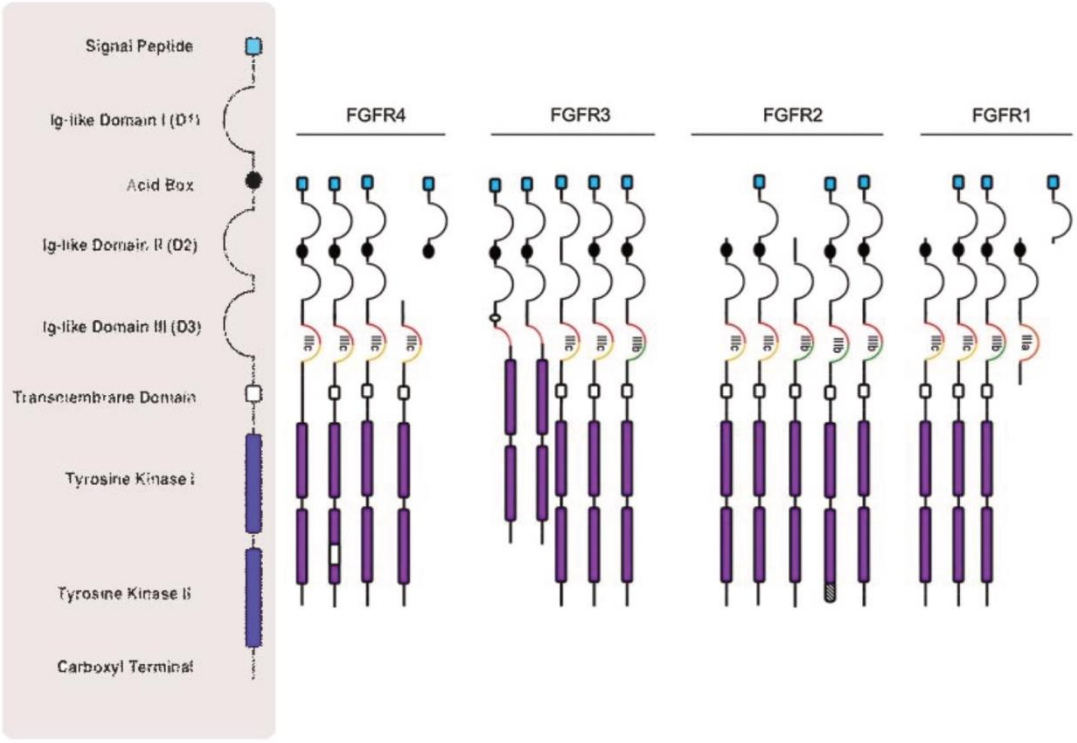

Figure 1: (modified from Tiong et al., 2013) **The basic structure of FGFR and splice variants.** The basic structure of a FGFR. The FGFRs are phylogenetically closely related to the VEGFRs and PDGFRs, consist of three extracellular immunoglobulin (Ig) domains (D1-D3), a single transmembrane helix, an intracellular split tyrosine kinase domain (TK1 and TK2) and an acidic box. D2 and D3 form the ligand-binding pocket and have distinct domains that bind both FGFs and heparan sulfate proteoglycans (HSPGs). Acidic box is required for binding of bivalent cations for optimal interaction between FGFRs and HSPGs. The FGFRs isoforms are generated mainly by alternative splicing of the Ig III domain (D3). The D3 could be encoded by an invariant exon 7 (red) to produce FGFR-IIIa isofom or spliced to either exon 8 (green) or 9 (yellow) to generate the FGFR-IIIb or FGFR-IIIc isoforms, respectively. Epithelial tissues predominantly express the IIIb isoform and mesenchymal tissues express IIIc. FGFR4 is expressed as a single isoform that is paralogous to FGFR-IIIc. Hatched box represents a truncated carboxyl terminal.

While FGFR2IIIb specifically binds FGF7/KGF (Rubin et al., 1989) and FGF10 (Igarashi et al., 1998), FGFR2IIIc binds FGF2 (Yayon et al., 1992) (Figure 2).

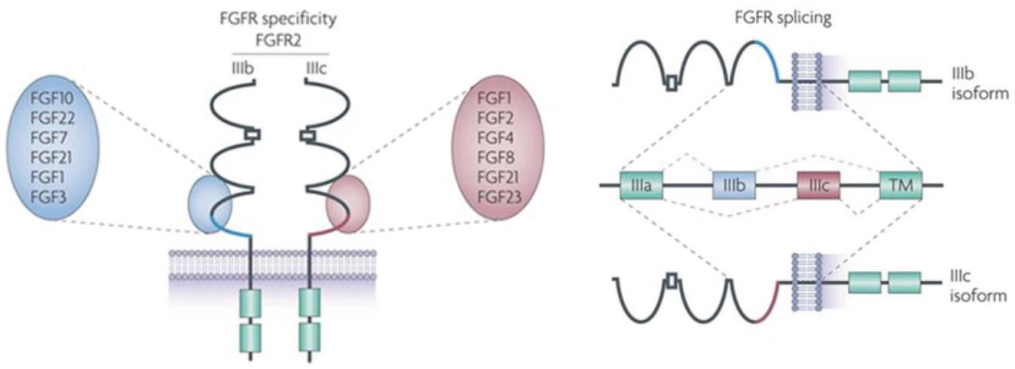

Figure 2: (Modified from Turner and Grose 2010) **FGFR2IIIb and FGFR2IIIb isoform specificity and structure.** The specificity of the FGF-FGFR interaction is established

partly by the differing ligand-binding capacities of the receptor but also by alternative splicing of FGFR, which substantially alters ligand specificity. The Ig III is encoded by an invariant exon (IIIa), and the alternative splicing of exon IIIb or IIIc, both of which bind the transmembrane (TM) region. Epithelial tissues predominantly express the IIIb isoform and mesenchymal tissues express IIIc. Different ligand specificity of FGFR2 isoforms is shown. The FGFR2IIIb ligands are shown in blue and the FGFR2IIIc ligands are shown in purple. For exemple, FGF2 binds specifically to FGFR2IIIc and have essentially no binding to FGFR2IIIb

The alternative splicing also affects the tissue specificity of the FGFRs. The FGFR2IIIb isoform is exclusively expressed in epithelial cells, while the FGFR2IIIc isoform is expressed physiologically in mesenchymal cells (Miki et al., 1992; Orr-Urtreger et al., 1993). FGFs-FGFRs binding induce receptor dimerization and the juxtaposition of the two intracellular kinase domains of the two receptors which brings to the phosphorylation of each other (Goetz and Mohammadi, 2013). The binding needs the presence of a cofactor, the heparan sulphate proteoglycan (HPSG), which enhances resistance to proteolysis and forming coupled ternary complex on the cell surface (Schlessinger et al., 2000). FGF/FGFR interaction induces receptor dimerization, which leads to a conformational change in receptor structure inducing the activation of the intracellular kinase domain, which triggers the intermolecular transphosphorylation of the tyrosine kinase domain and the carboxy-terminal tail (Turner and Grose, 2010; Brooks et al., 2012). Some tyrosine residues of the receptor can be auto phosphorylated (Lew et al., 2009) then acting as docks for adaptor proteins, they can lead to activation of various signaling pathways (Turner and Grose, 2010). The central downstream substrates of FGFRs are the FGFR substrate 2 (FRS2) and the phospholipase Cγ (PLCγ).

FRS2 family is composed by two members, FRS2α and FRS2β (Gotoh et al., 2008), both with a consensus sequence at the N-terminus for lipid binding in the plasma membrane (Gotoh et al., 2008), a phospho-tyrosine binding (PTB) domain and multiple tyrosine phosphorylation sites at the Cterminus, which need to bind RTKs. FRS2 proteins can bind a limited variety of RTKs, such as neurotrophic receptors, RET and ALK, but especially FGFRs. FRS2α acts as the most important mediator of intracellular signaling via FGFRs. The PTB domains of FRS2 binds constitutively to unphosphorylated peptides at the juxta membrane domain of the FGFR. FRS2α includes four tyrosine phosphorylation sites that

connect the adaptor protein growth-factorreceptor-bound protein 2 (Grb2) and has two binding sites for the SH2containing tyrosine phosphatase protein (Shp2). Grb2 can bind many proteins via two SH3 domains, such as Gab1, SOS and Cbl. The recruitment of Gab1 by Grb2 forms a ternary complex with FRS2α, which in turn recruits PI-3 kinase and allows activation of PI3K/AKT pathway (Figure 3) (Altomare and Testa, 2005). SOS is a guanine nucleotide exchange factor (GEF), which can activate Ras. The recruitment of Grb2-Sos on FGFR triggers Ras/mitogenactivated protein kinase (MAP kinase) pathway activation (Figure 3) (Eswarakumar et al., 2005). The FRS2α-Shp2 binding, caused by FGF stimulation, induces tyrosine phosphorylation on Shp2, that provokes a strong activation of ERK, a component of MAP kinases family (Hadari et al., 1998). The activation of ERK induced by growth factors can be transient or sustained (Marshall et al., 1995). A not related FRS2 signaling, downstream FGF/FGFR, is the PLCγ pathway (Figure 3). This phospholipase auto phosphorylates through its Src homology 2 (SH2) domain and induces its own activation. Particularly, it has been demonstrated that the tyrosine 766 residue in the Cterminal of FGFR1 is required for PLCγ protein binding (Mohammadi et al., 1991), which corresponds to tyrosine 769 in FGFR2b (Ceridono et al., 2005; Cha et al., 2009; Brooks et al., 2012).

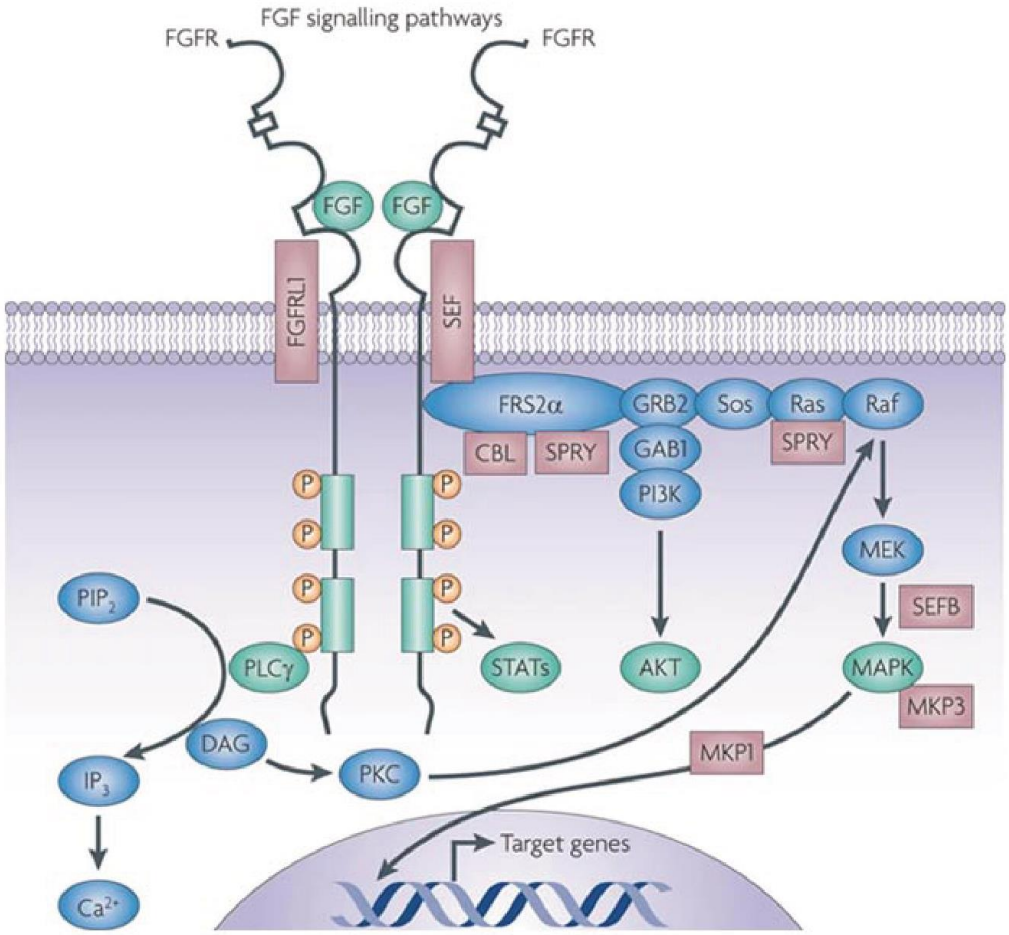

Figure 3: (modified from Turner and Grose 2010) **FGFR signaling pathway.** FGFs induces FGFE-mediated signaling pathway by interacting with specific FGFRs and HSPGs. The macromolecular interactions mediate FGFRs, dimerization or oligomerization and activate multiple signal trasduction pathways, including those involving FRS2, RAS, p38 MAPKs, ERKs, JNKs, Src, PLCγ, Crk, PKC and PI3K.

Several other pathways can be activated by FGFRs, such as signal transducer and activator of transcription signaling (STAT), ribosomal protein S6 kinase 2 (RSK2), the p38 MAPK and Jun Nterminal kinase pathways (Touat et al., 2015), which can be activated downstream RAS by the MAPKKs, MKK4 and MKK7 (Katz et al., 2007) but also via PKCδ (Liu et al., 2006; Chen et al., 2008).

Deregulation of FGFR2 signaling in cancer

Deregulation of the FGF-FGFR axis can be involved in several tumors, at different stages. It can produce cell transformation, tumor progression and resistance to anticancer therapy. FGFR signaling influences oncogenesis through ligand-dependent and independent mechanisms as autocrine and paracrine signaling, angiogenesis, and epithelial–mesenchymal transition (EMT) (Babina and Turner, 2017) (Figure 4).

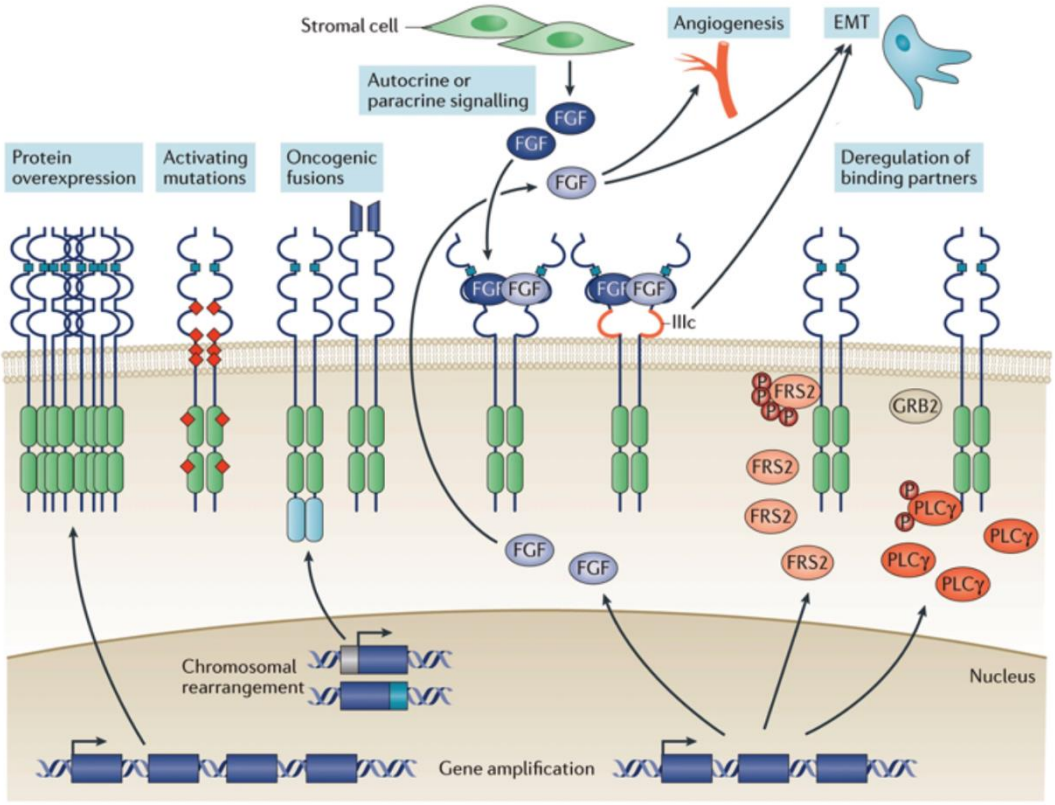

Figure 4: (modified from Babina and Turner 2017) **Deregulation of FGFR signaling in cancer.** Deregulation of FGFR signaling may contribute to oncogenesis through some genetic mechanism such as FGFR gene amplification and consequently protein overexpression, activating mutations resultant in receptor dimerization or kinase domain constitutive activation also in the absence of the ligand, chromosomal translocations that may create FGFR aberrant fusion with other proteins at any carboxy terminus, leading to encreased receptor dimerization (blue fusion), or amino terminus, leading to receptor hyperactivation in a ligand-indipendent manner (grey fusion). Otherwise FGFRs can be overstimulated by their ligands secreted by the tumor cells (light blue) or stromal compartment (dark blue); these ligands may also induce tumor-associated angiogenesis. As a conseguence of a genetic mechanism or an extracellular stimulus, the FGFR IgIII domain can be alternatively spliced from the epithelial IIIb to the mesenchymal IIIc isoform, altering the receptor ligand specify and leading to autocrine signaling and EMT induction. Oncogenic deregulation of FGFR signaling may also be ascribed to gene amplification and consequentlu protein overexpression of FGFR binding partners such as FRS2 and PLCγ.

FGFR gene amplification often turns into protein overexpression, causing an increased receptor accumulation and triggering the downstream signaling pathways. Amplification can occur quite frequently for FGFR1 in squamous non-small-cell lung carcinoma (NSCLC) (Weiss et al., 2010; Yang et al., 2014), small-cell lung carcinoma (Peifer et al., 2012) and is considered an adverse prognostic marker in earlystage NSCLC (Cihoric et al., 2014) and in a significant percentage of hormone receptor-positive and triple negative breast cancer (Courjal et al., 1997; ReisFilho et al., 2006; Lee et al., 2014). Amplification of FGFR2 is less frequent than FGFR1 but has been described in aggressive subtype of gastric cancer (Matsumoto et al., 2012) and in 2% of breast cancer (Turner et al., 2010). Amplification of FGFR3 and FGFR4 are not frequently reported. Alternatively, to receptor gene amplification, FGFR activating mutations can induce increased receptor dimerization in the absence of ligand or constitutive activation of the kinase domain. FGFR2 mutations are found in 10–12% of endometrial carcinomas (Dutt et al., 2008; Helsten et al., 2015), approximately 4% of NSCLCs and gastric cancers (Greenman et al., 2007), and 2% of urothelial cancers (Gao et al., 2013). An alternative splicing event with welldefined cell type-specificity and functional effects is the oncogenic mechanism that provokes the mutually exclusive splicing of the FGFR IgIII loop from the epithelial IIIb to the mesenchymal IIIc isoform. This switch modifies the ligand specificity of the receptor, causing in altered autocrine signaling.

The splicing from the epithelial FGFR2IIIb isoform to the aberrant mesenchymal FGFR2IIIc isoform makes the cell responsive to new ligands as FGF2 but not FGF7 and induce EMT in normal murine mammary cells (Shirakihara et al., 2011) and epithelial human cells (Ranieri et al., 2015; Ranieri et al., 2016) provoking tumorigenic features (Oltean et al., 2006; Zhao et al., 2013; Ranieri et al., 2015; Ranieri et al., 2016). FGFs may play an active role in oncogenesis overstimulating their receptors in an autocrine or paracrine approach. FGF2 can trigger pathological EMT, wound healing (Broadley et al., 1989; Ortega et al., 1998; Ranieri et al., 2016) and angiogenesis by promoting cancer progression (Zhao et al., 2013; Oltean et al., 2006; Shirakihara et al., 2011) of epithelial cells alone (Ranieri et al., 2018) or in combination with vascular endothelial growth factor (VEGF) (Pepper et al., 1998; Yan et al., 2008). It is known that the Epithelial Splicing

Regulatory Proteins 1 and 2 (ESRP1 and ESRP2) control alternative splicing events of several genes (Warzecha et al., 2009) including the FGFR2 (Warzecha et al., 2009). Splicing regulators are RNA binding proteins (RBPs) that exert alternative mRNAs transcripts from a single gene choosing between different exon sequences. This post-transcriptional regulation of the mRNAs encoding RBPs can also influence cell type-specific splicing decisions. The epithelial isoform FGFR2b is controlled by ESRP1 and ESRP2 (Warzecha et al., 2009). It is widely demonstrated as the expression of ESRP1 and ESRP2 correlate with the expression of FGFR2IIIb and its silencing force the FGFR2IIIc isoform switch (Ranieri et al., 2015). One of the causes in epithelial context of downmodulation of ESRP1/2 proteins and the consecutive FGFR2 isoform switch toward mesenchymal phenotype is the HPV16 infection and in particular the expression of the viral oncoprotein E5 (16E5) (Ranieri et al., 2015). FGFR2 aberrant isoform switch causes a modification in the downstream signaling that turns upside-down both morphology and cellular behavior. In the epithelial context, FGFR2b stimulated by FGF7 triggers divergent responses, such as autophagy through the JNK1-mediated signaling, MTOR-independent pathway (Russell et al., 2014; Nanni et al., 2018) as well as proliferation (Belleudi et al., 2011) and differentiation (Belleudi et al., 2011; Rosato et al., 2018). FGFR2b/FGF7 signaling controls the early, and the late steps of keratinocyte differentiation (Belleudi et al., 2011; Purpura et al., 2013) by the sequential involvement of PKCδ and PKCα respectively (Rosato et al., 2018). Instead, the isoform switch of FGFR2b and the consequent expression, out of context, of the FGFR2c changes the cellular responsiveness to the ligand FGF2. FGFR2c/FGF2 axis not only induces EMT as above mentioned (Ranieri et al., 2015; Ranieri et al., 2016) but inhibits differentiation, promotes invasion (Ranieri et al., 2018) and has a negative impact on autophagy (Nanni et al., 2019). It has been recently demonstrated that, after FGF2 stimulation, the FGFR2c does not induce the activation of PKCδ, probably counteracting differentiation process, but has an impact on the activity of PKCε (Ranieri et al., 2020). PKCs are a class of finely regulated serine-threonine kinases that are essential for the control of intensity as well as spatial distribution of the signals (Isakov et al., 2018; Rosse et al., 2010). PKCs can be recruited to membrane protein scaffolds,

where these may control the behavior of protein complexes influencing their assembly state, their subcellular localization and their ability to recruit downstream effectors (Rosse et al., 2010). Recent studies showed that the different specificity in PKC isoform activation displayed by FGFR2b and FGFR2c is consistent with the recent advanced hypothesis that the alternative splicing could represent a powerful mechanism to diversify FGFR signaling (Latko et al., 2019). PKCε has been found overexpressed in several carcinomas, including squamous cell carcinomas (SCCs) (Gorin et al., 2009; Isakov et al., 2018). Among PKC family members, PKCε shows the greatest oncogenic potential (Isakov et al., Semin, 2018), in fact its overexpression is a sufficient requirement to increase growth rate and motility in human keratinocytes (HKs) (Papp et al., 2004). PKCε regulates several cellular processes not only via the phosphorylation of its downstream substrates, but also through their intracellular re-localization (Newton et al., 2010) as well as it can induce EMT phenotype in normal breast cells (Jain et al., 2014a; Jain K et al., 2014b). Recently it has been demonstrated how PKCε, downstream FGFR2c, act upstream an oncogenic signaling network leading to the induction of EMT-related transcription factors such as STAT3, Snail1 and Fos-Related Antigen-1 (FRA1), and Signal Transducer and Activator of Transcription 3 (STAT3), which are probably sequentially induced and cooperate with each other in triggering and sustaining the EMT program (Figure 5) (Ranieri et al., 2020).

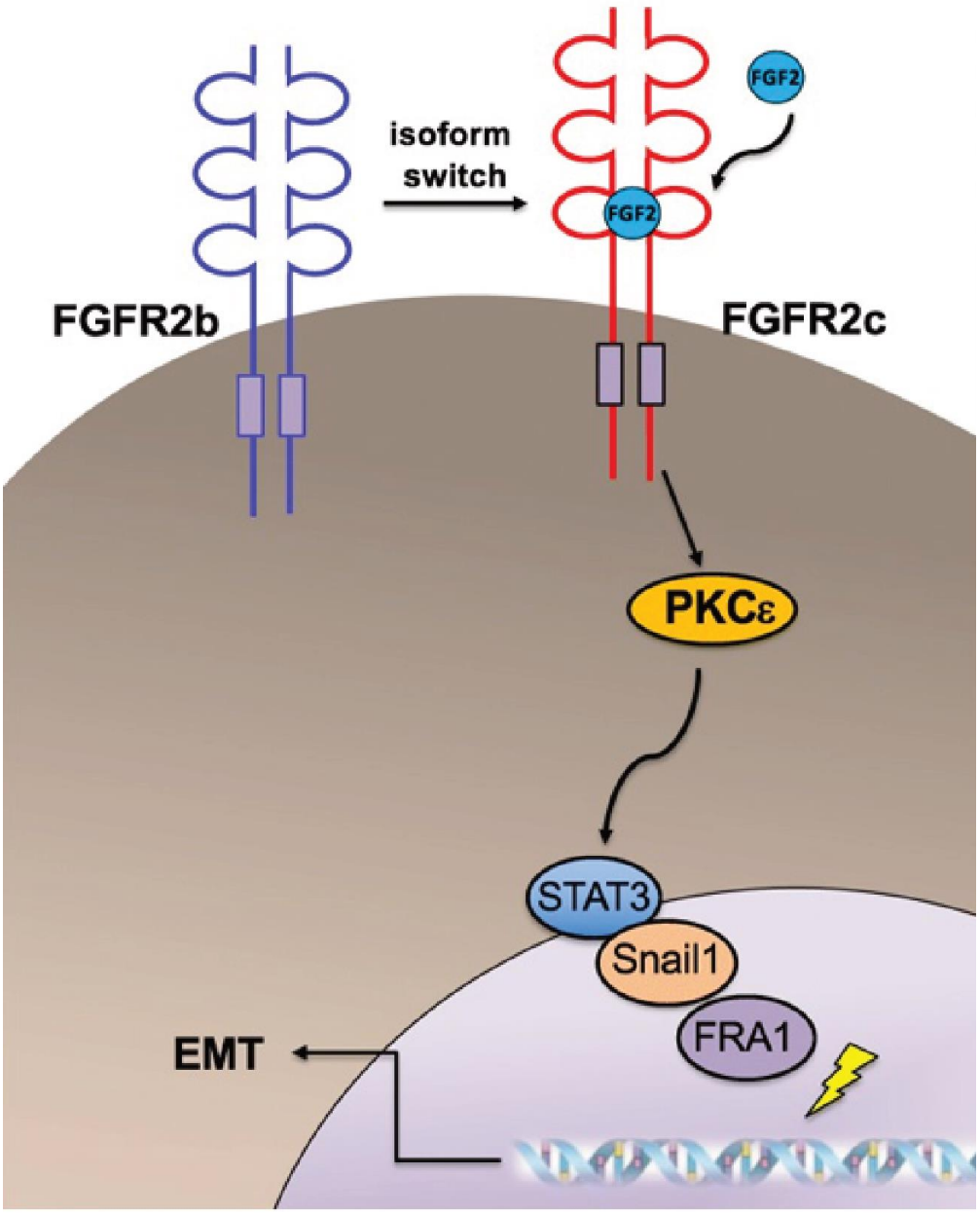

Figure 5: (modified from Ranieri et al., 2020) **Schematic drawing of FGFR2c downstream signaling of EMT induction.** PKCε acts downstream FGFR2c upregulating three EMT-related trascription factors STAT3, Snail1 and FRA1 which are induced in cascade and cooperate with each other in triggering and sustaining the EMT program.

Epithelial-Mesenchymal Transition

The epithelial cells plasticity allows reversibly to have a transition into mesenchymal cells, either partially or fully. Even if the EMT process may

present some variations, which depend on the cell type, tissue context and activating signals, several processes remain unchanged during the EMT process (Zada et al., 2021). The epithelial cell–cell contacts such as tight junctions, adherents' junctions, desmosomes and gap junctions disassembled and there is loss of cell polarity caused by the disruption of the Crumbs complex, a key regulator of cell polarity and cell shape (Lamouille et al., 2014). Cells that begin EMT reorganize their cortical actinic cytoskeleton in different structures such as actin stress fibers, sheet-like protrusions called lamellipodia and spike-like extensions called filopodia at the edge of lamellipodia. This new actin structure facilitates dynamic cell extension, motility and employs a proteolytic function in ECM degradation (Lamouille et al., 2014). The expression of epithelial genes is inhibited, including those that encode cell junction complexes and at the same time mesenchymal genes are activated. In addition, the integrin composition changes, the epithelial α6β4 integrin that links cells with the basement membrane is inhibited, and the mesenchymal α3β1, α5β1, α1β1 integrins, that bind laminin, fibronectin and type I collagen respectively are upregulated. Moreover, the intermediate filament composition changes from epithelial to mesenchymal type, with the expression of vimentin that replaces cytokeratin (Lamouille et al., 2014). Gene reprogramming for the switch from the epithelial to the mesenchymal phenotype involves some master regulators, including Snail, Twist and ZEB transcription factors (Lamouille et al., 2014). Snail is uniquely required for triggering EMT, while Twist1, that directly bind E-boxes in promoters, activating ZEB1 (Zinc Finger E-Box Binding Homeobox 1), is determinant in EMT duration (Tran et al., 2011). A negative feedback loop is demonstrated between ZEB1 and miR 200 expression, in fact if ZEB protein down modulates miR 200, on the other hand miR 200 suppresses ZEB expression too (Brabletz and Brabletz, 2010). Downmodulation in miR 200 expression results in increased ZEB1 and ZEB2 levels and EMT progression (Brabletz and Brabletz, 2010). A similar feedback loop also occurs between miR-203 and Snail (Moes et al., 2012). During EMT, Snail1 can be induced by the transcription factor STAT3 which is which is upregulated in several human carcinomas, including head and neck squamous cell carcinoma (HNSCC), breast, ovary, prostate, and lung cancer (Chan et al., 2004; Kobielak and Fuchs 2006). FRA1 is a

member of the FOS family of transcription factors, is a transcriptional target of Twist and Snail and acts as an effector of the EMT program and is required for its execution (Tam et al., 2013).

EMT has been classified into three different types depending on the tissue context and the type of cells that this transition generates (Debnath et al., 2021; Kalluri and Weinberg, 2009; Zeisberg and Neilson, 2009) (Figure 6).

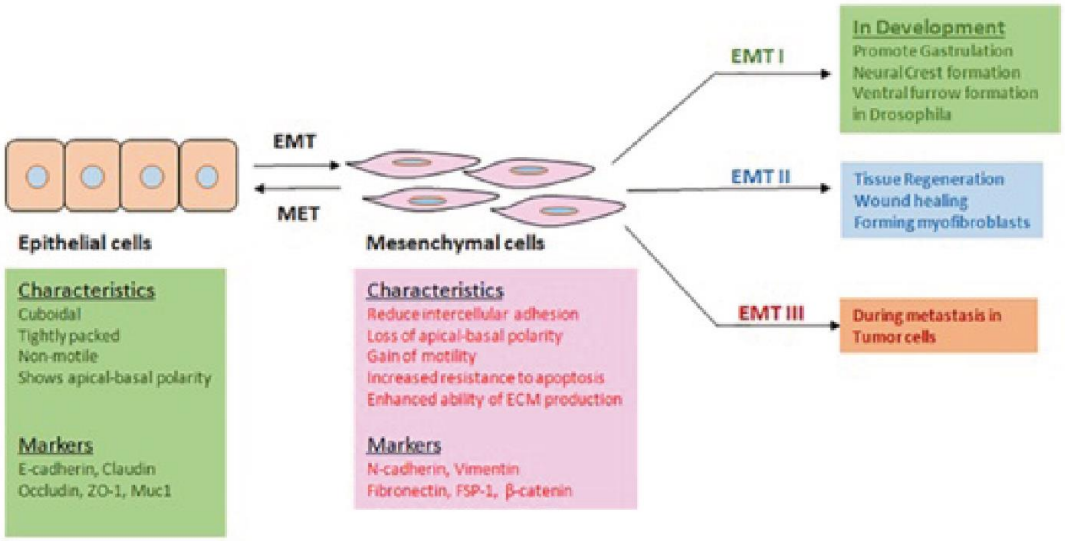

Figure 6: (modified from Debnath et al., 2021) **EMT: characteristics, markers and contexts.** EMT is activated in different physiological contexts, thereby facilitating cellular movement. It is associated with both morphological and characteristic changes

Type I EMT is associated with implantation, embryo formation and organ development; this process generates mesenchymal cells that have the potential to subsequently undergo the reverse process, MET, to generate secondary epithelia (Thiery et al., 2009). Type II EMT is associated with wound healing, tissue regeneration and organ fibrosis. In this case, the EMT program belongs to a series of repair-associated events that normally reconstruct tissues following trauma and inflammatory injury. In this case, cells undergoing EMT leave the epithelial layer, find their way through the basement membrane, and accumulate in the interstitial space of the tissue where they ultimately shed all their epithelial markers and gain a fully fibroblastic phenotype (Leopold et al., 2012). However, in contrast to type I EMT, type II EMT is associated with inflammatory stimulus and in physiological conditions, it ends once inflammation is attenuated. If inflammation persists, ongoing type II EMT continues unabated and the

process degenerates into a pathological condition, called fibrosis, leading eventually to organ destruction. EMT process is associated with fibrosis occurring in several organs like kidney, liver, lung, and intestine (Thiery et al., 2009; Kalluri and Weinberg, 2009; Zeisberg and Neilson, 2009). Type III EMTs has only a pathological implication since it occurs in neoplastic epithelial cells that have previously undergone genetic and epigenetic changes, affecting oncogenes and tumor suppressor genes.

This already transformed cell takes advantage of EMT mechanism to acquire a malignant phenotype in order to achieve the subsequent steps of the invasion-metastasis cascade, such as invasion through the basement membrane, intravasation, transport through the circulation, extravasation, and finally formation of micro metastasis with life-threatening consequences. Metastasizing cancer cells must shed their mesenchymal phenotype via a MET during secondary tumor formation (Thiery et al., Cell 2009; Kalluri and Weinberg, 2009; Zeisberg and Neilson, 2009).

There is still an open-question about the comparison between tumor EMT and wound healing related-EMT; since EMT types II and III share a set of genetic and biochemical elements, it's difficult to discriminate a physiological early event that transform a mature, differentiated keratinocyte into a migrating cell and the pathological initial transformation of a primary tumor cell into a metastatic tumor cell (Leopold et al., 2009). In both cases, cells must be able to change form, alter actin cytoskeleton, reprogram gene expression, modify the extracellular matrix and then, secrete proteases to help in the process. Rapid responses and ongoing development of the wound healing response is coordinated by a series of growth factors (Barrientos et al., 2008). In wounding of epidermal cells, platelet clot formation induces the release of a multitude of growth factors. These growth factors attract macrophages and leukocytes. They all together (keratinocytes, platelets, macrophages and leukocytes) release a variety of additional growth factors including epidermal growth factor (EGF), transforming growth factor α (TGFα), and transforming growth factor β1 and 2 (TGFβ1, TGFβ2). Later other growth factors act on keratinocytes including TGFβ3 from macrophages, insulin-like growth factor (IGF) or FGF7 from fibroblasts and epidermal cells and hepatocyte growth factor (HGF).

Previous studies demonstrated that FGF7 promotes keratinocyte migration (Tsuboi et al., 1993; Ceccarelli et al., 2007; Belleudi et al., 2011) confirming its important contribution in wound healing and repair (Finch and Rubin, 2004). In addition, several other growth factors such as platelet derived growth factor (PDGF) and TNFα are actively involved in wound healing (Leopold et al., 2012).

The process of EMT for cancer cells shares with wound healing the central role of growth factors. Like wound healing, EMT is provoked by increased levels of EGF, TGFβ1 and PDGF. In addition, hepatocyte growth factor (HGF) triggers the c-MET pathway that is often part of the EMT program (Leopold et al., 2012; Lamouille et al., 2014). In addition to the similarity of growth factors and receptors involved, the intracellular signaling pathways are also matched and they include Rho activity, Ras/ERK/MAPK pathway, the PI3K/Akt/mTOR axis, p21 axis and GSK3 activities (Leopold et al., 2012; Lamouille et al., 2014).

On the other hand, a crucial difference between the two processes is on the type of cell movement: while type III EMT is characterized by the migration of isolated mesenchymal-like cells, already lacking strong adherent junction, during wound healing cells show a collective movement in which cell-junctions (and consequently E-cadherin expression) are conserved (Friendl and Wolf, 2009; Friendl and Gilmour, 2009). An important hallmark of tumor EMT is not only the downregulation of E-cadherin, to bolster the destabilization of adherent junctions, but also the concomitant de novo expression of mesenchymal neural cadherin (N cadherin), the so-called “cadherin switch” (Wheelock et al., 2008; Yilmaz and Christofori, 2009). Through this switch, the tumor cell loses contact with adjacent epithelial cells and acquires an affinity for mesenchymal cells through homotypic N-cadherin interactions. These interactions are weaker than homotypic E cadherin interactions and facilitate cell migration and invasion (Lamouille et al., 2014). N-cadherin mediated cellcell adhesion causes the collective cell invasion mode in epithelial cells undergoing EMT (Shih et al., 2012).

In addition to “cadherin-switch”, a transcription factor belonging to Snail family, Snail1, allows to distinguish wound healing from tumor EMT: while Snail2 is up-regulated in both wounded epithelium and in tumor cells

undergoing EMT (Savagner et al., 1997; Savagner et al., 2005; Arnoux et al., 2009; Kusewitt et al., 2009, Hudson et al., 2009; Shirley et al., 2010), Snail1 is only involved in EMT (Ranieri et al., 2020) and has not been shown to be a major player in keratinocytes during wound healing (Savagner et al., 2005; Sou et al., 2010). Finally, while the hemidesmosome component α6β4-integrin persists during wound healing where it translocated at the leading edge of migrating cells (Santoro and Gaudino, 2005; Maschler et al., 2005; Sehgal et al., 2006; Margadant et al., 2006) it down modulated during EMT.

Previous works of our research group showed that while FGFR2b expression and signaling induce keratinocyte migration (Ceccarelli et al., 2007; Belleudi et al., 2011), the out-of-context expression of FGFR2c in these cells triggers tumorigenic features and EMT, demonstrated by the downmodulation of epithelial markers (E-Cadherin and β4-integrin) and the upregulation of mesenchymal markers (N-Cadherin) (Ranieri et al., 2015; Ranieri et al., 2016; Ranieri et al., 2018). More recently we better characterized the signaling pathway downstream FGFFR2c mainly involved, demonstrating that PKCε acts inducing in cascade the EMT-related transcription factors STAT3, Snail1 and FRA1 (Ranieri et al., 2020).

Autophagy

Autophagy is a degradative pathway conserved from yeast to mammalians, used for recycling or discarding cytoplasmic components, such as damaged organelles, membranes and molecules, in order to preserve the cellular homeostasis or to fit the cells to stress conditions (Feng et al., 2014). There are three forms of autophagy: macro autophagy, micro autophagy and chaperone-mediated autophagy (Zada et al 2021). In macro autophagy substrates are isolated in autophagosomes, composed of double-phospholipid membrane structure. This vesicle fuses with lysosome promoting the degradation of its inner membrane and the substrates on it by lysosomal enzymes (Mizushima et al., 2011). The second type of autophagy is chaperone-mediated autophagy (CMA). Here, the cytosolic

proteins are marked by a pentapeptide motif tag with a consensus sequence, then recognized and selectively caught to the lysosomal membrane by the chaperon protein Hsc70, which promotes the translocation into the lysosomal lumen through the binding to LAMP-2A. The third type is micro autophagy, and it is used when part of the cytoplasm is engulfed by direct invagination of the lysosomal membrane (Ravikumar et al., 2010; Mizushima et al., 2011).

Macro autophagy, commonly called autophagy, plays an important role in maintaining cellular homeostasis, is the most extensively studied membrane pathway and is present at basal level. This process can be up-regulated by several stimuli as a cytoprotective response against nutrient starvation (glucose or amino-acid withdrawal), hypoxia, oxidative stress, pathogen infection, radiation and anticancer drug treatment (Yang and Klionsky, 2010). Macro autophagy can also be a selective process to remove specific damaged organelles, such as mitochondria (mitophagy) and peroxisomes (pexophagy) (Deffieu et al., 2009; Dunn et al., 2005), or invasive pathogens (xenophagy) (Levine et al., 2011) to the lysosomal compartment (Figure 7).

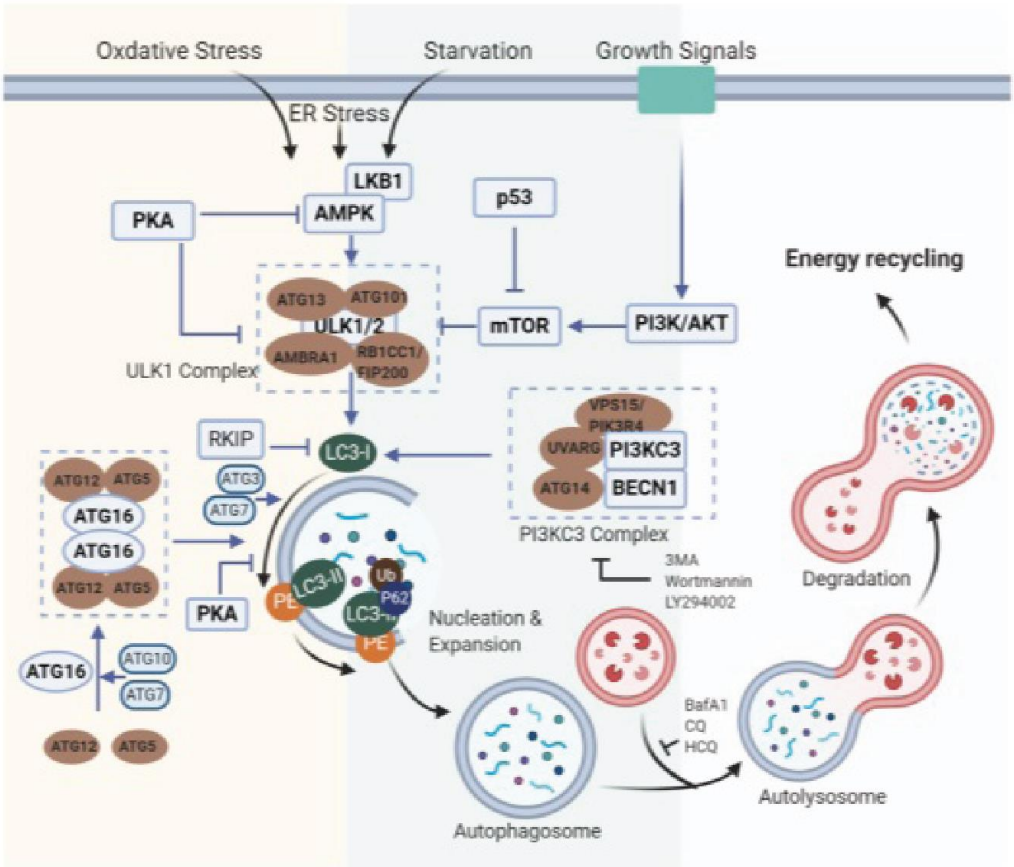

Figure 7: (modified from Zada et al., 2021) **Autophagy steps.** Autophagy involves multiple steps: induction, nucleation, vescical expansion and lysosomal fusion. A few critical signal complexes are involved in the regulation of autophagy induction. The ULK1/2 complex regulates the initiation of autophagy together with PI3KC3 (class III PI3K) complex. It is composed of several proteins, including ATG13, ATG10, AMBRA1 and FLIP200. The ULK1/2 complex is controlled by either activator (e.g. AMPK) or inhibitor signals (e.g. mTOR pathway or PKA). thePI3KC3 complex, comprising classIII PI3K with BECN1, UVRAG, PIK3R4 and ATG14 proteins, is specifically inhibited by chemical compounds such as 3.methylaldenine (3-MA) and wortmannin, as well as protein such as BL2 or RUBICON. LC3 is lipidated to phosphatidylethanolamine (PE) via two ubiquitin-like conjugation proteins. PE-conjugated LC3s (LC3-II form) are formed via nucleation and vesical expansion resulting in autophagosomes, followed by fusion with lysosomes facilitated by other proteins such as SNARE or Rabs to degrade its contents for energy recycling. The fusion with lysosomes is inhibited by compounds such as chloroquine (CQ), hydroxychloroquine (HCQ) and bafilomycin A1 (Baf-A1)

The autophagic machinery consists of about 40 autophagy-related genes (ATGs) originally identified in yeast. Many of these genes have orthologs in mammalians. Even if they show important differences in biology and architecture, they have been defined as the core autophagy genes, involved in autophagosome biogenesis (Lamb et al., 2013; Bento et al., 2016). The process for the formation of a mature autophagosome can be divided in three step: the initiation, that is the transmission of the signal to the membrane, with consequent recruitment of the initiating complexes; the nucleation, that leads to the formation of the isolation membrane from the membrane source of the signal; the expansion, where the isolation membrane expands until close completely, forming the autophagosome (Mizushima et al., 2011; Lamb et al., 2013; Feng et al., 2014).

The first involved in the stage of initiation is the ULK1/2 complex. The ULK complex is made of ULK1/2, ATG13, FIP200 and ATG101. ULK1/2 cooperates with ATG13, which directly binds FIP200 (Hosokawa et al., 2009; Jung et al., 2009). The ULK1/2 complex can be activated in an AMP-activated protein kinase (AMPK)-dependent way for glucose starvation in Ser317 and Ser777 or for amino acid starvation in AMPK-independent manner. Under fed conditions the ULK1/2 complex is inhibited by the binding with mTORC1 and phosphorylation on Ser757.

On the contrary, upon nutrient starvation or treatment with the mTOR inhibitor, rapamycin, mTORC1 (see below) is released from the ULK1/2 complex leading to its activation and autophagy initiation (Ganley et al., 2009; Hosokawa et al., 2009; Jung et al., 2009; Kim et al., 2011). Another

important complex required for the initiation step of autophagosome formation is the class III phosphatidylinositol (PI) 3-kinase (PI3K) complex made of p150, Beclin 1 and ATG14. This complex is essential for the generation of phosphatidylinositol 3-phosphate (PI3P) (Mizushima et al., 2011). Beclin 1 is a key protein of the PI3K complex since it interacts with Vps34 and enhances its activity. Many other proteins can interact with Beclin 1 positively regulating autophagy, such as AMBRA1 and Bif-1, or negatively like BCL-2 that inhibits autophagy by sequestering Beclin 1 from the PI3K complex (Funderburk et al., 2010). During the nucleation step ULK1/2 and the PI3K complexes are recruited to the membrane site of autophagosome initiation. Here the ULK1/2 complex can phosphorylate different proteins like Beclin 1 and its interacting protein AMBRA1 at Ser and Thr residues, which in turn enhance the activity of the PI3K complex (Di Bartolomeo et al., 2010; Russell et al., 2013). The PI3K complex generates a pool of PI3P in the membrane, necessary for the recruitment of other ATG proteins or autophagyspecific effectors. In the elongation, two ubiquitin-like proteins are involved in the autophagosome membrane expansion and closure: ATG12 and LC3.

ATG12 is conjugated to ATG5 through a mechanism that requires ATG7, an E1-like enzyme, and ATG10, an E2-like enzyme (Mizushima et al., 1998). The ATG12-ATG5 complex interacts with ATG16L1, forming the ATG12-ATG5ATG16L1 complex, which is recruited to the outer side of the isolation membrane. At the same time cytosolic LC3 is cleaved in the C-terminal by the cysteine protease ATG4, leaving a glycine residue, which is subsequently activated by ATG7. Finally, the E2-like enzyme ATG3 and the ATG12-ATG5- ATG16L1 complex promote the conjugation of LC3 to the phosphatidylethanolamine (PE), generating lipidated LC3 (LC3-II), which associates to the autophagosomal membrane (Kabeya et al., 2004; Sakoh-Nakatogawa et al., 2013). This complex is released upon the autophagosome closure (Mizushima et al. 2001), while the LC3-II localized in the inner membrane of the autophagosome is retained (Kabeya et al., 2004). Recent evidence has shown also a role for actin filaments in autophagosome shaping. During the nucleation step, the actin-capping protein (CapZ) binds to PI3P and stimulates actin polymerization and branching, in the inner face of the isolation membrane (Aguilera et al., 2012;

Mi et al., 2015). This evidence strongly suggests a new important role for actin in the autophagosome biogenesis. The origin of the autophagosomal membrane remains unclear. Among the different possible membrane sources, the ER seems to be the best candidate, while the other organelles may contribute to the autophagosomal membrane expansion during the elongation step (Lamb et al., 2013; Ktistakis and Tooze, 2016; Bento et al., 2016). Other membrane sources seem to be required for autophagosome formation like mitochondria and particularly the outer membrane of these organelles. In fact, during serum-starvation autophagy, ATG5 and LC3 colocalize with mitochondria, and their membranes seem to be in contact with the autophagosome (Hailey et al., 2010). Moreover, another theory about the autophagosome biogenesis proposes the ER-mitochondria contact site as a candidate in this process, since ATG14 and ATG5 were found localized in this site (Hamasaki et al., 2013).

Many signaling pathways can control the autophagic process after serum deprivation, hypoxia or stress conditions but the most important is the mTOR pathway (Ravikumar et al., 2010; Russel et al., 2014). mTOR (mammalian target of rapamycin) is a serine/threonine kinase, which can form two different complexes, mTORC1 and mTORC2, among which only the mTORC1 complex is involved in autophagy regulation (Jewell et al., 2013). mTORC1 complex consists of five subunits: TOR, RAPTOR (regulatory-associated protein of mTOR), mLST8 (mammalian lethal with SEC13 protein), DEPTOR (DEP domain-containing mTOR-interacting protein) and PRAS40 (40 kDa Pro-rich AKT substrate) (Laplante and Sabatini, 2012). Under nutrient-rich conditions mTORC1 is active and associated to lysosomes. Here mTORC1 is activated by the small GTPase Rheb (Garami et al., 2003; Inoki et al., 2003; Tee et al., 2003). During growth factors stimulation, the main pathway involved in Rhebmediated activation of mTORC1 is the PI3K/AKT pathway, (Inok et al., 2002; Potter et al., 2002). mTORC1 activation acts as a powerful inhibitor of the autophagic process. In fact, active mTORC1 can inhibit the two autophagic complexes involved in autophagy initiation, ULK1/2 complex and PI3K complex (Russel et al., 2014). A way to inhibit mTORC1 and induce the autophagic process is via the serine/threonine kinase AMPK. This kinase inhibits mTORC1 by the activation of TSC, a complex involved in the

release of mTORC1 from lysosomes and consequently in its inhibition. AMPK can also directly inhibit mTORC1 via its phosphorylation (Gwinn et al., 2008) or directly activating ULK1 on Ser317 and Ser777 (Kim et al., 2011; Bach et al., 2011), or Vps34, through Beclin 1 phosphorylation on Ser91 and Ser94 (Kim et al., Cell 2013). Upon serum starvation AMPK provokes a transient activation of MAPK/ERK pathway, the consequent increase of Beclin 1 expression, mTORC1 disassembly and so the autophagy induction as cytoprotective mechanism. Autophagy induced by serum starvation can also be activates via c-Jun N-terminal protein kinase 1 (JNK1) which phosphorylates BCL-2 and allows the release of Beclin 1 from the autophagic inhibitor complex BCL2/Beclin 1 (Wei et al., 2008).

Several GF/RTKs signaling pathways, including those activated by FGF/FGFRs can employ a regulatory control on the autophagic process. Insulin-like growth factor-1 (IGF1) (Sobolewska et al., 2009) as well as plateletderived growth factor (PDGF) (Takeuchi et al., 2004), negatively regulate autophagy through the activation of the PI3K/AKT/mTOR pathway. It has been defined that PDGF can induce autophagy in vascular smooth muscle cells (VSMCs) via a PI3K/AKT/mTOR-independent pathway (Salabei et al., 2013).

In addition, an inhibitor role on autophagy through a canonical mTORdependent mechanism has been shown also for the epidermal growth factor (EGF) (Sobolewska et al., 2009). A further mTOR-independent inhibitory mechanism on autophagy, which directly affects Beclin 1 inhibition, has been described for EGF/EGFR (Wei et al., 2013). Some evidence proved contrasting roles for FGFRs on autophagy regulation. It has been shown that FGFR2b has a key function in the regulation of epidermal early differentiation as mentioned above and downstream in this process is involved the phosphoinositide 3-kinase (PI3K/AKT) signaling (Belleudi et al., 2011). Much evidence demonstrated a close interplay between differentiation and autophagy for many cell types, including keratinocytes (Haruna et al., 2008;
Moriyama et al., 2014; Akinduro et al., 2016; Belleudi et al., 2014; Chikh et al., 2014). The activation of FGFR2b by FGF7 triggers autophagy in keratinocytes (Belleudi et al., 2014) and this effect depends on FGFR2b

expression and activation through a PI3K/AKT/mTOR-independent pathway (Belleudi et al., 2014; Belleudi et al., 2015). JNK1 is a signaling hub that regulates autophagy downstream FGFR2b signaling in keratinocytes (Nanni et al., 2018) and in other contexts as the bone growth (Cinque et al., 2015). JNK1 signaling triggers an mTOR-independent autophagy via BCL-2 phosphorylation and consequent beclin-1 release from its inhibitory complex (Wei et al., 2008; Russell et al., 2014; Zhou et al., 2015). However, autophagy not only regulates several biological functions, such as cell differentiation, but can also play either onco-suppressive or oncogenic roles in cancer, depending on its stage (see below). In particular, while during the initial steps of tumorigenesis autophagy appears to be linked to a negative loop to EMT, in established tumors this process has a pro-survival effect and its possible interplay with EMT remains still debated (see below). Therefore, keeping in mind the role of FGFR2c in driving EMT (Ranieri et al., 2015; Ranieri et al., 2016; Ranieri et al., 2018; Ranieri et al., 2020), a recent work of our research group established the role of the aberrant expression of FGFR2c in autophagy, demonstrating that this receptor and its signaling in epithelial context negatively interfere with the autophagic process, also suggesting that this interference could significantly contribute to cancerogenesis (Nanni et al., 2019). FGFR2c-induced repression of autophagy involves the AKT/MTOR pathway, which also inhibits JNK1 signaling (Nanni et al., 2019) another pathway which activates autophagy via BCL-2 phosphorylation and consequent direct release of Beclin-1. In fact, JNK1 and BCL-2 phosphorylation appeared repressed by FGFR2c activation, but strongly activated upon AKT/MTOR shut-off resulting in autophagy stimulation (Nanni et al., 2019). Indeed, the obtained observations are in line with previous findings showing that AKT and JNK can be interplaying pathways, with AKT signaling able to inhibit JNK activation (Zhao, 2015), possibly antagonizing the formation of the MAPK8IP1/JIP1-JNK complex (Kim et al., 2002; Pan et al., 2006).

Crosstalk between autophagy and EMT in cancer

Context-dependent opposite roles in cancer have been recently proposed for the degradative pathway of autophagy (reviewed in Chen et al., 2019; Zada et al., 2021; Gundamaraju et al., 2022). In fact, this process can prevent cancer initiation by removing intracellular mutagens and damaged mitochondria (which release high quantity of mutagenic ROS), but it can also adjuvate cancer development, for instance providing nutrients for tumor cell metabolism and precursors for the rapid macromolecule biogenesis, required during cancer cell proliferation. Also, in the specific context of pancreatic adenocarcinoma (PDAC), most evidence point to autophagy as survival strategy contributing to the malignant progression (Piffoux et al., 2021; Mollinedo and Gajate 2019; New and Tooze 2019), even if some findings have suggested a tumor suppressive role for this process, preventing cancer development at its early stages (Rosenfeldt et al., 2013; New and Tooze 2019). Autophagy and EMT are recognized as the major key biological events in cancer pathogenesis and several observations have highlighted that they are linked by a tumor type- and tumor stage-dependent crosstalk, which involve several intersecting oncogenic signaling pathways (reviewed in Chen et al., 2019; Zada et al., 2021; Gundamaraju et al., 2022) (Figure 8 A). For these reasons, to clarify the molecular mechanisms underlying this crosstalk is a very hard work, but it represents an essential goal to identify novel targets, for more effective cancer therapies. The relationship between EMT and autophagy is complex because accumulating findings have recently suggested that autophagy may be activated or inhibited by EMT-related signaling pathways. On the other hand, autophagy may also play an important “double edge sword” role in the initiation and in the suppression of EMT (reviewed in Chen et al., 2019; Zada et al., 2021; Gundamaraju et al., 2022) (Figure 8 B). In fact, autophagy has been found to be able to selectively degrade key transcription factors of EMT and to stabilize cell-cell tight junctions in transitioning cells (Figure 8 B). Thus, at the early stage of tumor development, active autophagy appears to play a tumor suppressive role counteracting the transition to mesenchymal phenotype. By contrast, in some advanced tumors, sustained

autophagy appeared not only to improve cell survival and metabolism, as reported above, but also to govern the balance between EMT and the opposite process MET via the regulation of EMT protein levels (reviewed in Chen et al., 2019; Zada et al., 2021; Gundamaraju et al., 2022) (Figure 8 B). Therefore, in these cancer types, autophagy inhibition might result in an useful strategy to counteract advanced EMT and tumor metastatic progression.

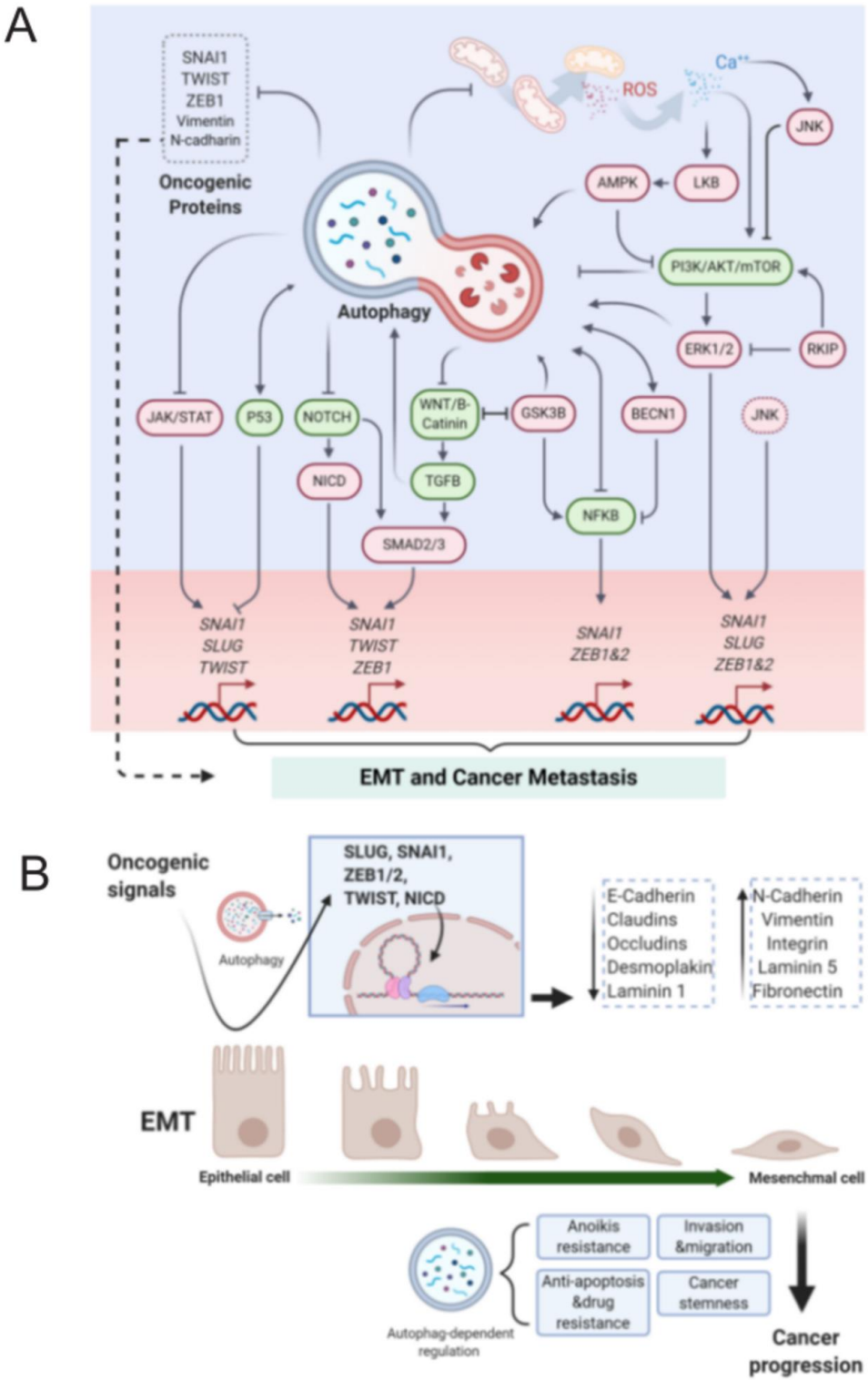

Figure 8: (modified from Zada et al., 2021) **The crosstalk between autophagy and cancer-associated signaling pathways in cancer.** A. During cancer progression (EMT and cancer metastasis), any signaling pathways are functionally connected during the inhibition or activation of autophagy. Activation and inhibition of proteins or pathways is indicated by arrows and bar-heads, respectively. B. Epithelial-like cells are transformed to mesenchymal-like cells by oncogenic signals via the activation of EMTassociated transcription factors such as SNAI1, SLUG, ZEB1, TWIST, and NOTCH1. The intracellular level or activation of these mediators is partly regulated by autophagy. EMT induces an increase in mesenchymal markers such as N-Cadherin and Vimentin and a decrease in epithelial markers such as E-Cadherin and Claudins. Mesenchymal-like cells exhibit specific cellular properties: Anoikis resistance, invasion and migration, anti-apoptosis, drug resistance, and cancer stemness. These mesenchymal properties are functionally associated with the regulation of autophagy in cancer cells.

On the base of its ability to differently regulate EMT, now several drugs targeting autophagy (activators and inhibitors) are under investigation for clinical trials in cancer diseases. Among them, autophagy inhibitors as adjuvants for classical chemotherapies is the most experimented. In particular, hydroxychloroquine (HCQ) (which is a derivative of chloroquine showing less toxicity), that inhibits autophagy by preventing lysosomal acidification, is widely studied in a variety of solid and blood tumors.

On the light of previous data collected by our research group demonstrating the ability of the aberrant expression of the mesenchymal FGFR2c isoform in inducing EMT signature (Ranieri et al., 2016; Ranieri et al., 2018), as well as the inhibition of autophagy (Nanni et al., 2019), in this project we wondered if this receptor might be able to upstream activate a complex oncogenic signaling network, resulting in the establishment and in the control of a negative crosstalk between EMT and autophagy and we investigated this possibility in both human keratinocyte and PDAC context.

Pancreatic Ductal Adenocarcinoma (PDAC) and FGFR2 isoform switch

Tumors affecting the exocrine glands are called adenocarcinomas. The majority of pancreatic cancer (about 95% of pancreatic cancers) involves the exocrine pancreas and initiates in the ducts when the exocrine cells start to grow out of control (Singhi and Wood 2021). Pancreatic ductal adeno

carcinoma (PDAC) is the most lethal of all common cancers, with the highest mortality-to-incidence ratio, being an indolent tumor difficult to treat that shows a rapid progress from diagnosis to death. Pancreatic cancer is a deadly disease with a poor prognosis, with a 5-year survival rate of only 10%, the lowest of common solid tumors. Almost 90% of PDACs are diagnosed after they have spread beyond the pancreas, with systemic metastases in >50%. PDAC is most often diagnosed at a late stage, after it has spread beyond the pancreas and is no longer curable by surgery (Singhi and Wood 2021). Complete surgical resection remains the only potential therapeutic treatment, but only 10–20% of pancreatic cancers are resettable at the time of diagnosis, and even the 5-year survival rate for PDAC after surgery remains rather low (15–20%), mainly due to metastatic disease or local recurrence.

PDAC arises from non-invasive precancerous lesions, that have not yet invaded beyond the basement membrane and represent a key target for early detection approaches. There are two categories of precancerous lesions in the pancreas: microscopic and macroscopic. The majority of PDACs arise from microscopic pancreatic intraepithelial neoplasia (PanIN). These neoplasms are confined to the pancreatic ductal system, most frequently the small pancreatic ducts. On the other hand, an important minority of PDACs arise from larger macroscopic precursor lesions (IPMN), which are frequently identified on imaging studies as pancreatic cysts. In addition, a less common type of precancerous macroscopic lesion, mucinous cystic neoplasm (MCN), does not involve the duct system. The grade of dysplasia in PanIN, IPMN and MCN is determined based on the architectural and cytological atypia of the epithelial cells. Precancerous lesions are graded based on the highest (rather than the predominant) grade of dysplasia present in the neoplasm. KRAS hotspot mutations and telomere shortening are the earliest alterations found in pancreatic tumorigenesis, occur at high prevalence even in early lesions, with >90% of low-grade PanINs. Alteration in key oncosuppressor genes as CDKN2A and TP53 rises with increasing dysplasia grade and occurs at significant prevalence in high-grade PanINs. More than 90% of PDAC cases at all grades carry a defective KRAS gene, and none of the most commonly

mutated genes in PDAC [KRAS, CDKN2A (encoding p16), TP53 and SMAD4] are currently druggable (Kleeff et al., 2016). Palliative gemcitabine has been the standard treatment for pancreatic cancer for many years with a modest survival benefit of about 3 months. At present the first-line therapy in pancreatic cancer includes FOLFIRINOX and nab-paclitaxel plus gemcitabine, but in all cases the survival outcomes of pancreatic cancer remain poor.

PDAC is the epitome of a treatment-resistant malignancy, driven by a so far “undruggable” oncoprotein, KRAS (Papke and Der, 2017; Hajatdoost et al., 2018). Activating mutations in KRAS are a hallmark in PDAC, occurring in 90– 95% cases of the deadly and highly metastatic adenocarcinoma. KRAS encodes a small GTPase that is activated through binding of GTP and translocation to the plasma membrane, in a cycling way of active (GTP) and inactive (GDP) form. The majority of KRAS mutations occur at codons 12, 13 and 61, leading to constitutive activation; the protein becomes insensitive to GTPase-activating proteins (GAPs), that let the protein inactivation. It is widely demonstrated that the downstream signaling of KRAS involves both the RAF/MEK/ERK signaling and PI3K/AKT/mTOR. In cooperation these two pathways strongly intersect with each other and are involved in the control of several oncogenic outcomes, including cell growth dysregulation, EMT induction and autophagic enhancement (Butler et al., 2015; Mollinedo et al., 2019; Safa 2020).

Focusing on the impact on EMT signature, Hotz and coworkers suggest that the lethal behavior in PDAC is at least partially correlated to EMT activation, in fact, IHC on resected PDAC tissues showed altered expression of EMT-TFs in tumor compared to parenchyma (Hotz et al., 2007). A study of 174 patients with PDAC showed a strong link between high levels of EMT-TFs and the incidence of lymph node metastasis or portal vein invasion (Yamada et al., 2013). Meta-analysis has corroborated the evidence of the vital role of EMT in the tumor budding (TB) and confirmed the considerable association between high grade TB and enhanced risk of death or disease recurrence. (Lawloret al., 2019; Chouat et al., 2018). Transforming growth factor-β (TGF-β) is the most important EMT-activator in many cancer subtypes, including PDAC (Alvarez, et al., 2019) and its role in pancreatic

cancer progression is cellular context dependent. Induction of TGF-b β directly stimulates Slug, Snail, Twist, and Zeb1 expression allowing to EMT initiation, and consequently reducing proliferation by cell-cycle arrest and promoting invasion (Siegel and Massague, 2003; Lamouille et al., 2009). Moreover, EMT seems to be equally important in all stages of tumors progression because it is a hub process of cellular plasticity and intra-tumoral heterogeneity. Single cell analyses of tumors obtained from human PDAC surgical samples, found many phenotypic cancers cell clusters, them were classified into groups along the epithelial-tomesenchymal process based on their gene expression profile. The epithelial clusters revealed an increase of the genes linked to differentiation and proliferation, but the mesenchymal one showed an EMT-related profile (Palamaris et al., 2021).

FGFR2 isoform switch is an additional oncogenic event occurring during pancreatic carcinogenesis, whose contribution in EMT induction and cell invasion (Ishiwata et al., 2012; Ueda et al., 2014; Ishiwata et al., 2018) as well as in the dysregulartion of autophagy still appears controversial.

The importance of the deregulation of FGF/FGFR axis and possibly of FGFR2 isoform switch in PDAC is suggested by the relevance demonstrated by FGF2, secreted by CAFs, in the modulation of both cell survival and motility (Awaji et al., 2019). In addition, several reports pointed on the aberrant expression of FGFR2c as important event in pancreatic carcinogenesis.

In fact, while the expression of either the epithelial FGFR2b isoform or the FGFR2c mesenchymal variant has been reported in PDACs (Cho et al., 2007; Ishiwata et al., 2012) and FGFR2c expression was detected in several pancreatic cancer cell lines (Ishiwata et al., 2012), the transfection of FGFR2c in whose Pancreatic cancer cell lines expressing low levels of this receptor increased cell proliferation as well as sphere formation ability in vitro, as well as tumour growth in vivo (Ishiwata et al., 2012). However, the treatment with anti-FGFR2c antibody in those cell lines expressing high levels of FGFR2c failed to interfere with their invasive behavior (Ishiwata et al., 2012), condition that was reached, on the other hand, by the transfection of ESRP1 (Ueda et al., 2014), the epithelial splicing regulatory protein

responsible for FGFR2 isoform splicing in favor of FGFR2b expression (Warzecha et al., 2009). Therefore, even if the role of FGFR2c expression on cancer cell and tumor growth appear to be assessed, its relevance in the control of cell invasion remains to be further defined. Further investigations will be also required to establish the possible role of FGFR2c and its downstream oncogenic signaling mediated by PKCε in the control of both EMT and autophagy, two key events involved in PDAC development and progression, whose complex crosstalk we have recently proposed to be under the control of FGFR2c, at least in the context of human keratinocytes.

Aims

On the light of all these evidences, in this thesis project we have proposed:

To further characterize the molecular mechanisms underlying EMT/autophagy negative crosstalk during carcinogenesis, investigating the possible involvement of the aberrant FGFR2c expression and signaling in this interplay;

To investigate the role of FGFR2c and Its PKCε downstream signaling in the control of EMT and autophagy in the context of the Pancreatic Ductal Adenocarcinoma (PDAC).

Materials and Methods

Cells and treatments

The human keratinocyte cell line HaCat, stably expressing FGFR2c (pBp-FGFR2c), or the empty vector (pBp) and the pancreatic adenocarcinoma cell line PANC-1 and MIA PaCa-2, purchased from American Type Culture Collection (ATCC), were cultured in DMEM 10% bovine fetal serum (FBS) plus antibiotics. For MTOR, PKCε and FGFR2 silencing, clones were transiently transfected with MTOR small interfering RNA (MTOR siRNA) (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA; SC35409), or stably transfected with PKCε Plasmid shRNA (h) vector (Santa Cruz Biotechnology, Inc., CA, USA; SC-36251-SH), with Bek/FGFR2 shRNA (h) (Santa Cruz, SC-29218-V) or an unrelated siRNA/shRNA as a control, using Lipofectamine 2000 transfection reagent (Life Technologies, Carlsbad, CA, USA; 11668-019). For growth factor stimulation, cells were left untreated or incubated with FGF2 (PeproTech, London, BFGF 100-188) 100 ng/mL for 24 hours at 37°C. For inhibition of FGFR2 tyrosine kinase activity, cells were pre-incubated with a specific FGFR2 tyrosine kinase inhibitor, SU5402 25 μmol/L (Calbiochem, Nottingham, UK; 572 630) for 1 hour before treatments with growth factors (GFs). To inhibit AKT or MTOR, cells were incubated with AKT-specific inhibitor AKT-I-1/2 (1 μmol/L; Calbiochem, 124 005) or with the specific MTOR inhibitor rapamycin (100 nmol/L; Cell Signaling Technology, Beverly, MA, USA; 9904), respectively, for 1 hour at 37°C before being treated with FGF2 in the presence of each inhibitor. For RNA interference and consequent specific FGFR2b or FGFR2c silencing, cells were transfected with a FGFR2b siRNA sequence (5'-AATTATATAGGGCAGGCCAAC-3') (Qiagen, Valencia, CA, USA) or FGFR2c siRNA sequence (5'-GGAATGTAACTTTTGAGGA-3') (Qiagen) or with a control sequence (5'-AATTCTCCGAACGTGTCACGT-3')
(Qiagen) using Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA 11668030) according to the manufacturer's protocol.

Immunofluorescence

Cells were grown on coverslips, fixed with 4% paraformaldehyde in PBS for 30 min at 25 °C followed by treatment with 0.1 M glycine for 20 min at 25 °C and with 0.1% Triton X-100 for an additional 5 min at 25 °C to allow permeabilization. Cells were then incubated with the following primary antibodies: mouse monoclonal anti-LC3 (1:100 in PBS, 5F10 Nanotools, Teningen, Germany, 0231), polyclonal anti-vimentin (1:50 in PBS; Dako, Glostrup, Denmark; M0725) for 1 h at 25 °C and then with a goat anti-mouse IgG-Alexa Fluor 488 (1:200 in PBS, Life Technologies, Carlsbad, CA, USA; A11001) for 30 min at 25 °C. Nuclei were stained with DAPI (Sigma-Aldrich, Saint Louis, MO, USA; D9542). All fluorescence signals were analyzed by scanning cells in a series of sequential sections with an ApoTome System (Zeiss, Oberkochen, Germany) connected with an Axiovert 200 inverted microscope (Zeiss); image analysis was performed by the Axiovision software (Zeiss), and images were obtained by 3D reconstruction of the total number of the serial optical sections. Quantitative analysis of LC3-positive dots per cell was performed analyzing 100 cells for each sample in 5 different microscopy fields from 3 different experiments. Results are shown as means ± standard deviation (SD).

Western blot analysis

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 MgCl2, 5 mM EGTA, supplemented with protease inhibitors (10 g/mL aprotinin, 1 mM phenylmethylsulphonyl fluoride
[PMSF], 10 µg/mL leupeptin) and phosphatase inhibitors (1 mM sodium orthovanadate, 20 mM sodium pyrophosphate, 0.5 M NaF). A range of 20 to 50 µg of total protein was resolved under reducing conditions by 8 or 12%

SDS-PAGE and transferred to reinforced nitrocellulose (BA-S 83; Schleicher & Schuell, Keene, NH, USA; BA-S83). The membranes were blocked with 5% nonfat dry milk (Bio-Rad Laboratories, Hercules, CA, USA, 170-6404) in PBS 0.1% Tween 20 (Bio-Rad, 170-6531) and incubated with anti-SQSTM1 (BD Bioscience, San Josè, CA, USA, 610 833), anti-LC3 (MBL, Woburn, MA, USA; PD014), anti-p-FGFR (55H2, Y653/654, Cell Signaling, 3476S), anti-p-AKT (Ser 473; Cell Signaling, 9271), anti-p-MTOR (Ser 2448; Cell Signaling, 5536S), anti-p-S6K (ser 371, Cell Signaling, #9208), anti p-PKCε (Ser729, Abcam, Cambridge, UK; ab63387), anti-p-p44/42 mitogen-activated protein kinase (MAPK) (p-ERK1/2) (Thr202/Tyr204; Cell Signaling, 9101S), anti-E- cadherin (Dako, Carpinteria, CA, USA; NCH-38) and anti-vimentin (M0725, Dako, Glostrup, Denmark) , followed by enhanced chemiluminescence (ECL) detection (Thermo Scientific, Rockford, IL, USA; 34 580). The membranes were rehydrated by washing in PBS/Tween-20, stripped with 100 mmol/L β-mercaptoethanol and 2% SDS for 30 minutes at 55°C and probed again with, anti-FGFR2 (C17, Santa Cruz Biotechnology, sc-122), anti-PKCε (Abcam, ab124806), anti-AKT (H-136; Santa Cruz Biotechnology, sc-8312), anti- α/β- Tubulin (Cell Signaling, 2148S), anti-S6K (Cell Signaling, #9202) polyclonal antibodies or with anti-MTOR (7C10, Cell Signaling, 2983S), anti-ACTB (Sigma-Aldrich, A5441), anti-GAPDH (6C5, Santa Cruz Biotechnology, sc-32233) monoclonal antibody to estimate the protein equal loading. Densitometric analysis was performed using Quantity One Program version 4.6.8 (Bio-Rad). The resulting values from three different experiments were normalized, expressed as fold increase respect to the control value and reported in graph as mean values ± standard deviation (SD). Student's t test was performed, and significance levels have been defined as $P < .05$.

RNA extraction and cDNA synthesis

Total RNA from HaCat, PANC-1 and MiaPaCa-2 cells was extracted using the TRIzol method (Invitrogen, 15596018) according to the manufacturer's instructions and eluted with 0.1% diethylpyrocarbonate (DEPC)-treated water. Each sample was treated with DNase I (Invitrogen, 18068-015). The total RNA concentration was quantitated by spectrophotometry; 1 µg of total RNA was used for reverse transcription using the iScriptTM cDNA synthesis kit (Bio-Rad, 170-8891) according to the manufacturer's instructions.

Primers

Oligonucleotide primers necessary for target genes and the housekeeping gene were chosen by using the online tool Primer-BLAST (Ye J. et al., 2012) and purchased from Invitrogen. The following primers were used: for the Ecadherin target gene: 5′-TGGAGGAATTCTTGCTTTGC-3′ (sense), 5′CGCTCTCCTCCGAAGAAAC-3′(antisense); for the vimentin target gene: 5′-AAATGGCTCGTCACCTTCGT-3′(sense), 5′-AGAAATCCTGCTCTCCTCGC -3′ (antisense); for the Snail1 target gene: 5′GCTGCAGGACTCTAATCCAGA-3′ (sense), 5′-ATCTCCGGAGGTGGGATG3′ (antisense); for the STAT3 target gene: 5′-CAGAGATGTGGGAATGGGGG3′ (sense), 5′-TGGCAAGGAG TGGGTCTCTA-3′ (antisense); for the FRA1 target gene: 5′-GCAGGCGGAGACTGACAAA-3′ (sense), 5′-GATGGGTCGGTGGGCTTC-3′; for FGFR2b target gene: 5′CGTGGAAAAGAACGGCAGTAAATA-3′ (sense), 5′-GAACTATTTATCCCCGAGTGCTTG-3′ (antisense); for FGFR2c target gene: 5′-TGAGGACGCTGGGGAATATACG-3′ (sense), 5′-TAGTCTGGGGAAGCTGTAATCTCCT-3′ (antisense) ; for the 18S rRNA housekeeping gene: 5′-CGAGCCGCCTGGATACC-3′ (sense) and

5′CATGGCCTCAGTTCCGAAAA-3′ (antisense). For each primer pair, we performed no-template control and no-reverse-transcriptase control (reverse transcription [RT]-negative) assays, which produced negligible signals.

PCR amplification and Real-Time quantification

Real-time RT-PCR was performed using the iCycler real-time detection system (iQ5 Bio-Rad) with optimized PCR conditions. The reactions were carried out in a 96-well plate using iQ SYBR green super mix (Bio-Rad, 1708882), adding forward and reverse primers for each gene and 1 µL of diluted template cDNA to a final reaction mixture volume of 15 µL. All assays included a negative control and were replicated three times. The thermal cycling program was performed as described previously (Persechino et al., 2021). Real-time quantitation was performed with the help of the iCycler IQ optical system software, version 3.0a (Bio-Rad), according to the manufacturer's manual. Results are reported as mean values ± SD from three different experiments in triplicate.

Transmission electron microscopy

PANC-1 and PANC-1 PKCε shRNA cells left untreated or stimulated with FGF2 for 24 h as above were washed three times in PBS and fixed with 2% glutaraldehyde (Electron Microscopy Science, 16300) in PBS for 2 h at 4 °C. Samples were postfixed with 1% osmium tetroxide in veronal acetate buffer (pH 7.4) for 1 hr at 25 °C, stained with uranyl acetate (5 mg/mL) for 1 h at 25 °C, dehydrated in acetone and embedded in Epon 812 (EMbed 812, Electron Microscopy Science). Ultrathin sections were examined unstained

or poststained with uranyl acetate and lead hydroxide, under a Morgagni 268D transmission electron microscope (FEI, Hillsboro, OR, USA) equipped with a Mega View II charge-coupled device camera (SIS, Soft Imaging System GmbH, Munster, Germany) and analyzed with AnalySIS software (SIS).

Invasion assay

Migration assay was performed using 24-well transwell migration Boyden chambers (8 μm pore size; Costar, Cambridge, MA, USA) pre- coated with matrigel (dilution 1:2 in DMEM; BD Biosciences, Bedford, MA, USA) as reported (Ranieri et al., 2016). Quantitative analysis was assessed counting for each sample the migrated cells in 10 microscopic fields (objective used: 20X) from three independent experiments. Results have been expressed as mean values ± SD. p values were calculated using Student's t test, and significance level has been defined as $P > .05$.

Statistics

The data were analyzed using analysis of variance (ANOVA) to test for differences amongst all means. A Tukey’s multiple comparison test was used to determine differences between selected groups. The significance levels were defined as p values ≤ 0.05.

Results

Part 1

The forced reactivation of autophagy reverses FGFR2c-induced EMT program and inhibits receptor-mediated cell invasion.

Since MTOR is the main pathway involved in the inhibition of autophagy induced by aberrant FGFR2c expression in human keratinocytes (Nanni et al., 2019), we first assessed if a negative crosstalk between receptor-controlled autophagy and EMT does exist in these cells using rapamycin, the widely accepted general inhibitor of MTOR-dependent autophagy. To this aim, we assessed Western blot analysis in HaCaT human keratinocytes stably espressing FGFR2c (HaCaT pBp-FGFR2c) and in HaCaT clones transduced with the empty vector (HaCaT pBp), used as control. The cultures were left untreated or stimulated with FGF2, the ligand which does not bind to FGFR2b, but is able to activate other FGFRs including FGFR2c. The results showed that rapamycin was able to interfere with the phosphorylation of MTOR at Ser 2448, as well as with that of its downstream substrate S6K, at Ser 371, both induced only in pBp-FGFR2c clones by ligand stimulation (Figure 9 A). In these cells, rapamycin also turned the decrease of the widely recognized autophagic marker LC3-II into a significant increase (Figure 9A). As previously speculated by us, this effect is possibly attributable to the negative interplay between MTOR pathway and JNK1 signaling, that causes JNK1-dependent activation of autophagy in consequence of MTOR signaling shut-off (Nanni et al., 2019). In addition to the impact on LC3-II decrease, rapamycin also reversed the accumulation of the autophagic substrate SQSTM1/p62, detectable only in HaCaT pBp-FGFR2c clones stimulated by FGF2, confirming the reactivation of the autophagic flux (Figure 9A). Thus, rapamycin efficiently counteracted the repression of autophagy orchestrated by FGFR2c activation.

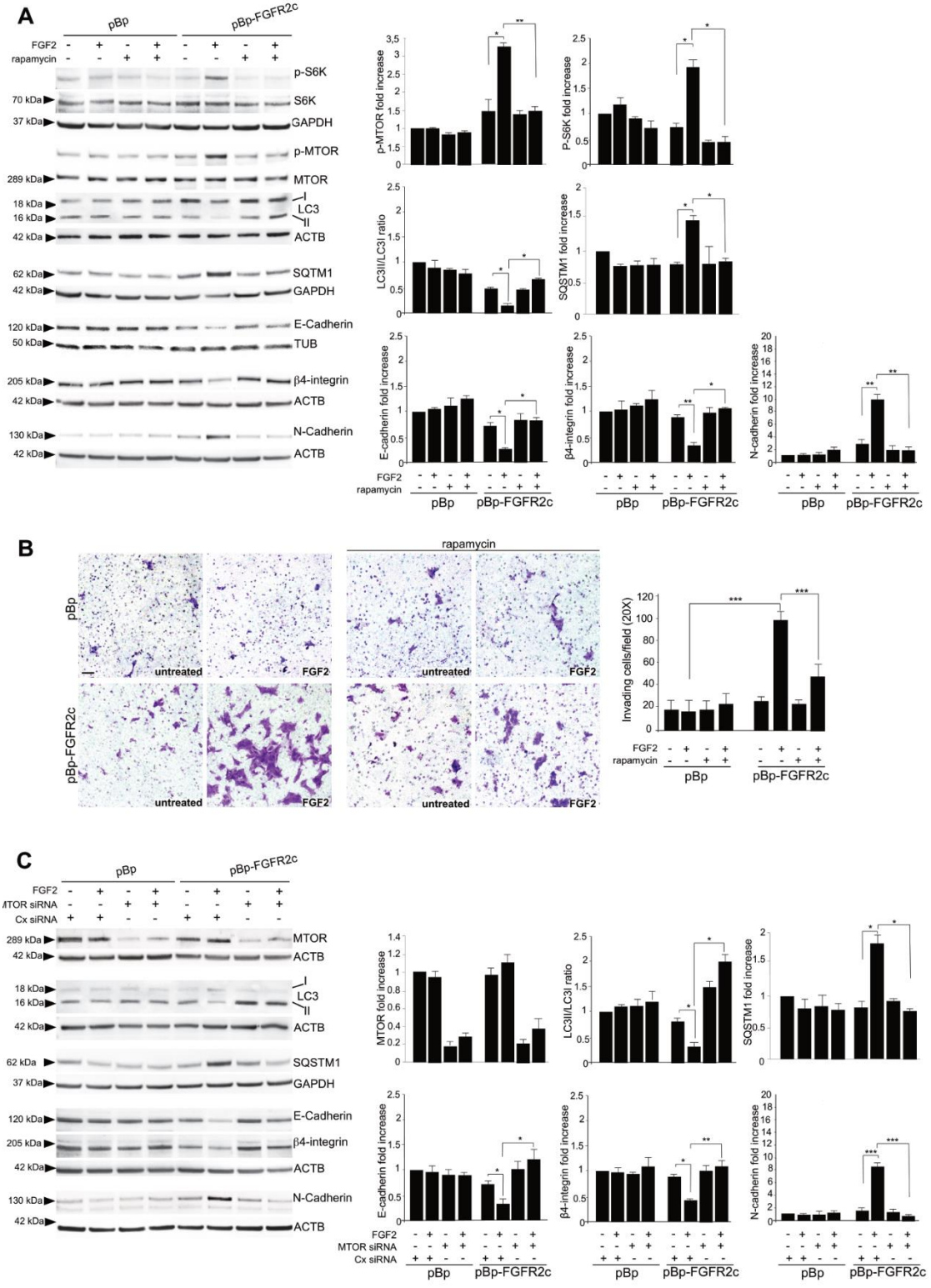

Figure 9: Reactivation of MTOR-dependent autophagy negatively impact on FGFR2c-mediated EMT and cell invasion. A, HaCaT pBp-FGFR2c and HaCaT pBp clones were left untreated or stimulated with FGF2 in presence or not of rapamycin. Western blot analysis shows that, only in FGF2-stimulated pBp-FGFR2c clones, rapamycin negatively interferes with MTOR and S6K phosphorylation, with LC3-II decrease (turning it into an increase) and SQSTM1 accumulation and reverses the repression of the epithelial markers E-cadherin and β4-integrin, as well as the appearance of the mesenchymal marker N-cadherin. For the densitometric analysis, the values from 3 independent experiments were normalized, expressed as fold increase and reported in graph as mean values ± standard deviation (SD). Student t test was performed as reported in Materials and Methods, and significance levels

have been defined as P < .05: *P < .05, ** P < .01. B, HaCaT clones were seeded on matrigel pre-coated transwell Boyden chamber filters and FGF2 was added in the bottom chamber, in the presence or not of rapamycin, to stimulate cell chemotaxis. Results shows that, in FGFR2c cultures, the increase of the number of invading cells induced by FGF2 is counteracted by rapamycin. Quantitative analysis was assessed as reported in Materials and Methods. Results are expressed as mean values ± standard deviation (SD). Student's t test was performed as reported in Materials and Methods, and significance level has been defined as P < .05: ***P < .001. Bar: 50 μm. C, HaCaT clones were transiently transfected with MTOR siRNA or with an unrelated siRNA(Cx RNA), as negative control, and then left untreated or stimulated with FGF2 as above. Western blot analysis shows that MTOR silencing reverses the decrease of LC3-II, the accumulation of SQSTM1 and the modulation of the epithelial markers E-cadherin and β4-integrin and that of the mesenchymal marker N-cadherin induced by FGF2 stimulation in FGFR2c expressing clones. Densitometric analysis was performed as above. *P < .05, ** P < .01

Then, we analyzed the impact of autophagy reactivation on FGFR2c-controlled EMT program. To this aim, we focused our attention on EMT markers expression, observing that rapamycin efficiently reversed the decrease of the epithelial markers E-cadherin and β4-integrin, as well as the appearance of the mesenchymal marker N-cadherin, both caused in pBp-FGFR2c clones by FGF2 treatment (Figure 9 A). We then investigated the impact of MTOR inhibition on the invasive behavior of HaCaT pBp-FGFR2c clones, previously described by us (Ranieri et al., 2016; Ranieri et al., 2018), using the in vitro assay of matrigel pre-coated transwell Boyden chambers. Upon cell seeding, FGF2 was added in the bottom chamber, in the presence or not of rapamycin, to stimulate cell chemotaxis. The results showed that the significant increase of invading cells, observed in pBp-FGFR2c cultures only in response to FGF2 (Figure 9 B), was clearly impaired by the presence of rapamycin (Figure 9 B). In order to further assess the outcome of forced reactivation of MTORdependent autophagy on FGFR2c-mediated EMT in epithelial context, MTOR protein depletion was carried out in HaCaT clones by specific siRNA transfection. Western blot analysis showed that, similarly to what observed in the presence of rapamycin, in HaCaT pBp-FGFR2c cells stimulated with FGF2, MTOR silencing was not only sufficient to reverse the decrease of LC3II in an increase and to block SQSTM1 accumulation (Figure 9 C), confirming the reactivation of autophagy, but also was effective in counteracting the FGF2-mediated modulation of the epithelial/mesenchymal markers (Figure 9 C), confirming the impairment of EMT program.

Since AKT is the substrate acting upstream MTOR in FGFR2c-mediated inhibition of autophagy (Nanni et al, 2019), we also checked the effects of its

inhibition on FGFR2-driven EMT. Western blot analysis showed that AKT signaling shut-off by the previously tested AKT-I-1/2 inhibitor (Nanni et al, 2019) efficiently reversed the decrease of LC3-II levels, the accumulation of SQSTM1 and the modulation of the EMT-related markers induced by FGF2 only in FGFR2c expressing clones (Figure 10). These results confirm the involvement of the entire AKT/MTOR signaling cascade at the crossroad between autophagy regulation and EMT processes.

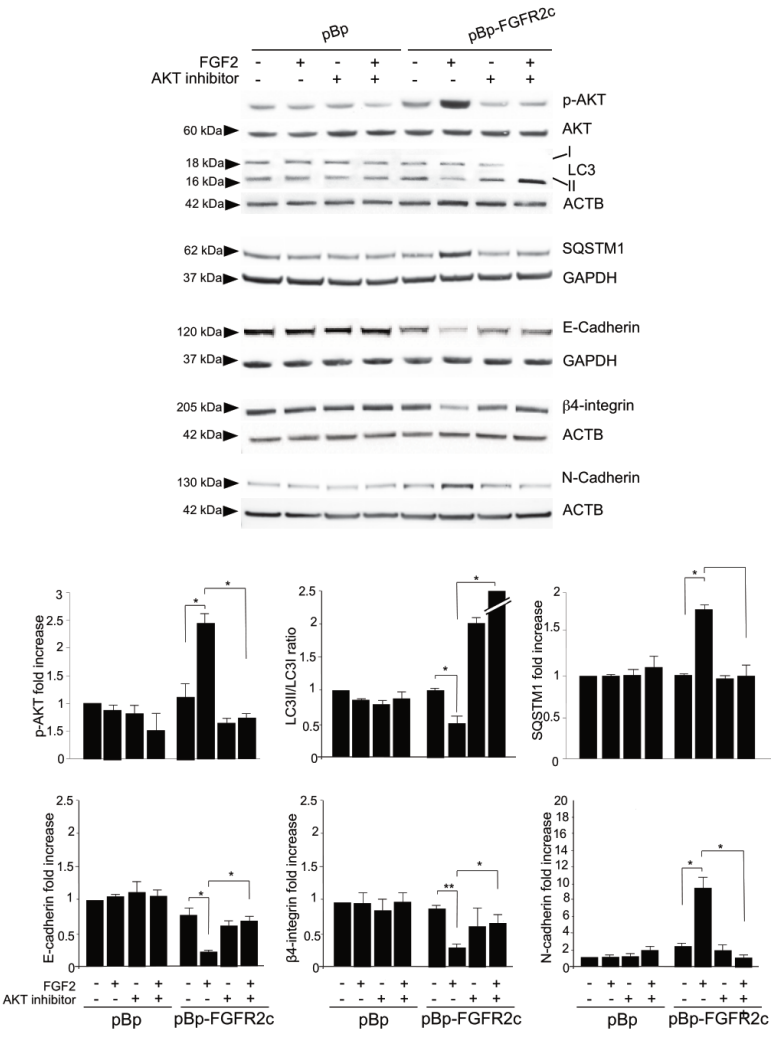

Figure 10: The inhibition of AKT signaling represses FGFR2c-induced EMT. HaCaT pBp-FGFR2c and HaCaT pBp clones were left untreatedor stimulated with FGF2 in presence or not of AKT-I-1/2 inhibitor. Western blot analysis shows that AKT inhibitor reverses the decrease of LC3-II levels, the accumultion of SQSTM1 and the modulation of all the EMT-related markers induced by FGF2 stimulation in FGFR2c expressing clones. The densitometric analysis and Student t test were performed as reported in Figure 1a: *p<0.05, ** p<0.01.

Since FGFR2c-triggered EMT is driven by PKCε-dependent induction of Snail1, STAT3 and FRA1 (Ranieri et al., 2016; Ranieri et al., 2018), we wondered if and how the forced reactivation of autophagy could impact on the

expression of these EMT-related transcription factors. Real-time RT-PCR showed that both MTOR silencing via siRNA (Figure 11 A) and PKCε stable depletion by shRNA (Figure 11 B) were able to counteract the increase of mRNA levels of all these transcription factors, evident in pBp-FGFR2c clones in response to FGF2 (Figure 11 A, B). Thus, the forced reactivation of the autophagic process appears to negatively affect the induction of EMT-related transcription factors in a comparable way to PKCε signaling shut-off.

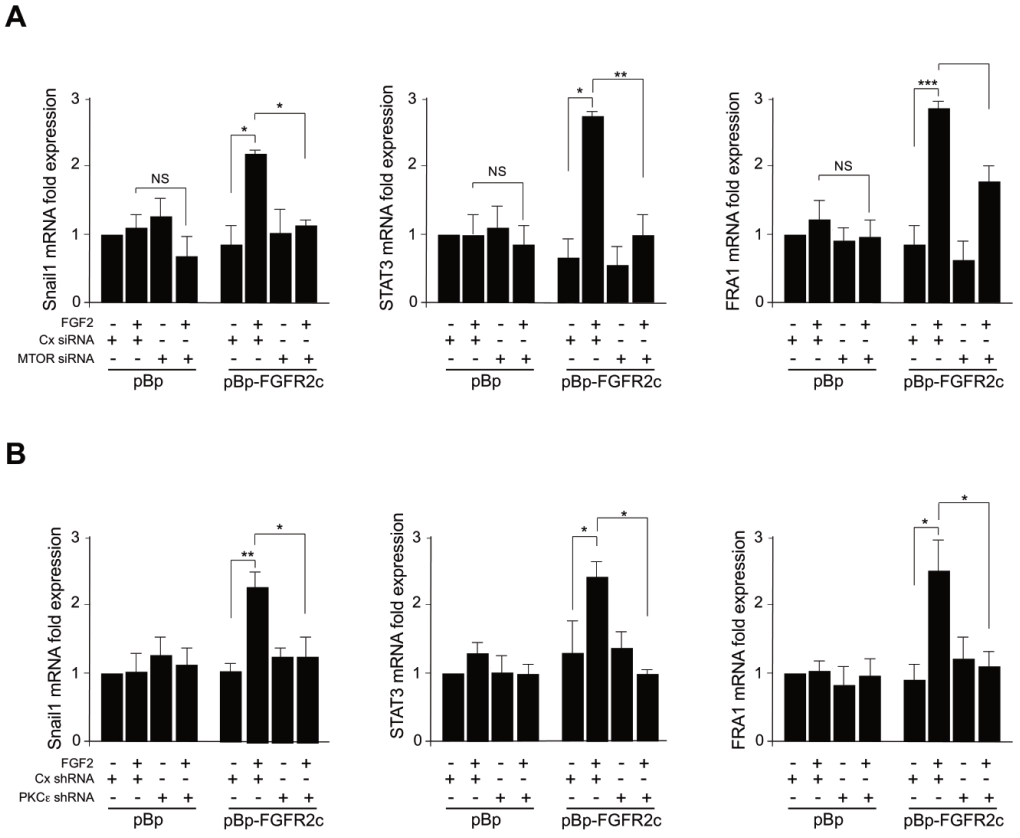

Figure 11 : mTOR silencing and PKCε depletion. mTOR silencing and depletion similarly interfere with FGFR2c mediated induction of the EMT-related transcription factors STAT3, Snail and FRA1. HaCat pBp and HaCat pBp-FGFR2c clones were transiently transfected with mTOR siRNA (A) or stably trasfected with PKCεshRNA (B) and then left untreated or stimulated with FGF2. Unrelated siRNA (Cx siRNA) and shRNA (Cx shRNA) were respectively used as negative controls. Both mTOR transient silencing (A) and PKCε depletion (B) counteract the increase of mRNA Snail1, FRA1 and STAT3 levels induced in FGFR2c expressing clones in response to FGF2 stimulation. Results are expressed as mean value ± SD. Student's test reported was performed as in materials and Methods and significance level was defined as $p<0.05$*; $p<0.005$; $p<0.01$**.

Selective PKCε shut-off efficiently reverses the negative impact of FGFR2c signaling on autophagy.

The possibility that FGFR2c could orchestrate a fine interplay between autophagy and EMT in epithelial context is also sustained by the evidence that protein kinase C isozymes, including PKCε, are also key regulators of the autophagic pathway (Kaleli et al., 2020) Thus, we investigated the possible contribution of PKCε signaling on FGFR2c-mediated repression of the autophagic process in human keratinocytes by shRNA approaches. The impairment of FGF2-induced EMT program in FGF2-stimulated FGFR2c clones after PKCε depletion was confirmed by the recovery of the epithelial marker E-cadherin (Figure 12A), while LC3-II increase indicated the activation of autophagy (Figure 12A). In addition, the expected accumulation of the autophagy substrate SQSTM1 in FGFR2c clones stimulated by FGF2, was significantly dampened by PKCε depletion (Figure 12A), suggesting a reactivation of the autophagic flux. Finally, quantitative immunofluorescence approaches showed that the expected reduction of LC3 positive dots per cell in HaCaT pBp-FGFR2c clones stimulated with FGF2 (Figure 12B) was completely reversed by stable depletion of PKCε, resulting in a visible increase (Figure 12B). Thus, PKCε signaling appears to be involved in the inhibition of autophagy orchestrated by FGFR2c, when this receptor is aberrantly expressed in human keratinocytes.

As last step, to confirm the central role of FGFR2c in regulating all the observed, intersected effects between autophagy and EMT, we used the FGFR2 tyrosine kinase inhibitor SU5402. In HaCaT pBp-FGFR2c clones the presence of SU5402 was sufficient to abolish all the responses to FGF2, not only in terms of FGFR2c, PKCε and MTOR/S6K phosphorylation (Figure 12C), as expected (Nanni et al., 2019; Ranieri et al., 2020), but also in term of Ecadherin/N-cadherin modulation and repression of LC3 -II, as well as SQSTM1 accumulation (Figure 12C). Overall, our findings appear to confirm that a crosstalk between autophagy and EMT does exist in human keratinocytes, which appears to be upstream established by the aberrant expression and signaling of FGFR2c.

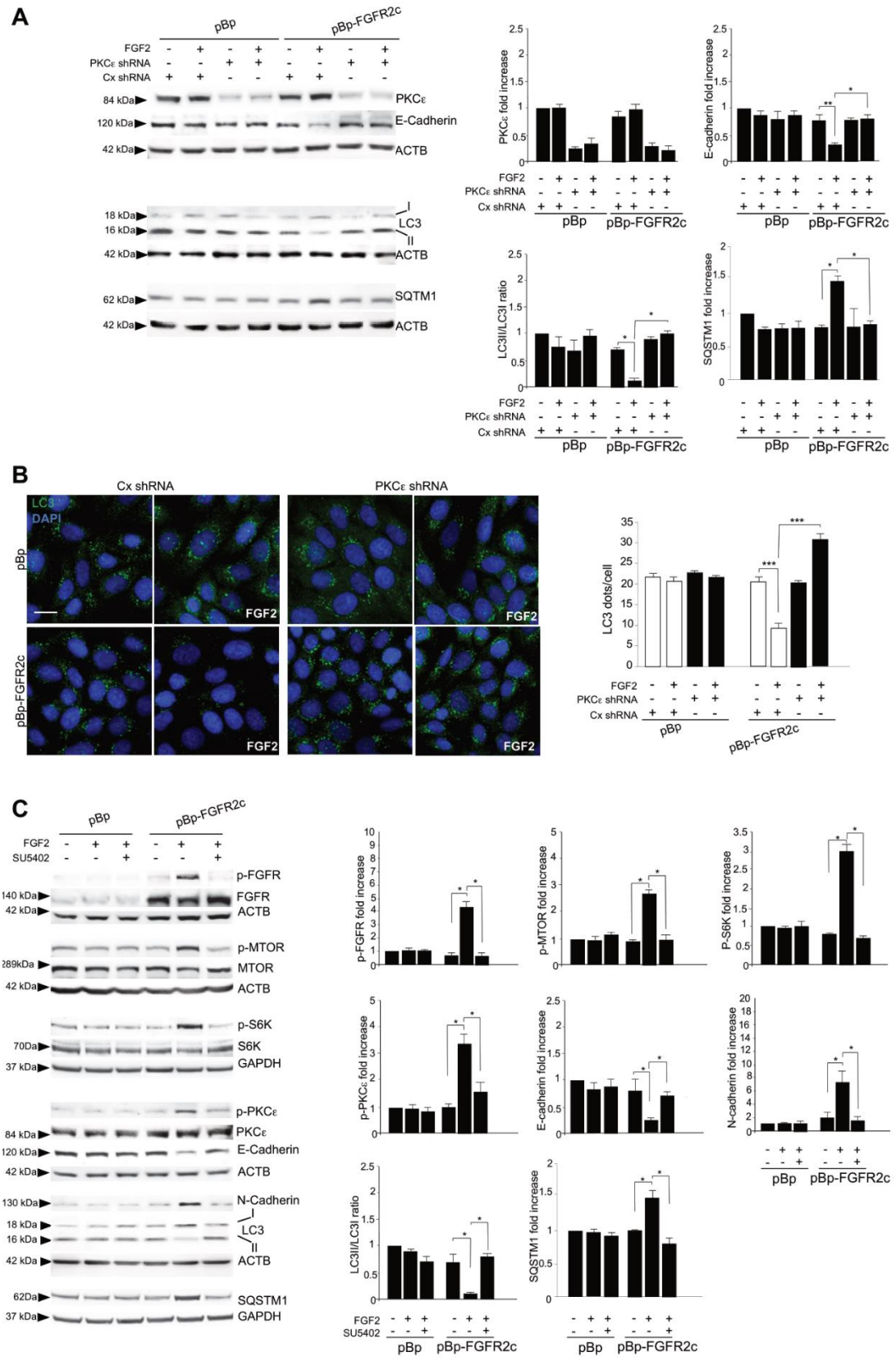

Figure 12. PKCε signaling shutoff restores the autophagic process and FGFR2c signaling underly EMT/autophagy negative crosstalk. HaCaT pBp-FGFR2c and HaCaT pBp clones were stably transfected with PKCε shRNA or with an unrelated shRNA, as negative control, and then left untreated or stimulated with FGF2 as above. A, Western blot analysis shows that PKCε depletion reverses all the effects induced by FGF2 on the expression of E-cadherin, LC3-II and SQSTM1 in HaCaT pBp-FGFR2c clones. Densitometric analysis and

Student t test were performed as reported in Figure 1A. *P < .05, **P < .01. B, Quantitative immunofluorescence analysis shows that PKCε depletion reverses the decrease of LC3 positive dots per cell induced in HaCaT pBp-FGFR2c cells by the stimulated with FGF2. Quantitative analysis of LC3 positive dots per cell was performed as described in Materials and Methods, and the results are expressed as mean values ± standard errors (SE). Student's t test was performed, and significance level was defined as P < .05: *** P < .001. Bar: 20 μm. C, HaCaT pBp-FGFR2c and HaCaT pBp clones were left untreated or stimulated with FGF2 in presence or not of the FGFR2 kinase inhibitor SU5402. Western blot analysis shows that SU5402 abolishes FGFR2c, PKCε, MTOR and S6K phosphorylation, and reverses both the modulation of E-cadherin and N-cadherin markers, the repression of LC3-II and the accumulation of SQSTM1 induced by FGF2 in HaCaT pBp-FGFR2c clones. Densitometric analysis and Student t test were performed as above. *P < .05, ** P < .01

Part 2

FGFR2c Aberrant Expression Affects the Intracellular Signaling

We first investigated to what extent the aberrant expression of the mesenchymal FGFR2c isoform in PDAC cell lines could impact on the intracellular signaling activation in response to FGFs. To this aim, we assessed the expression levels of the epithelial and the mesenchymal variants of FGFR2 (FGFR2b and FGFR2c, respectively) in PANC-1 and MiaPaCa-2 pancreatic tumor cell lines, selected for different levels of FGFR2c (Ishiwata et al., 2012; Ueda et al., 2014), and we compared them with those observed in human keratinocyte HaCaT cell line and normal human fibroblasts (HFs), used as positive controls for FGFR2b and FGFR2c expression, respectively. mRNA levels were assessed by real time RT-PCR and normalized respect to 18SrRNA. Results showed that FGFR2c expression was significantly higher in PANC-1 cells, compared to Mia-PaCa-2 cells (Figure 13A, right panel), while no appreciable levels of FGFR2b mRNA were detected in both PDAC cell lines, compared to HaCaT cells (Figure 13A, left panel). Then, in the two selected PDAC cells expressing different levels of FGFR2c, we investigated the activation of the intracellular signaling in response to FGF2, the FGF family member, which does not bind the epithelial FGFR2b, but interacts with other FGFRs, including FGFR2c. Particular attention was paid to MEK/ERK and AKT/MTOR, which are the two main signaling pathways responsible not only for cell growth deregulation and survival, but also for EMT induction (Huang et al., 2021; Safa 2020) and for the modulation of autophagy (Mollinedo and Gajate 2019) in pancreatic cancer cells. Western blot analysis showed that an enhancement of the basal phosphorylation of ERK1/2 after FGF2 stimulation was higher in PANC-1 respect to Mia PaCa-2 cells (Figure 13B) while that of AKT was exclusively in PANC-1 cells (Figure 13C). The treatment with the FGFR2 kinase inhibitor SU5402 was able to abrogate these effects (Figure 13B,C), confirming their dependence from FGFR2c activation. The higher sensitivity of PANC-1 cells to FGF2 was also evident, downstream AKT, as it

increased phosphorylation of MTOR (Figure 13D) and of its substrate S6K (Figure 13E), both events that were abolished by the presence of SU5402 (Figure 13D,E). Therefore, a higher expression of FGFR2c resulted in a more pronounced responsiveness of tumor cells to FGF2 in terms of intracellular signaling activation.

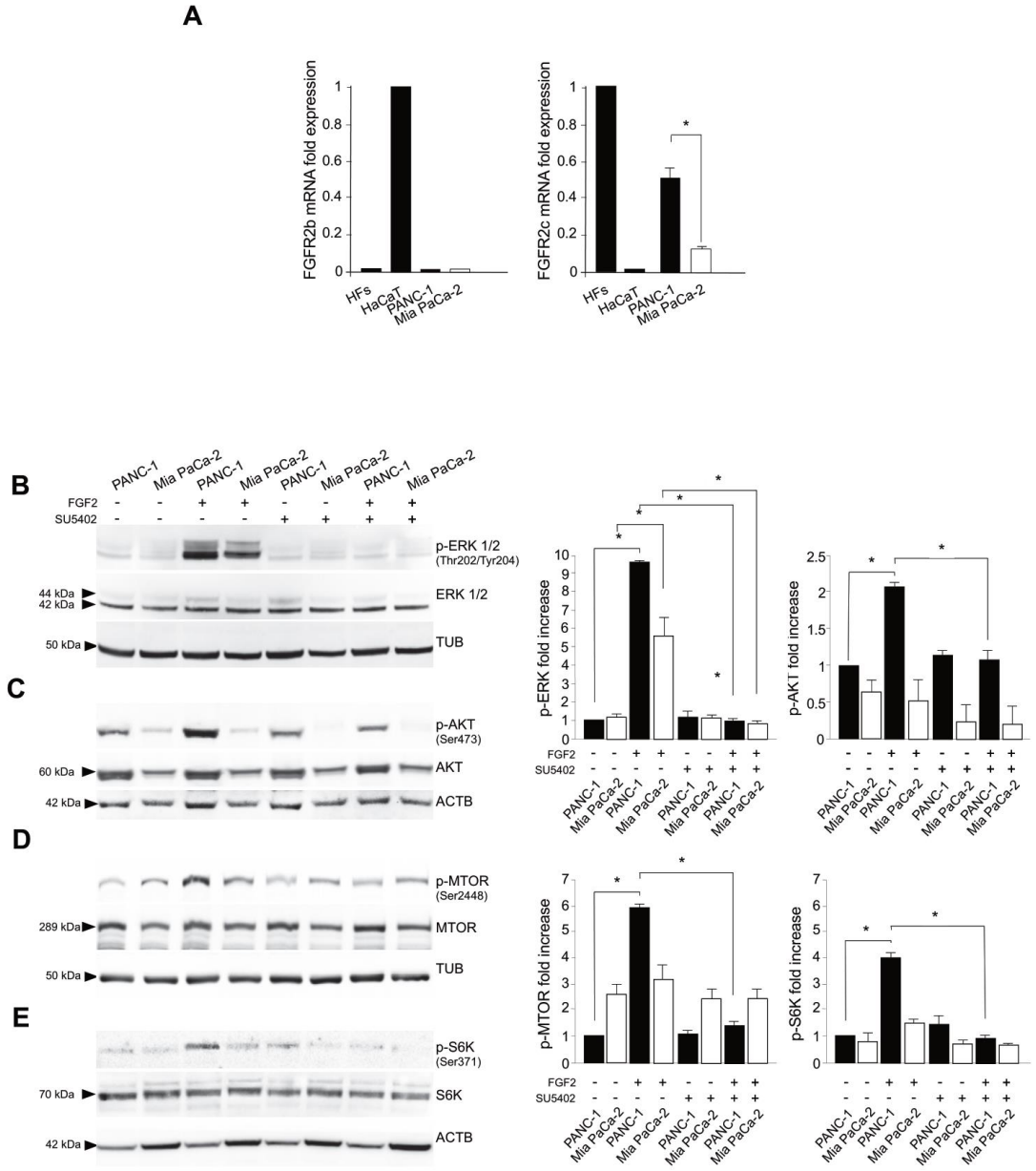

Figure 13. FGFR2c expression affects the susceptibility of ERK1/2 and AKT signaling to FGF2. PANC-1 and Mia PaCa−2 pancreatic tumor cell lines were left untreated or stimulated with FGF2 in the presence or absence of the FGFR2 tyrosine kinase inhibitor SU5402, as described in material and methods. (A) Real−time RT−PCR was performed normalizing mRNA levels respect to 18SrRNA. FGFR2c mRNA levels are significantly higher in PANC−1 cells compared to Mia PaCa−2. No appreciable levels of FGFR2b mRNA are detected in both PDAC cell lines. Human HaCaT keratinocyte cell line and normal human fibroblasts (HFs) are used as positive controls for FGFR2b and FGFR2c expression, respectively. Results are expressed as mean value ± SD (n = 3). ANOVA with Tukey's multiple comparison test: * $p \leq 0.05$. (B–E) Western blot analysis shows that the enhancement of ERK1/2 phosphorylation after FGF2 stimulation is higher in PANC−1 than in Mia PaCa−2 cells (B), while that of AKT was exclusively visible in PANC−1 cells (C). The treatment with SU5402

abrogates these effects (B, C). An increase of both MTOR and S6K phosphorylation upon FGF2 treatment is detectable only in PANC−1 cells and it is abolished by SU5402 (D, E). Equal loading was assessed with anti−actin or tubulin antibodies. Results are expressed as mean value ± SD (n = 3). Densitometric analysis was performed as reported in material and methods. ANOVA with Tukey's multiple comparison test: * $p \leq 0.05$.

FGFR2c Expression Enhances the EMT Phenotype in Response to FGF2

Then, we shifted our attention to EMT-related gene profile expressed in PDAC cells expressing different levels of FGFR2c. We found that the expression levels of the transcription factors Snail1, FRA1 and STAT3, which we previously identified as involved in FGFR2c-mediated EMT (Ranieri et al., 2020; Ranieri et al., 2021) overlapped with those of FGFR2c, appearing significantly higher in PANC-1 cells, compared to MiaPaCa-2 cells (Figure 14A).

Consistent with what was observed for the EMT-related transcription factors, the modulation of epithelial/mesenchymal markers compatible with EMT also appeared to overlap FGFR2c expression, displaying a more pronounced downregulation of the epithelial markers E-cadherin and a higher expression of the mesenchymal marker vimentin in PANC-1 cells compared to Mia PaCa-2 cells (Figure 14B). HaCaT cells and the primary culture of human fibroblasts (HFs) were used as positive controls for the expression of epithelial and mesenchymal markers, respectively (Figure 14B) Thus, in PDAC cells, the EMT expression profile appears to be related to the extent of FGFR2c expression.

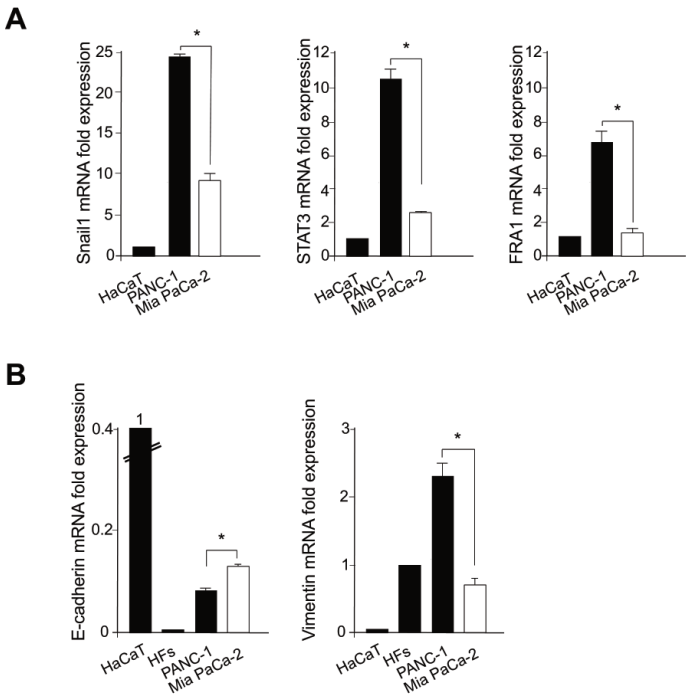

Figure 14: EMT-related expression profile in PDAC cells expressing different levels of FGFR2c. (A) Realtime RT-PCR demonstrated that the expression levels of EMT-related transcriptio factors Snail1, FRA1 and STAT3 are significantly higher in PANC-1 cells, compared to MIA PaCa-2 cells. (B) The expression of the epithelial marker E-cadherin is lower and the mesenchymal marker Vimentin is higher in PANC-1 cells compared to MIA PaCa2 cells. HaCaT cells and primary culture of human fibroblast (HFs) are used as positive controls for the expression of epithelial and mesenchymal markers, respectively. Results are expressed as mean value ± SD (n=3). ANOVA with turkey's multiple comparison test: *p<0.05

To assess to what extent the expression level of FGFR2c could impact on the enhancement of EMT features in response to microenvironmental factors, we analyzed the modulation of the EMT-related transcription factors Snail1, FRA1 and STAT3 after FGF2 stimulation. Real time RT-PCR showed that all the three transcription factors were highly induced by growth factor stimulation in PANC-1, but not in MiaPaCa-2 cells (Figure 15A), and this effect was efficiently counteracted by SU5402 (Figure 15A) confirming its dependence on FGFR2 signaling. Biochemical analysis was performed to assess the contribution of FGFR2c expression and signaling on epithelial/mesenchymal marker modulation. The results showed that, only in PANC-1 cells, the very low levels of the epithelial marker E-cadherin and the high levels of the mesenchymal marker vimentin appeared further decreased and increased, respectively, by FGF2 stimulation (Figure 15B). Again, the efficiency of SU5402 in reversing these effects (Figure 15B) confirmed the dependence on FGFR2c activation and signaling. In contrast, the hardly detectable levels of E-cadherin, as well

as the lower levels of vimentin observed in Mia PaCa-2 cells compared to PANC-1 cells (Figure 15B), appeared not significantly affected by FGF2 treatment (Figure 15B). Our biochemical findings were also validated by immunofluorescence approaches, which showed how FGF2 stimulation did not substantially impact on Mia PaCa-2 morphology (Figure 15C), while it forced PANC-1 cells to detach from each other and to assume a spindle shape (Figure 15C). In addition, the immunostaining with anti-vimentin appeared significantly increased by FGF2 and abrogate by SU5402 only in PANC-1 cells (Figure 15C).

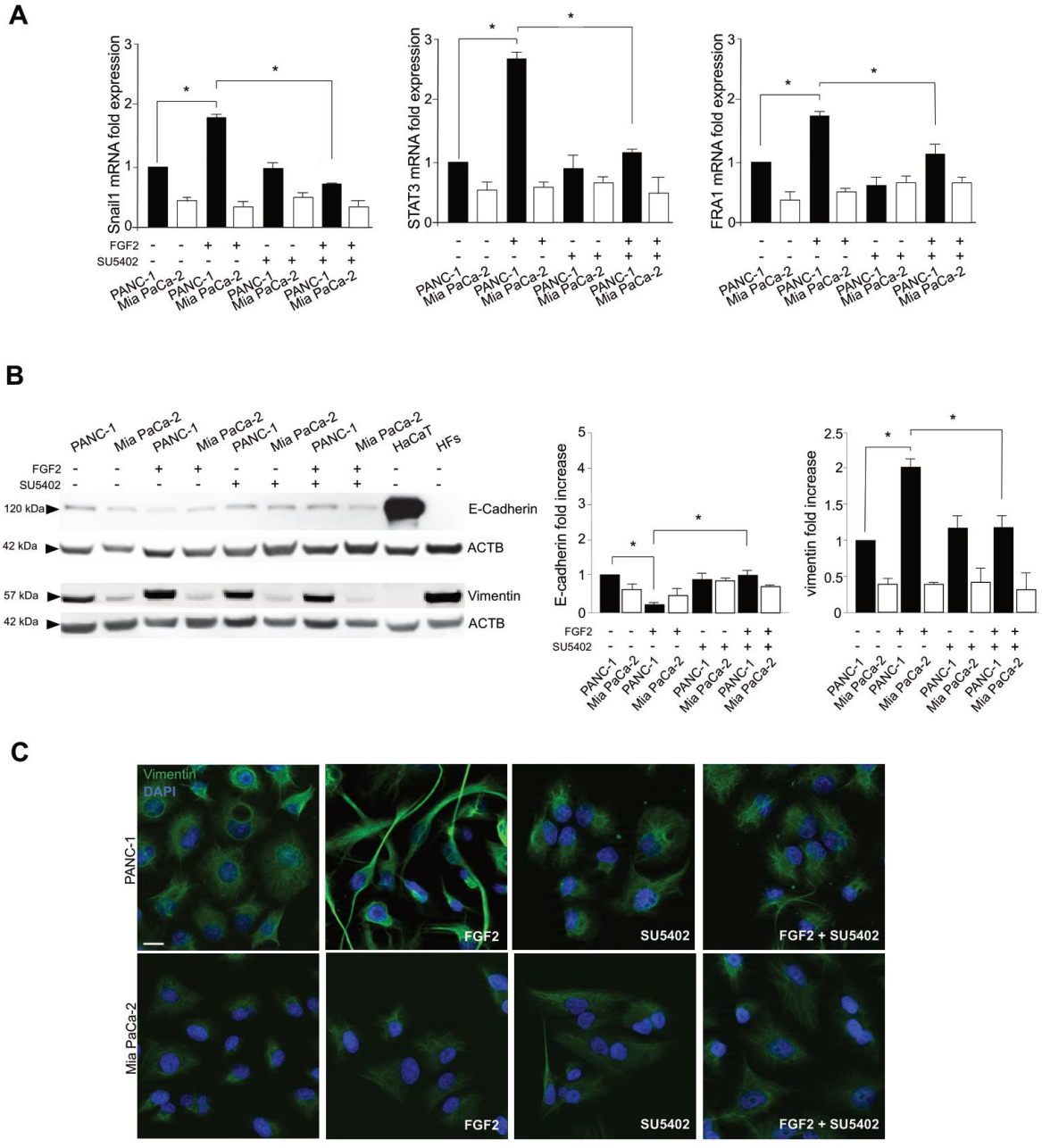

Figure 15. FGFR2c expression impacts on the enhancement of EMT phenotype in response to FGF2. PANC−1 and Mia PaCa−2 cells were left untreated or stimulated with FGF2 in the presence or absence of SU5402, as above. HaCaT cells and HFs were used as controls for the expression of E−cadherin and vimentin, respectively. (A) Real−time RT−PCR

shows the induction of the EMT−related transcription factors Snail1, STAT3 and FRA1 by FGF2 stimulation only in PANC−1 and its reversion by SU5402. Results are expressed as mean value ± SD (n = 3). ANOVA with Tukey's multiple comparison test: * p ≤ 0.05. (B) Western blot analysis shows that, only in PANC−1 cells, the very low levels of the epithelial marker E−cadherin and the high levels of the mesenchymal marker vimentin are further decreased and increased, respectively, by FGF2 stimulation. The presence of SU5402 reverses these effects. E−cadherin and vimentin expression are not significantly changed by FGF2 treatment in Mia PaCa−2 cells. Equal loading was assessed with the anti−actin antibody. Results are expressed as mean value ± SD (n = 3). The densitometric analysis was performed as reported above. ANOVA with Tukey's multiple comparison test: * p ≤ 0.05. (C) Immunofluorescence analysis shows that FGF2 stimulation leads PANC−1 cells to detach from each other and to assume a spindle shape, while Mia PaCa−2 morphology remains unchanged. The anti−vimentin immunostaining is increased by the stimulation only in PANC−1 cells. All the effects are abrogated by SU5402. Nuclei were stained with DAPI. Bar: 10 μm.

To definitely confirm that the greater responsiveness displayed by PANC-1 cells to FGF2 in terms of acquisition of a more pronounced mesenchymal expression profile and morphology might be ascribed to a higher expression of FGFR2c, we performed a stable protein depletion of FGFR2 by specific short hairpin RNA (FGFR2 shRNA) transfection, whose efficiency was verified by biochemical approaches (Figure 16A). Parallel experiments performed using isoform specific small interfering RNAs showed that, especially in PANC1 cells, the transfection with FGFR2c siRNA, but not that with FGFR2b siRNA, induced a decrease of the band at the molecular weight of 100kDa (Figure 16B), which was comparable to that obtained using the generic FGFR2 shRNA (Figure 16A). These results confirmed that the transfection with the FGFR2 shRNA actually results in an efficient depletion of the receptor mesenchymal variant. It is worth noting that, as expected (Ishiwata 2018) PDAC cell lines and HaCaT keratinocytes, used as positive control for FGFR2b expression, expressed two different variants of FGFR2 isoforms, differing for the presence or not of the first Ig loop in the extracellular domain, which implies different molecular weights (100 and 140 kDa, respectively) (Tiong et al., 2013). Then, analyzing the expression of the EMT-related transcription factors Snail1, STAT3 and FRA1 by real-time RT-PCR, we found that their increase, induced only in PANC-1 by FGF2 stimulation (Figure 16C), was abolished by the receptor depletion (Figure16C).

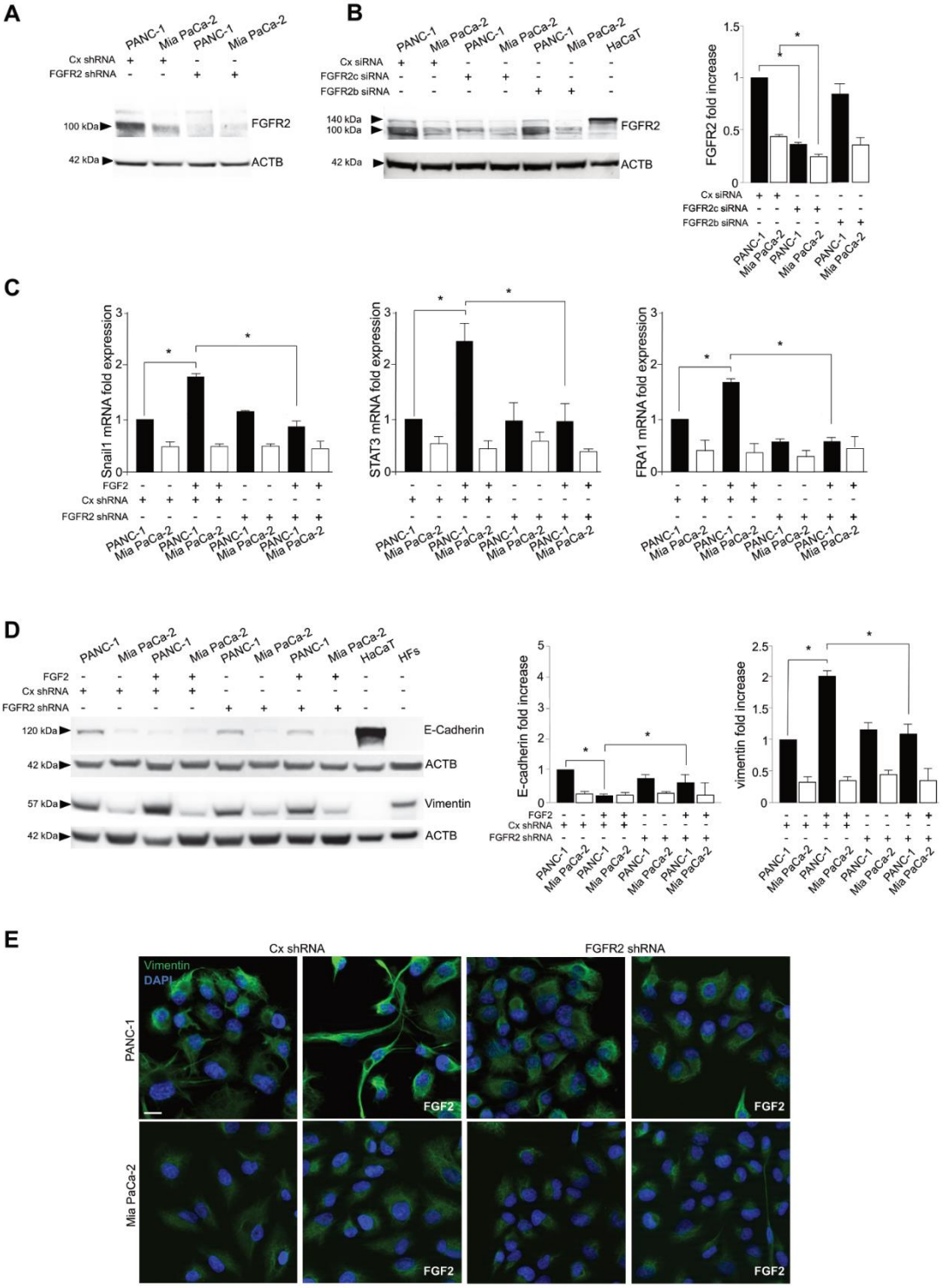

Figure 16. FGFR2c depletion affects the responsiveness of PANC−1 cells to FGF2 in terms of enhancement of the mesenchymal expression profile. PANC−1 and Mia PaCa−2 cells were stably transduced with FGFR2 shRNA or alternatively transfected with specific FGFR2b siRNA or FGFR2c siRNA. Unrelated shRNA (Cx shRNA) or siRNA (Cx siRNA) were used as negative control. Cells were left unstimulated or stimulated with FGF2 as above. HaCaT cells were used as positive control for the expression of FGFR2 and E−cadherin, while HFs for that of vimentin. (A) Western blot analysis shows the efficiency of the stable protein depletion of FGFR2 by shRNA transduction. (B) Especially in PANC−1 cells, the transfection with FGFR2c siRNA, but not that with FGFR2b siRNA, induces a decrease of FGFR2 band, which is comparable to that obtained using the generic FGFR2 shRNA (shown in A). Equal loading was assessed with the anti−actin antibody. Results are expressed as mean value ± SD (n = 3). The densitometric analysis was performed as reported above. ANOVA with Tukey's multiple comparison test: * $p \leq 0.05$. (C) Real−time RT−PCR shows that FGFR2 depletion abolishes the increase of Snail1, STAT3 and FRA1 induced only in PANC−1 cells

by FGF2 stimulation. Results are expressed as mean value ± SD (n = 3). ANOVA with Tukey's multiple comparison test: * p ≤ 0.05. (D) Western blot analysis shows that the stable depletion of FGFR2 makes PANC−1 unresponsive to FGF2, in terms of further repression of E−cadherin and vimentin enhancement. Equal loading was assessed with the anti−actin antibody. Results are expressed as mean value ± SD (n = 3). The densitometric analysis was performed as reported above. ANOVA with Tukey's multiple comparison test: * p ≤ 0.05. (E) Immunofluorescence analysis shows that, in PANC-1 cells, the enhancement of vimentin immunostaining and the tendency to assume a spindle−shaped morphology in response to FGF2 are abolished by FGFR2 depletion. Bar: 10 μm.

Comparable results were obtained in biochemical and immunofluorescence experiments, performed to assess the impact of FGFR2c depletion on the modulation of the epithelial/mesenchymal markers and on the changes in cell morphology in response to FGF2. In fact, in line to what was observed in the presence of SU5402, the stable depletion of FGFR2 abolished PANC-1 responsiveness to FGF2, in terms of E-cadherin repression and vimentin enhancement (Figure 16D,E). In addition, these cells lost the tendency to assume a spindle-shaped morphology in response FGF2 stimulation, conserving their cobblestone shape and the ability to growth in packed colonies (Figure 16E). Thus, the different responsiveness of PANC-1 and Mia PaCa-2 cells to paracrine FGFs in terms of acquisition of EMT phenotype appears to be mostly attributable on their different expression of FGFR2c.

The Activation of PKCε Is the Key Molecular Event Downstream FGFR2c Underlying EMT Induction

Since we recently found that PKCε is the main signaling substrate contributing to FGFR2c-mediated induction of EMT in human keratinocytes (Ranieri et al.,2020; Ranieri et al 2021), the possible involvement of this signaling substrate also in the context of pancreatic cancer has been investigated in this work. To this aim, the extent of PKCε activation in the selected PDAC cell lines was firstly assayed by analyzing the phosphorylation of its Ser 729 site, which depends on the internal catalytic activity and is a widely recognized indicator of PKCε activation (Lau et al., 2012; Karavana et al., 2014). Western blot analysis showed that an appreciable increase of phosphorylation of PKCε at this autophosphorylation site was detected only in PANC-1 cells upon FGF2

stimulation (Figure 17A), which was abolished by SU5402 (Figure 17A), confirming its close dependence on FGFR2c activation. In addition, the absence of a detectable increase of phosphorylation in MiaPaCa-2 cells also suggests that PKCε activation could be dependent on FGFR2c expression levels. On the other hand, differently from what observed in human keratinocytes (Ranieri et al.,2020),

FGF2 stimulation did not induce appreciable changes in PKCε protein levels (Figure 17A). Then, we analyzed the role exerted by PKCε in the establishment of EMT phenotype, generating PANC-1 and Mia PaCa-2 cell lines stably depleted for PKCε by transfection with specific shRNA. The efficiency of PKCε gene silencing was confirmed by Western blot analysis (Figure 18). Real time RT-PCR showed that the induction of the three EMT-related transcription factors downstream FGFR2c, induced in PANC-1 cells by FGF2 (Figure 17B), was significantly repressed by PKCε depletion (Figure 17B).

In addition, biochemical experiments highlighted that PKCε knockdown also counteracted the repression of E-cadherin, as well as the upregulation of vimentin induced by FGF2 in these cells (Figure 17C), confirming the interference with EMT induction. Finally, immunofluorescence approaches showed how PKCε depletion was able to counteract either the enhancement of vimentin expression (Figure 17D) or the morphological changes in favor of the mesenchymal feature displayed by PANC-1 cells in response to FGF2 (Figure 17D). These results indicated that PKCε-mediated signaling downstream FGFR2c significantly contribute to the establishment of receptor-dependent EMT phenotype.

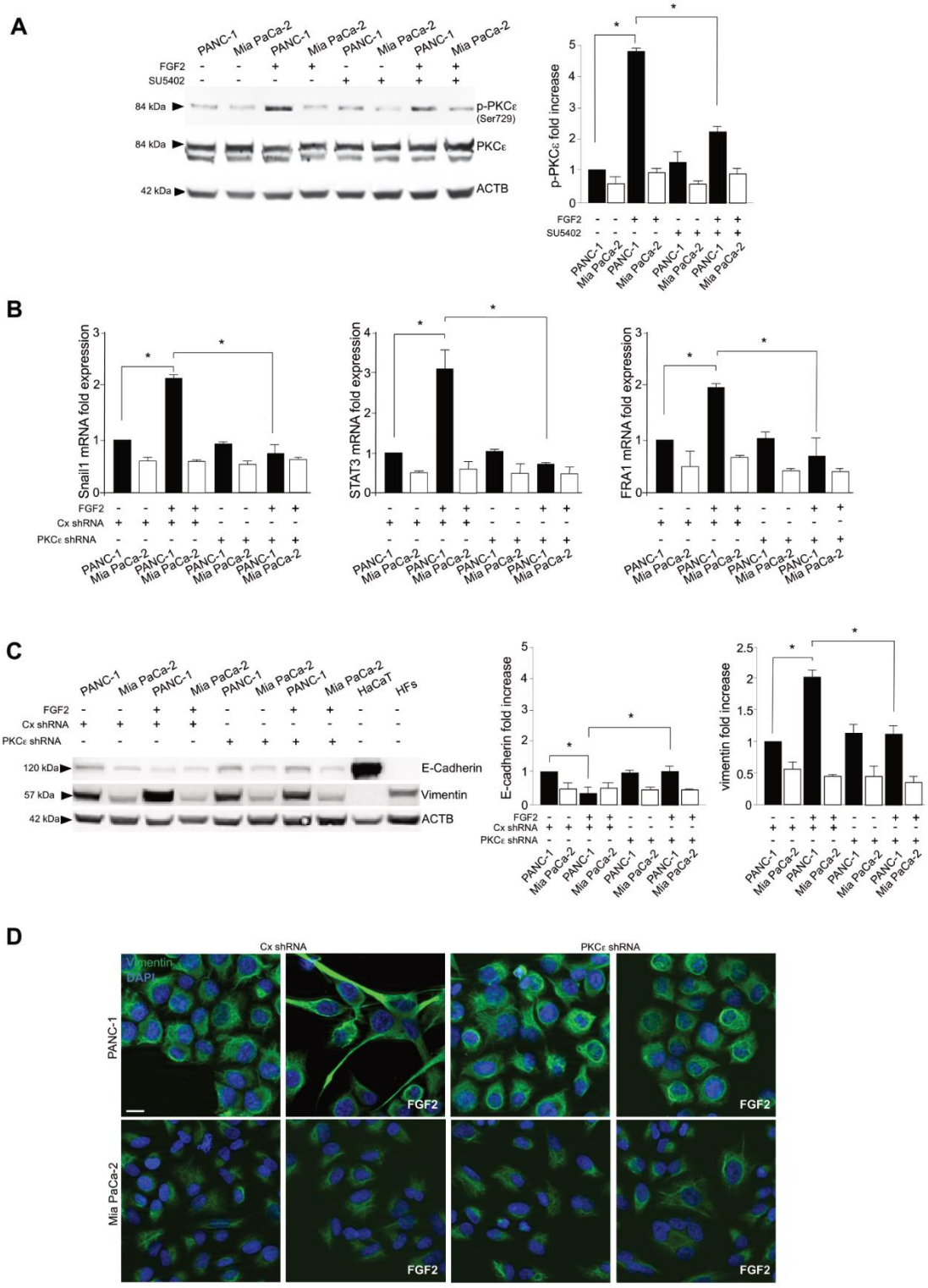

Figure 17: The depletion of PKCε interferes with FGF2−triggered EMT phenotype. PANC−1 and Mia PaCa−2 cells were left untransduced or stably transduced with PKCε shRNA or with an unrelated shRNA, as negative control. Cells were left untreated or stimulated with FGF2 in presence or absence of SU5402 as above. HaCaT cells and HFs were used as positive controls for epithelial/mesenchymal marker expression, as reported above. (A) Western blot analysis shows that the increase of phosphorylation of PKCε is observed upon FGF2 stimulation only in PANC−1 cells and this effect is abolished by SU5402. Equal loading was assessed with the anti−actin antibody. Results are expressed as mean value ± SD (n = 3). The densitometric analysis was performed as reported above. ANOVA with Tukey's multiple comparison test: * $p \leq 0.05$. (B) Real−time RT−PCR shows that the induction of Snail1, STAT3 and FRA1 only in PANC−1 cells in response to FGF2 is repressed upon PKCε depletion. Results are expressed as mean value ± SD (n = 3). ANOVA with Tukey's multiple

comparison test: * p ≤ 0.05. (C) Western blot analysis highlights that PKCε knockdown also counteracted the repression of E−cadherin, as well as the upregulation of vimentin induced by FGF2 in PANC−1 cells. Equal loading was assessed with the anti−actin antibody. Results are expressed as mean value ± SD (n = 3). The densitometric analysis was performed as reported above. ANOVA with Tukey's multiple comparison test: * p ≤ 0.05. (D) Immunofluorescence analysis shows that PKCε silencing interferes with the enhancement of vimentin expression, as well as with the tendency of PANC−1 cells to assume the mesenchymal morphology in response to FGF2. Bar: 10 μm.

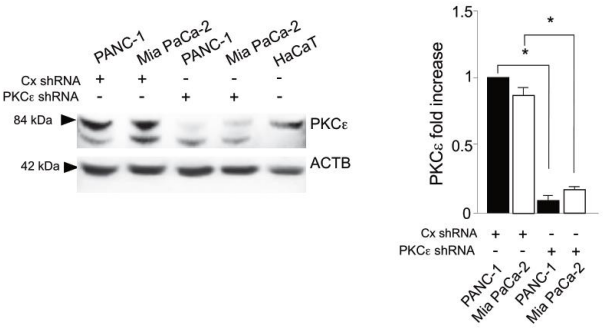

Figure 18: Efficiency of PKCε depletion by specific shRNA. PANC-1 and MIAPaCa-2 cells were stably trasduced with PKCε shRNA or with an unrelated shRNA, as negative control. HaCaT cells were used as positive control for PKCε expression. Western blot analysis shows the efficiency of PKCε gene silencing. Equal loading was assessed with anti actin antibody. Results are expressed as mean value ± SD. The densitometric analysis was performed as reported in materials and methods. ANOVA with Tukey's multiple comparison test: * p ≤ 0.05

PKCε Signaling Negatively Impacts on the Autophagic Process

We have recently proposed a role of PKCε-mediated signaling not only in FGFR2c-mediated induction of EMT, but also in FGFR2c-dependent inhibition of the autophagic process in human keratinocytes (Ranieri et al., 2021). Therefore, we investigated here the possible contribution of PKCε on autophagy also in the specific context of pancreatic cancer. Western blot analysis showed that PKCε knockdown abolished the decrease of the widely recognized autophagic marker LC3-II, induced by FGF2 stimulation exclusively in PANC-1 cells (Figure 19A). In addition, in these cells, PKCε depletion also counteracted the accumulation of the autophagy substrate

SQSTM1 in response to FGF2 (Figure 19A), confirming the efficient reactivation of the autophagic flux. Parallel quantitative immunofluorescence analysis showed that the reduction of LC3 positive dots per cell, evident only in PANC-1 cultures stimulated with FGF2 (Figure 19B), was efficiently reversed by the stable depletion of PKCε (Figure 19B). Comparable results were obtained counteracting FGFR2c signaling and expression by SU5402 or FGFR2 shRNA transfection, respectively (Figure 20A,B), demonstrating that the negative effects on autophagy exerted by PKCε upstream requires FGFR2c activation. The role played by PKCε in the repression of autophagy was further confirmed by electron microscopy studies, performed in PANC-1 cells stably transfected with PKCε shRNA or with control shRNA (Cx shRNA). Ultrastructural examination, performed by transmission electron microscopy (TEM), revealed that the reduction of autophagic vacuoles, triggered by FGF2 stimulation in control cells (Figure 19C,D) was counteracted by PKCε depletion, which enabled cells to maintain a higher number of autophagic structures in the cytoplasm also after FGF2 stimulation (Figure 19E). In addition, PANC-1 Cx shRNA cells, but not PANC-1 PKCε shRNA cells, appeared elongated in response to FGF2 treatment and their cytoplasm resulted enriched in vimentin filament bundles (Figure 19C, arrows). These ultrastructural observations are consistent with our immunofluorescence data (see Figure 17D) and confirm the ability of PKCε knockdown in reversing FGF2-induced mesenchymal phenotype. Thus, in agreement with our previous observations in human keratinocytes (Ranieri et al., 2020; Ranieri et al., 2021), at least in PANC-1 cells, PKCε-mediated signaling activated downstream FGFR2c appears not only to be involved in EMT induction, but also to exert a not negligible inhibitory effect on autophagy.

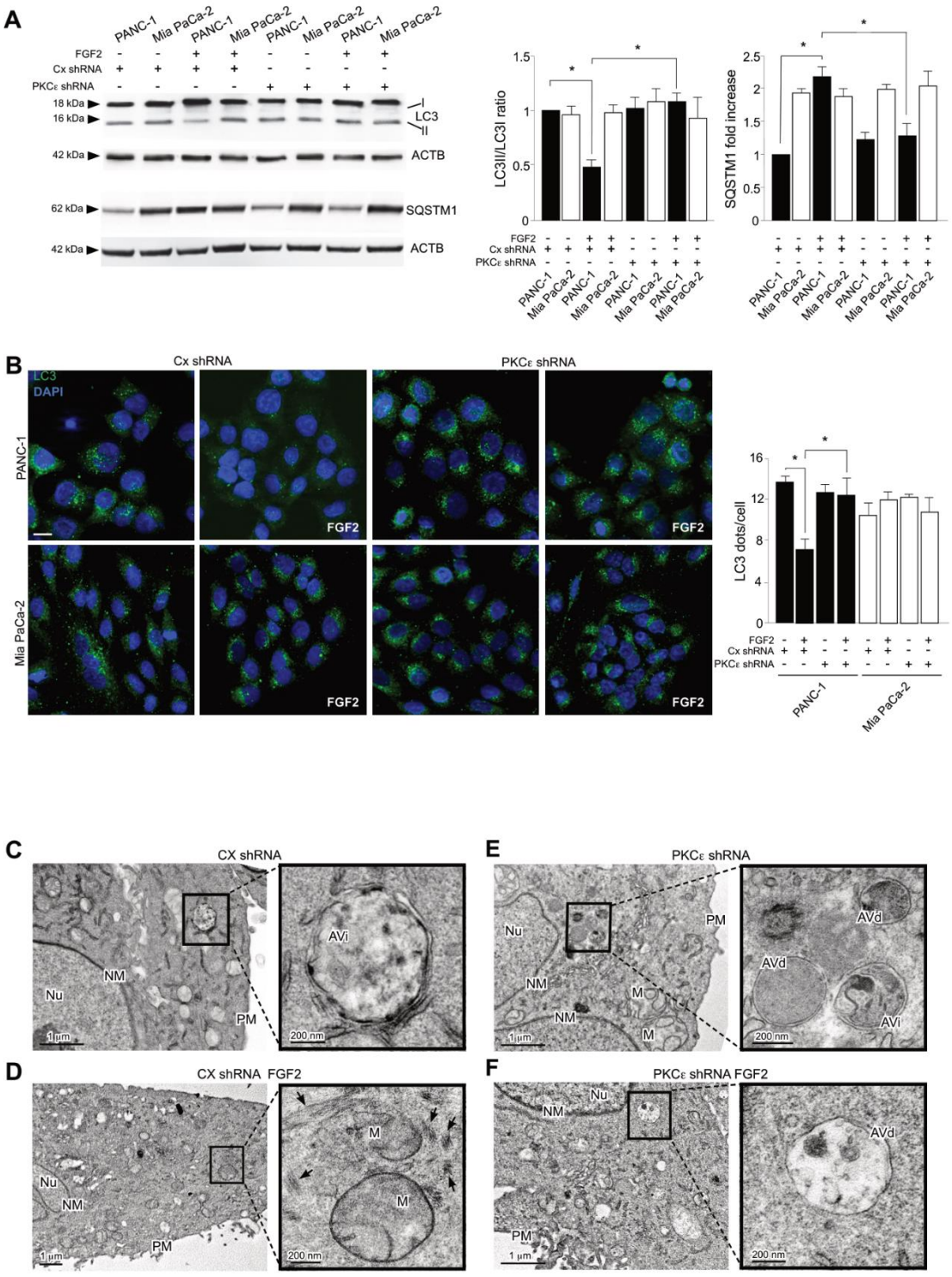

Figure 19. PKCε depletion also negatively impacts on FGF2−dependent inhibition of autophagy. PANC−1 and MiaPaCa−2 cells stably transduced with PKCε shRNA or with an unrelated shRNA were left untreated or stimulated with FGF2 as above. (A) Western blot analysis shows that PKCε knockdown abolishes the decrease of the autophagic marker LC3−II, as well as the increase of the autophagic substrate SQSTM1, induced by FGF2 stimulation exclusively in PANC−1 cells. Equal loading was assessed with the anti-actin antibody. Results are expressed as mean value ± SD (n = 3). The densitometric analysis was performed as reported above. ANOVA with Tukey's multiple comparison test: * $p \leq 0.05$. (B) Quantitative immunofluorescence analysis shows that the reduction of LC3 positive dots per cell, evident only in PANC−1 upon FGF2 is reversed by PKCε depletion. Quantitative analysis was performed as described in Materials and Methods, and results are expressed as mean

values ± SD (n = 3). ANOVA with Tukey's multiple comparison test: * p ≤ 0.05. (C–F) Ultrastructural analysis by transmission electron microscopy (TEM) shows initial autophagic vacuoles (AVi) with double isolation membrane in the cytoplasm of unstimulated PANC−1 Cx shRNA cells (C, magnification box). The examination of PANC−1 Cx shRNA stimulated with FGF2 shows a spindle−like shape, a reduced presence of AVs compared to unstimulated cells, and a higher cytoplasmatic complexity, with several intracellular filaments (D), arrows in the magnification box, possibly corresponding to vimentin bundles (D). AVi and degradative (AVd) autophagic vacuoles in the cytoplasm of both unstimulated and FGF2−stimulated PKCε shRNA cells (see magnification boxes). AVi: Initial autophagic vacuole; AVd: degradative autophagic vacuole; M: mitochondrion; Nu: nucleus; NM: nuclear membrane; PM: plasma membrane. Bars: 1 μm, 200 nm.

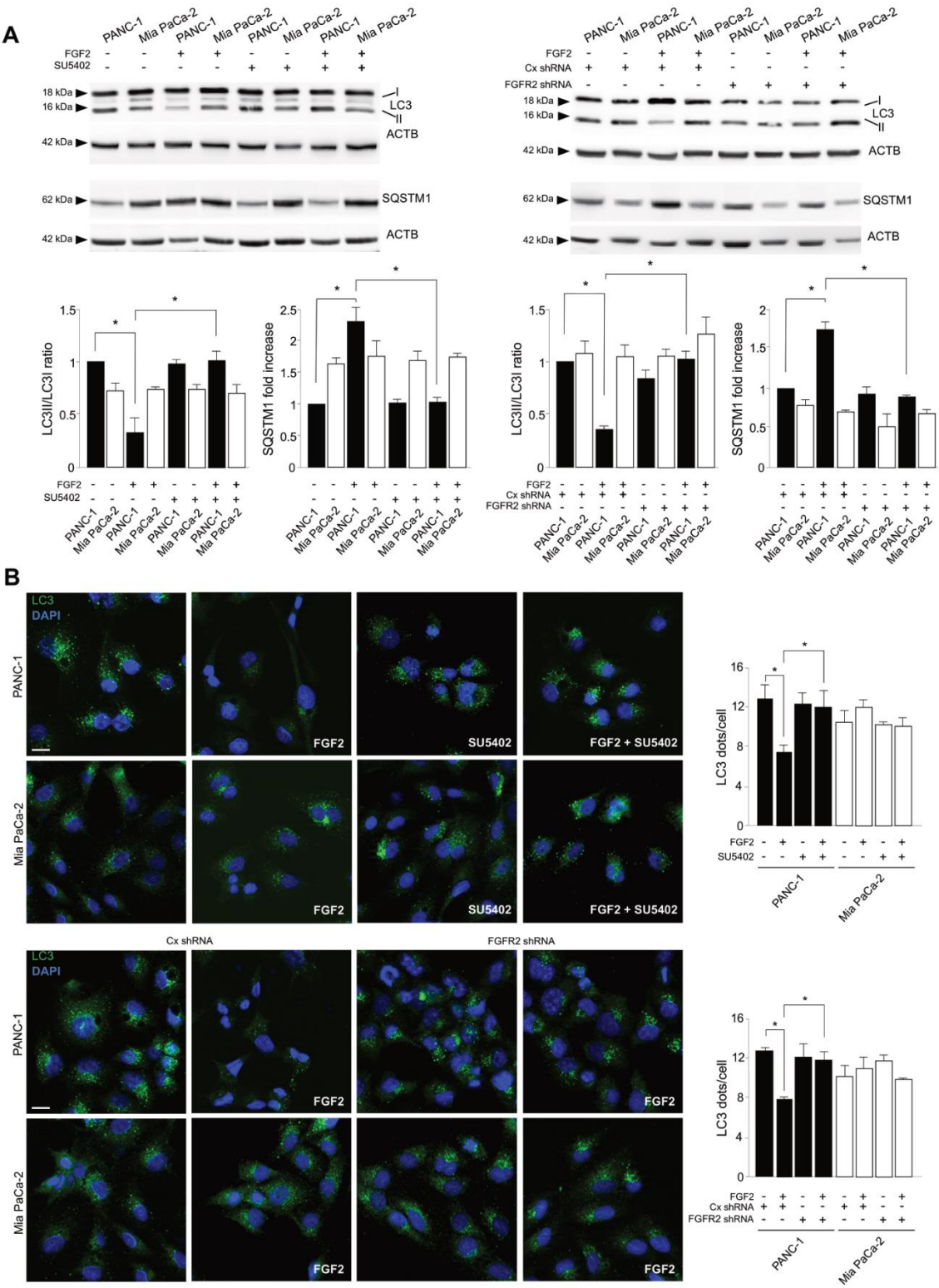

Figure 20. Impact of FGFR2 shut-off or depletion on the autophagic process. PANC-1 and MiaPaCa-2 cells stimulated with FGF2 in the presence or absence of the FGFR2 inhibitor SU5402. Alternatively, cells were transduced with FGFR2 shRNA or with an unrelated shRNA, as negative control and then stimulated with FGF2 (A) Western blot analysis shows that both the presence of SU5402 (left) and FGFR2 protein depletion by shRNA (right) inhibit both the decrease of the autophagic marker LC3II and the accumulation of SQSTM1 induced by FGF2 stimulation exclusively in PANC1 cells. Equal loading was assessed with the anti-actin antibody. Results are expressed as mean value ± SD. ANOVA with Tukey's multiple comparison test: * $p < 0.05$. (B) Quantitative immunofluorescence analysis shows that the decrease of LC3II positive dots per cell, evident only in PANC-1 upon FGF2 is switched by FGFR2 signaling shut-off or depletion. Results are expressed as mean values ± SD. ANOVA with Tukey's multiple comparison test: * $p < 0.05$

PKCε Signaling Interferes with Autophagy Converging on ERK1/2 Pathway

To clarify the molecular mechanisms underlying the involvement of PKCε in the autophagic process, we focused our attention on MTOR, which is considered the main negative regulator of autophagy also in pancreatic cancer cells (Mollinedo and Gajate 2019; Piffoux et al., 2021). Western blot analysis revealed that the phosphorylation of MTOR, as well as that of its substrate S6K, evident after FGF2 stimulation particularly in PANC-1 cells (Figure 21A), were strongly dampened by PKCε knockdown (Figure 21A). Surprisingly, no corresponding effects were observed on the AKT phosphorylation (Figure 21B). Since AKT is the upstream substrate generally responsible for MTOR activation, our unexpected results indicated that PKCε might activate MTOR through an alternative pathway. This possibility appears to be consistent with the peculiar ability, previously described for PKCε in other cellular contexts, to converge on MTOR through the activation of Raf/MEK/ERK signaling (Moschella et al., 2007). Actually, the important contribution of ERK1/2 signaling in MTOR activation and consequent autophagy inhibition has been widely described in pancreatic cancer cells (Mollinedo and Gajate 2019). Based on these assumptions, we investigated the impact of PKCε signaling on ERK1/2 pathway. Biochemical analysis showed that, in consequence of PKCε depletion, the increase of ERK1/2 phosphorylation in response to FGF2, visible in both pancreatic cell lines (Figure 21C), was reduced in Mia PaCa-2, which maintained a significant residual ERK phosphorylation (Figure 21C), but completely abolished in PANC-1 (Figure 21C). These results indicate that the

different expression of FGFR2c displayed by the two PDAC cell lines impact on the dependence on PKCε of ERK1/2 signaling. It is also worth noting that shFGFR2c transduced MiaPaCa-2 cells displayed a higher responsiveness to FGF2 in terms of ERK1/2 phosphorylation compared to non-transduced ones (see Figure 13 B in comparison with Figure 21C), even if this phosphorylation remains significantly lower than that shown by PANC-1 cells.

This variability of MiaPaCa-2 cell response to FGF2 might be the consequence of different culture conditions. These results indicated that, only in PANC-1 cells, the activation of ERK1/2 pathway upstream depends on PKCε activation. Since ERK1/2 is also a well-known pathway involved in EMT of PDAC cells (Huang et al., 2021), our results suggest the possibility that, in this tumor context, PKCε signaling, when activated in consequence of highly expression of FGFR2c, could simultaneously repress autophagy and orchestrate the EMT program directly converging on ERK1/2 pathway.

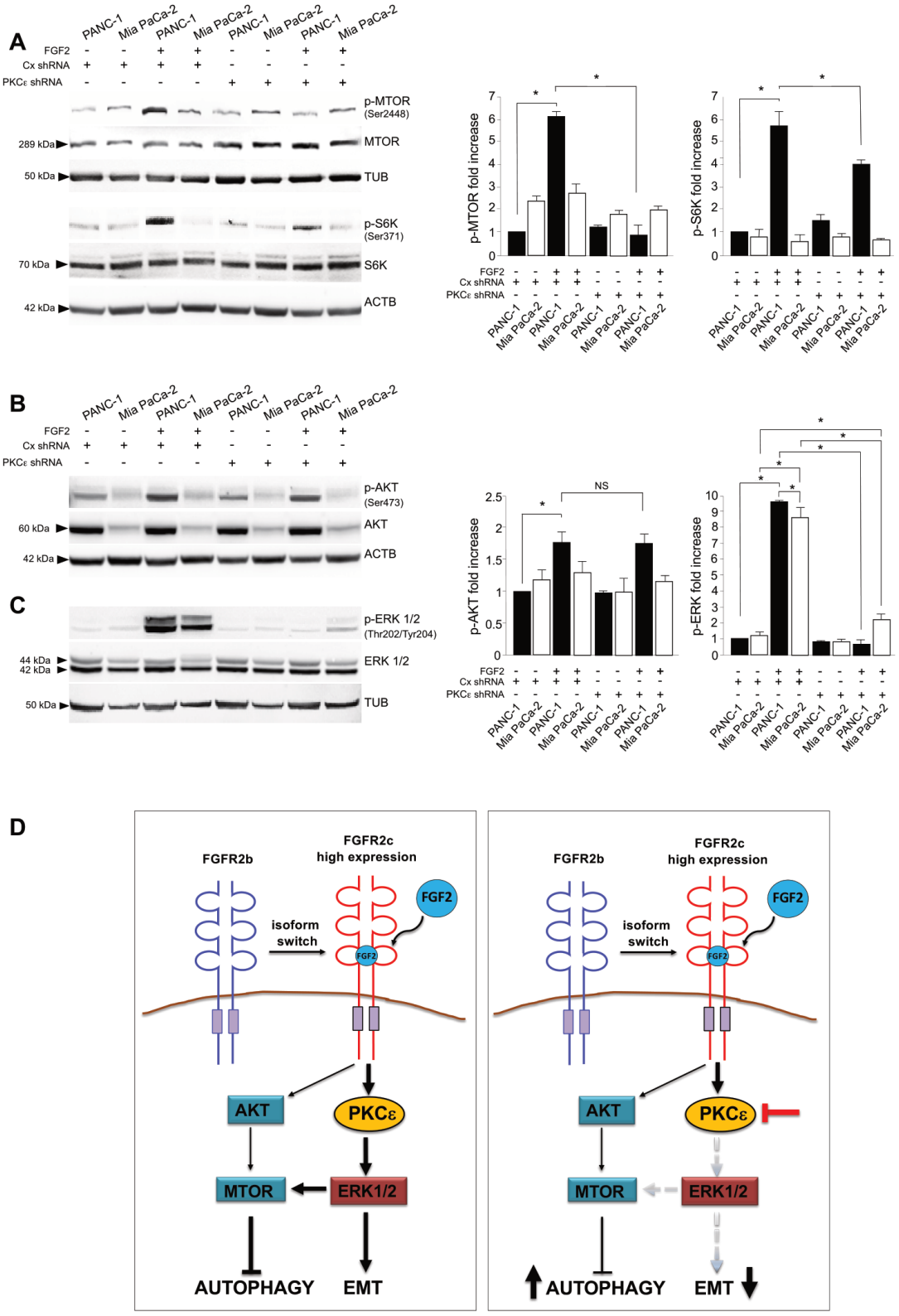

Figure 21. PKCε signaling shut−off by PKCε protein depletion interferes with both MTOR and ERK1/2 signaling pathways. PANC−1 and Mia PaCa−2 cells stably transduced with PKCε shRNA or with an unrelated shRNA were left untreated or stimulated with FGF2 as above. (A) Western blot analysis shows that the increase of phosphorylation of MTOR and S6K, evident after FGF2 stimulation only in PANC−1 cells, are strongly dampened by PKCε knockdown. (B) No corresponding effects are observed on the AKT phosphorylation. (C) The increase of ERK1/2 phosphorylation in response to FGF2, visible in both pancreatic cell lines, is significantly greater in PANC−1 cells and it is reduced in Mia PaCa−2 and completely abolished in PANC−1 by PKCε depletion. Equal loading was assessed with tubulin and anti-actin antibodies. Results are expressed as mean value ± SD (n = 3). The densitometric

analysis was performed as reported above. ANOVA with Tukey's multiple comparison test: * $p \leq 0.05$. (D) Schematic drawing representing the role of PKCε as key hub signaling molecule downstream FGFR2c, whose activation simultaneously counteracts autophagy and drives EMT bypassing AKT and directly converging on ERK1/2. PKCε knockdown results in a simultaneous reversion of these effects.

Discussion

The isoform switch is one of the molecular mechanisms involved in the dysregulation of receptor tyrosine kinase (RTK) signaling in cancer. Concerning this topic, recent studies of our research group have demonstrated that FGFR2 isoform switch and the consequent aberrant expression of the mesenchymal FGFR2c isoform in epithelial context induces the impairment of differentiation, EMT and tumorigenic features (Ranieri et al., 2016; Ranieri et al., 2018) mainly involving PKCε signalling (Ranieri et al., 2020). Contextdependent different roles in cancer have been recently also proposed for the degradative pathway of autophagy. (Kimmelman and White, 2017). In fact, while during metastatic spreading autophagy appears to sustain EMT, during the early steps of tumorigenesis the two processes appear to be linked to a negative loop (Chen et al, 2019). On the light of these background, for a long time our research group focused his attention on this degradative process, highlighing that, while FGFR2b signalling enhances the physiological, positive interplay between autophagy and keratinocyte differentiation (Nanni et al., 2018a; Nanni et al., 2018b), FGFR2 isoform switch and the consequent aberrant expression and signalling of FGFR2c inhibit the autophagic process, via the activation of the canonical AKT/MTOR pathway (Nanni et al., 2019). On the light of all this evidence, in this thesis project we investigated if FGFR2c aberrant expression might lead to the acquisition of EMT tumorigenic features not only by activating a complex oncogenic signalling network engaging several players, including PKCε, but also by upstream establishing and controlling a negative crosstalk between EMT and autophagy.

Since MTOR is the main pathway involved in the inhibition of autophagy induced by aberrant FGFR2c expression in human keratinocytes, (Nanni et al., 2019) we first assessed the impact of a forced reactivation of autophagy on FGFR2c-mediated oncogenic effects, using rapamycin, the widely accepted general inhibitor of MTOR-dependent autophagy. Using human keratinocytes stably expressing the aberrant FGFR2c, we demonstrated that rapamycin not only turned the FGF2-induced decrease of the widely

recognized autophagic marker LC3-II into an increase (as expected by autophagy reactivation), but also efficiently reverses FGFR2c-induced EMT program and inhibits receptormediated cell invasion. Our observations are consistent with the recent findings by Bell and coworkers, highlighting how the inhibition of autophagy, which impairs Met receptor traffic, is required for HGF-dependent invasive behaviour in several tumor contexts (Bell et al., 2019). Similar results were obtained testing the effects of autophagy reactivation on FGFR2c-mediated EMT via gene silencing of MTOR or AKT (which is the upstream regulator of MTOR) by small interfering RNA.

Since FGFR2c-triggered EMT is driven by PKCε-dependent induction of Snail1, STAT3 and FRA1 (Ranieri et al., 2016; Ranieri et al., 2020), we also found that the forced reactivation of the autophagic process by MTOR siRNA appears to negatively affect the induction of these EMT-related transcription factors in a comparable way to PKCε signalling shut-off, obtained by stable transfection of shRNA. On the other hand, the selective PKCε shut-off by shRNA resulted not only to inhibit FGFR2c-induced EMT, as expected (Ranieri et al., 2020), but also to reverse the negative impact of FGFR2c signalling on autophagy, inducing a recovery of LC3-II levels and a repression of the accumulation of the autophagy substrate SQSTM1, suggesting a reactivation of the autophagic flux. Thus, when FGFR2c is aberrantly expressed in epithelial context, the aberrant signaling downstream PKCε appears to be involved not only in EMT-induction and invasion, but also in the inhibition of autophagy orchestrated by FGFR2c. Our results are consistent with previous data showing that PKCε is involved in the suppression of the autophagic process in glioblastoma cells (Toton et al., 2018). In addition, other previous work has demonstrated that the transcription factor STAT3, activated downstream FGFR2c and PKCε, has been found to be involved not only in the triggering of EMT program (Ranieri et al., 2020; Wendt et al., 2014), but also in the inhibition of autophagy. (You et al., 2015). Despite these supporting studies, our current results seem apparently in contrast with the recent findings reported by Basu, which indicate a key role of PKCε in promoting autophagic process in metastatic breast cancer cells (Basu 2020). This discrepancy would be explained considering the hypothesis that FGFR2c plays its oncogenic role in the early

steps of tumour development (Ranieri et al., 2016; Ranieri et al., 2018; Ranieri et al., 2020) further confirming the dual and opposite contribution of autophagy in different (early and advanced-metastatic) steps of carcinogenesis (Chen et al., 2019).

As last aim, performing selective FGFR2 signaling shut-off using the FGFR2specific kinase inhibitor SU5402, we found that all the responses to FGF2, in terms of EMT induction and autophagy repression closely depend on FGFR2c activation, confirming the upstream role of FGFR2c in the regulation of EMT/autophagy crosstalk. Overall, our results represent the first indication that, at least in the context of human keratinocytes, the aberrant expression of FGFR2c, usually stemming from altered FGFR2 isoform switch, could be the upstream event leading to the activation of oncogenic signalling pathways intersecting with each other and cooperating in the establishment of the negative loop between EMT and autophagy, which contributes to the early steps of tumor development.

In the second part of this study the focus shifted our investigations to PDAC context, characterized by a strong EMT signature and deregulated autophagy. PDAC is a malignant tumor that presents KRAS constitutive activation (Mollinedo et al., 2019). Research efforts recently focused on the discovery of new molecules and pathways, to bypass RAS, and whose inhibition might significantly effect on PDAC malignant phenotype. FGFR2 isoform switch is considered an oncogenic event also during pancreatic carcinogenesis but its contribution in EMT induction and cell invasion still appears controversial (Ishiwata et al., 2012; Ueda et al., 2014; Ishiwata 2018). For this study has been used two PDAC cell lines (PANC-1 and Mia PaCa-2 cells) with undetectable levels of the epithelial FGFR2b isoform and different levels of the mesenchymal FGFR2c variant. It has been investigated the responsiveness to FGF2 in terms of AKT/MTOR and ERK1/2 signaling activation confirming close dependence on FGFR2c expression and activation. Focusing on the impact on EMT, resulted in PANC-1, which express higher levels of FGFR2c compared to Mia PaCa-2 cells, higher expression of the transcription factors related to EMT and modulation of epithelial and mesenchymal marker toward pathological EMT. A clear increase of EMT profile and mesenchymal morphology, after

FGF2 stimulation, appears exclusively in PANC-1 and it is abolished by FGFR2c kinase activity shut-off or depletion, confirming their dependence on receptor signaling and expression. In the in vivo cancer context, FGFR2c aberrant expression could strongly affect tumor cell sensitivity to paracrine factors liberated by microenvironmental cells, such as cancer associated fibroblasts (CAFs). This higher sensitivity could cause a strong activation of intracellular signaling and enhancement of malignant features. These results are conforming with previous studies, focusing on CAFs and CAF-released factors, such as FGF2, in inducing a more aggressive behavior in PDAC (Von Ahrens et al., 2017; Awaji et al., 2019). The next step has been to investigate the signaling pathways and substrates of downstream FGFR2c possibly involved in the EMT-related phenotype having a special consideration for PKCε, whose oncogenic role in epithelial cells has been widely described (Isakov 2018). In keratinocytes we have recently described PKCε as a key event downstream FGFR2c for EMT induction (Ranieri et al., 2020; Ranieri et al., 2021) The involvement of PKCε was examined using specific shRNA and showed that PKCε depletion strongly compromise the EMT signature, as well as the morphological changes triggered by FGF2 in PANC-1 cells. Interestingly, only PANC-1, PKCε phosphorylation is detectable, suggesting that PKCε activation could be dependent on FGFR2c expression. PKCs are considered “RAS-independent” substrates stimulated by several membrane receptors, including FGFRs (Touat et al., 2015), for this reason the identification of one of PKC family members as a fundamental signaling effector in the EMT phenotype induction (and possibly a higher aggressive behavior) could represent a fundamental improvement towards new therapeutic strategies aimed to bypass the “undruggable” target RAS. Interestingly, PKCε silencing abolished the ability of FGF2 to inhibit autophagy, another important process contributing to PDAC development and progression (Mollinedo et al., 2019; Piffoux et al., 2021; New and Tooze 2019). Autophagy and EMT in cancer are associated by a complex crosstalk (Chen et al., 2019). It has been demonstrated above that this crosstalk is regulated by FGFR2c and its downstream PKCε signaling, at least during the early steps of human epidermal carcinogenesis (Ranieri et al., 2020; Ranieri et al., 2021). It has been proved a negative impact of PKCε downstream

FGFR2c on autophagy at least in PANC-1, which expresses high level of the receptor. Autophagy is repressed in the early stages of tumorigenesis of advanced and aggressive cancers, such as PDAC cell lines, like PANC-1 and MIA PaCa-2, and it is widely described as an oncogenic event sustaining cell survival and metabolism (New and Tooze 2019). Alike to what has been proposed for MEK/ERK signaling in PDAC (Piffoux et al., 2021), these results can be explained counting that a negative modulation of autophagy results in an oncogenic outcome, as an autonomy to autophagy for survival. The specific shut-off of PKCε not only induces an inversion on EMT phenotype, but also increases autophagy, up-modulating tumor cell dependence on this survival strategy and thus their predisposition to autophagic inhibitors. Examining the molecular mechanisms for the inhibitory effect exerted by PKCε on autophagy, it has been found that the shut-off of PKCε suppressed the phosphorylation of the autophagic inhibitor mTOR, only in PANC-1 cells in response to FGF2. These results showed that, as recently suggested in breast cancer (Basu 2020), PKCε could inhibit autophagy triggering the canonical mTOR pathway also in PDAC. Furthermore, PKCε depletion strongly suppressed ERK1/2 phosphorylation in both PDAC lines, even if Mia PaCa-2 cells seem to hold a residual ERK1/2 phosphorylation, indicating that the dependence of ERK1/2 signaling on PKCε activation is resultant on FGFR2c expression levels. PKCε shut-off was ineffective on the activation of the canonical activator of mTOR of AKT, suggesting that, as suggested for cardiomyocytes (Moschella et al., 2007), PKCε might bypass AKT and directly converging on mTOR via ERK1/2. ERK1/2 is also a well-known pathway regulating EMT in PDAC (Huang et al., 2021; Safa 2020) so it could hypothesize that PKCε represents a hub signaling downstream highly expressed FGFR2c, whose activation might contribute to simultaneously inhibit autophagy and induce EMT bypassing AKT and converging on ERK1/2 (see schematic draw, Figure 21D). In this encouraging scenario, additional investigations will be required to assess the efficacy of PKCε inhibition, alone or with FGFR, ERK1/2 and autophagy inhibitors, as innovative therapeutic approaches to prevent and/or reverse tumor aggressive phenotypes.

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