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Development of Cdc25^{Mm} derivatives as anticancer agents

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1. Introduction

1.1 Signal transduction in eukaryotic cells

During their evolutionary process, all living organisms have been selected on the basis of their capability to survive, adapt and proliferate under different external conditions. In order to success, the living cells developed efficient and reliable mechanisms to sense and to respond in the most desirable way to several external stimuli. Talking about the most simple organisms, these stimuli are represented essentially by the presence or the absence of nutrition factors and by the chemical and physical conditions of the environment.

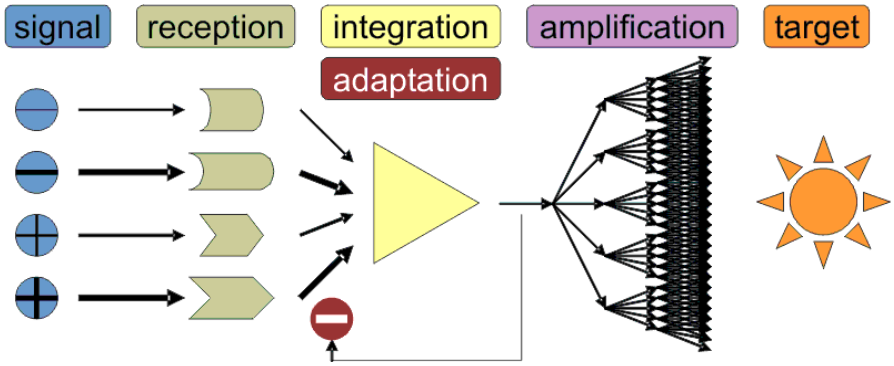

Fig 1.1: Schematic representation of signal transduction events [Max Planck Institute website]

If we think about the extraordinary complexity that can be found in multicellular organisms, however, it is clear that this communication machinery must be really fine tuned and regulated. As a matter of fact all the structural variety and

functional capacity of multicellular organisms is due to their ability to coordinate the biochemical reactions of the various cells of the total organism. The basis for this coordination is the intercellular communication, which allows single cells and all the external conditions to influence the behavior of other cells in a specific manner. In particular all these signals are able to influence crucial aspects of the living cells, regulating complex phenomena like proliferation, differentiation, cell motility, etc. etc… It's quite clear that any deregulation of such important events could be very dangerous and eventually lead to the arising of numerous pathologies.

For this reason, a better understanding of such important mechanisms could be crucial for the design and development of pharmaceutical molecules and therapeutics strategies.

1.2 Signal transduction and Cancer

It is easy to understand that one of the most important disease that could arise from misregulation of the signaling machinery is cancer. As a matter of fact, during the establishment, growth and spread of tumors, several step of miscommunication are often necessary. In normal cells we can find a complex network of interacting and partially redundant signaling pathways that are able to confer robustness to the correct behavior of each cell. In cancer cells, instead, impaired or defective signaling can lead to abnormal proliferation, blocked differentiation, enhanced motility or defective death induction. Moreover during the course of its evolution, each cancer cell accumulates several mutations and chromosomal aberrations so that some signaling pathway can be ablated, while others can become hyper-activated. Beyond single cell events, interaction between different kind of cells can also interfere with the normal cell behavior. Lack or excess of paracrine or endocrine growth factor signaling can play an important role in oncogenesis and tumor progression.

Several pathway are often involved in cancer disease. The Ras pathway, for example, plays a key role in regulating several aspects related to the establishment and the progression of proliferative disorders, as proliferation, survival, cell growth, cell motility and angiogenesis. Several evidences underline such an important role of Ras in cancer diseases. First of all *ras* genes have been identified as the human counterpart of

the transforming agents present in Harvey and Kirsten rat sarcoma viruses (*v-H-ras* [Dhar et al., 1982 and *v-K-ras* [Tsuchida et al., 1982]). Moreover, if transfected into immortalized mouse fibroblast NIH-3T3, these genes are able to confer a transformed phenotype to the cell line [Goldfarb et al 1982; Parada et al,1982; Santos et al 1982;] and the expression of a mutated *K-ras* gene alone in transgenic mice is sufficient to cause or to enhance tumor formation [Caulin et al 2004; Tuveson et al 2004; Vitale-Cross et al 2004]. Finally, the mutation incidence in *ras* genes is particularly high in several kinds of human tumors (Table 1.2)

Tumour type		Predominant ras mutation	Frequency ras mutation (%)
Pancreatic adenocarcinoma		K	78
Thyroid carcinoma			
	Follicular	H,K,N	53
	Papillary	[a]	25
	Undifferentiated	H,K,N	58
Colonic adenocarcinoma		K	36
Cholangiocarcinoma		N	56
Lung			
	Large cell carcinoma	K	21
	Adenocarcinoma	K	22
Endometrial carcinoma		K	21
Ovarian carcinoma		K	23
Seminoma		K,N	43
Dermatological			
	Keratoacanthoma	H	26
	Oral squamous carcinoma	[a]	23
Haematological			
	Multiple myeloma	N,K	30–40
	Acute myeloid leukaemia	N,K	20–30
	Chronic myelomonocytic leukaemia	N,K	50–70
	Plasma cell leukaemia	N,K	60–70

[a] Type of ras mutation not fully defined.

Table 1.2: Frequencies of *ras* mutations (Midgley et al, 2002)

Several growth factor receptors are also involved in the development and progression of neoplastic disease. As a matter of fact alterations of growth factor signaling can lead to increase proliferation, as well as suppression of apoptosis (especially under anchorage-independent conditions) and enhanced cell motility thereby favoring metastatic spread.

For these reasons, growth factor receptor can be considered a rational target for therapeutic strategies in cancer treatments. This would represent a more targeted therapy, in comparison to low specific conventional treatments, that can produce heavy side effects on normal cells.

Two different approaches have been taken into account when trying to develop an efficient growth factor targeted cancer therapy. First of all the inhibition of growth factor-growth factor receptor interaction, for example blocking the target receptor through specific monoclonal antibodies. On the other hand, a similar effect can be obtained by blocking the downstream signaling inside the cells, for example using small molecules capable to interfere with the kinase activity of tyrosine kinase receptor.

The first antireceptor drug has been developed against the epidermal growth factor receptor 2 (Erb2). Erb2 is a transmembrane tyrosine kinase receptor, responsive to the EGF signaling. This receptor has been found to be amplified in numerous cancers, including breast, ovarian and stomach cancer [Mendelsohn and Baserga, 2000; Holbro et al., 2003]. Moreover, its over-expression strongly correlates with a more

aggressive phenotype and a poor clinical outcome. The first drug arisen from all the studies in this direction was Herceptin (Trastuzumab; Genetech) in 1998. This drug is a humanized monoclonal antibody (MAb) against Erb3. Also the epidermal growth factor receptor 1 (EGFR1) and its ligands are often over-expressed in several types of human tumors. Beside against EGFR2, MAb against the EGFR capable of blocking the receptor-ligand interaction or to directly down-regulate the receptor levels have been developed [Shawver et al., 2002; Arteaga, 2003]. However, a second generation of inhibitors have been designed against this target: small molecules interfering with the tyrosine kinase activity of the receptor by blocking the ATP binding site have entered the late stage of clinical development.

Iressa (gefitinib, ZD1839; AstraZeneca), for example has been already approved in Japan for the treatment of non-small-cell lung cancer (NSCLC) and its efficacy as a single agent or in combination with other therapies to treat NSCLC and other cancers is under evaluation in the US clinical trials domain (www.clinicaltrials.gov).

1.3 Ras signaling pathway

Ras is the major representative of a large monomeric G protein superfamily, including more that 150 human protein [Wennerberg et al. 2005].

The key role played by Ras and its pathway in cell proliferation and cancer origin and progression was first discovered during the 80s, when it was noticed that about 30% of all human solid tumors bears at least one mutation in Ras protein encoding genes. These genes were first identified in the retroviruses causing murine sarcomas like Harvey's (*h-ras* [Dhar et al., 1982]) and Kirsten's sarcoma virus (*k-ras* [Tsuchida et al. 1982]). Subsequently the human homologous of such viral genes were identified, together with a third (*r-ras*) and a forth oncogene (*n-ras*) sharing high similarity with the first two genes [Shimizu et al. 1983].

The four major protein encoded by *ras* genes in mammalian cells are K-RasA and K-RasB (coming from alternative splicing of the same transcript), H-Ras and N-Ras. All the four protein have a very highly conserved amino-acidic sequence. The primary sequence of Ras protein can be divided into three different regions. A first region, spacing from the N-terminus until aa 86 and comprising the nucleotide binding site (aa 32-40) is perfectly conserved among all the four proteins. A very high sequence similarity (nearly 85% of identity) is visible in the subsequent 80aa too. The only exception is represented by the third and last region, thereby called hypervariable region,

located at the very end of the protein. As a matter of fact, in the C-terminus the only part conserved among the different Ras isoforms is the CAAX motif [Willingham et al, 1983], a crucial sequence responsible for the isoprenylation of the protein and its plasma membrane localization [Pronk et al. 1994]. During this process the cysteine residue of such motif (aa 186) is linked to a farnesyl group by a family of enzymes named farnesyl-transferase. The last 3 aa after the cysteine 186 are then cleaved and the same cysteine is carboxymethylated [Gutierrez et al 1989]. All these post translational modification are required for Ras sub cellular localization. However these are not enough. In fact H-Ras, K-RasA e N-Ras need to get linked to one or two others hydrophobic elements (typically palmitic acid) to achieve the correct membrane targeting [Hancock et al. 1989].

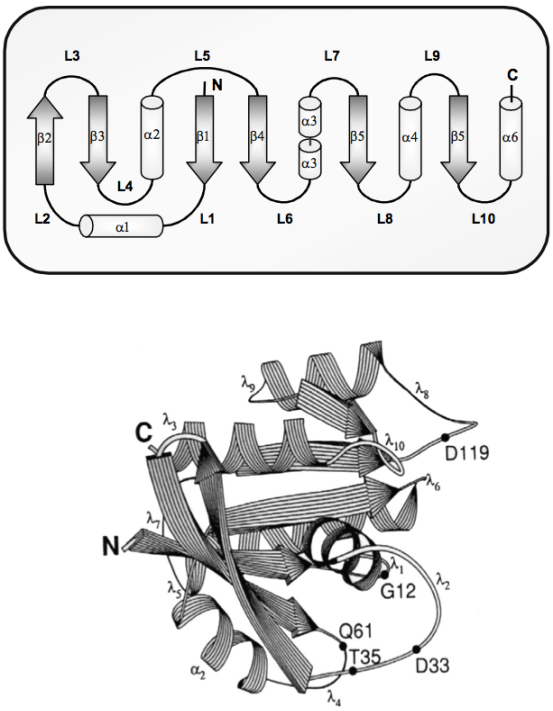

Fig 1.3: Ras secondary and tertiary structure

As seen before Ras proteins play a key role in many important cellular processes, including proliferation, migration, metabolism and differentiation.

Ras and Ras-related protein are able to carry out the regulation of such important events by their ability to act as a molecular switch, cycling between two different activation states set by its binding to either GDP (inactive state) or GTP (active state) (see fig. 1.4) [Wittinghofer et al. 1998].

However the spontaneous switch from the active to inactive conformation and vice versa are very slow. For this reason the regulation of Ras activity is tightly dependent upon the activity of two other classes of protein: a first one, whose members are named GEFs (Guanine nucleotide Exchange Factors), comprises the proteins able to stimulate the nucleotide dissociation from Ras, allowing the GDP to GTP exchange and thereby contributing to the positive regulation of Ras pathway. A second class, formed by the proteins named GAPs (GTPase Activating Proteins), is instead able to catalyse the GTP to GDP hydrolysis and act thereby as a negative regulator, shutting down Ras signaling. Two different elements are responsible for the Ras cycle to be "one-way" only. First of all the GTP to GDP hydrolysis is an irreversible reaction. On the other hand the intracellular concentration ratio GTP:GDP is so high (nearly 10 times) that it assures that every Ras-GEF complex, after resulting nucleotide free, will most probably interacts with GTP instead of GDP.

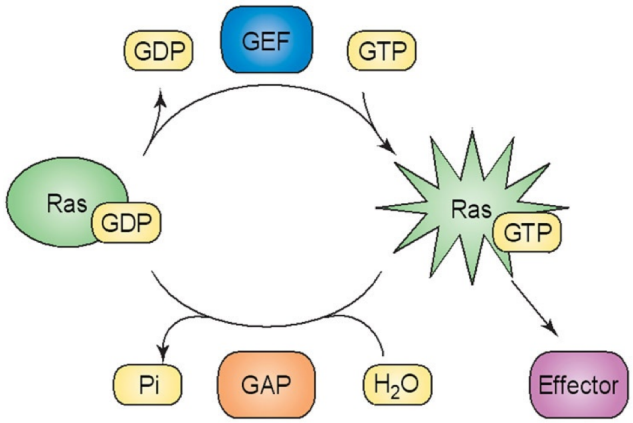

Fig 1.4: Ras functional cycle [Repasky et al, 2004]

When in the active form (complexed with GTP) Ras is able to bind and activate several effectors, leading to the numerous cellular responses we have seen before. The best-characterized Ras effector pathway implies the activation the three Raf serine/threonine kinases, A-Raf, B-Raf and Raf-1, leading to stimulation of the MEK1/MEK2 and ERK1/ERK2 mitogen-activated protein kinase (MAPK) cascade [Chong et al. 2003]. As a matter of fact, Raf proteins contain a specific domain, named RBD (Ras Binding Domani), wich is able to interact, with both high affinity ad high specificity, selectively with the active form of Ras. This binding triggers Raf activation and the subsequent protein kinase cascade. This cascade ultimately lead to the phosphorilation of transcription factors as c-Jun, c-

Fos or c-Myc and to the regulation of the expression of genes involved in cell proliferation, migration and differentiation.

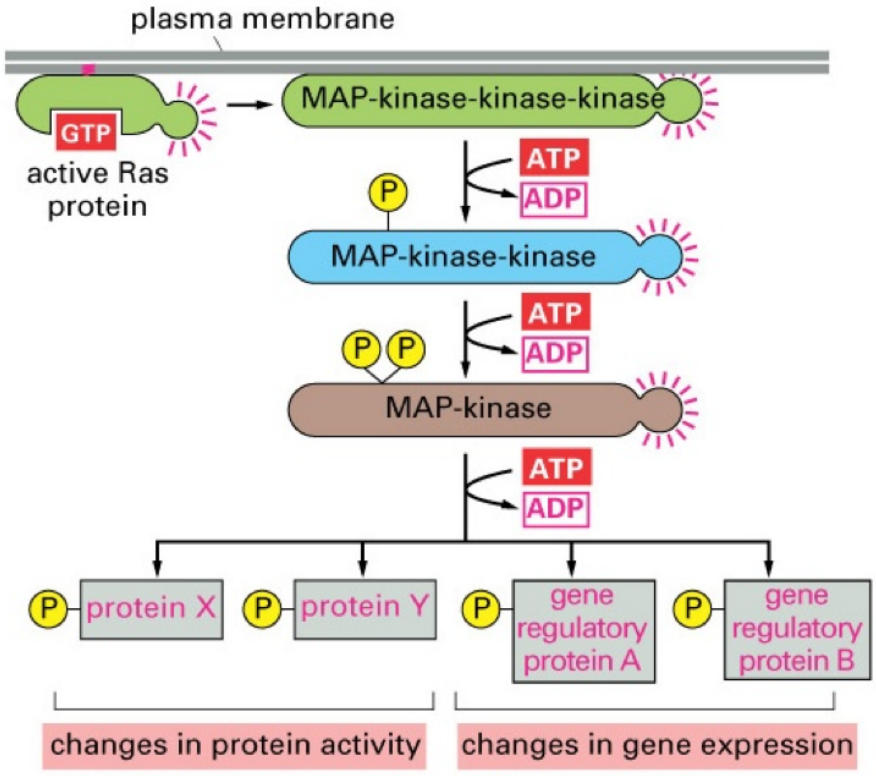

Fig 1.5: Ras induced MAPK phosphorylation cascade [Albetrs et al, *Molecular Biology of the Cell*]

Although much attention has been focused on the Raf–MEK–ERK pathway, recent research efforts have expanded the diversity of Ras effector pool and have identified a continually growing group of proteins with many diverse functions. For example another important pathway whose activation can be triggered by Ras is the Phosphoinositide 3-kinase (PI3K) pathway that plays a critical role transducing the cell survival signal [Vivanco et al, 2002].

PI3K enzyme comprises two different subunits, named respectively p85 and p110. The larger one retains the catalytic activity and thereby is responsible for the production of the second messenger IP3. The smaller subunit has instead a regulatory role, leading to the translocation of the enzyme to the plasma membrane through the interaction of its SH2 domains with the phosphorilated residues of specific receptors or adaptor proteins. Have been however proved that Ras itself is able to bind and activate p110-p85 complex in a GTP dependent manner [Rodriguez-Viciana et al, 1994].

Ras signaling can this way lead to an increase of intracellular levels of IP3 and the subsequent activation of downstream effectors recruited to the plasma membrane through their PH domains. One of the most characterized downstream pathway involves the protein kinase AKT that regulates several important processes like survival, proliferation and cell growth. AKT's crucial role in cell survival is mostly due to its anti-apoptotic properties. As a matter of fact, AKT mutants that results in enanched kinase activity can lead to the

suppression of PTEN dependent apoptosis [Li et al, 1998]. On the other hand, dominant negative AKT mutants can interfere with insulin-like growth factor 1 (IGF-I) survival signaling.
As said before AKT is also involved in cell proliferation, promoting cell cycle progression by preventing the proteasome dependent degradation of cyclin D1 [Diehl et al, 1998]. Moreover AKT can promote increased mammalian cell size both by stimulating protein synthesis and inhibiting protein degradation. These are just some examples of the different pathways that can be affected by Ras activity, but it appears clear that Ras is a key hub in signal transduction involving cell proliferation, growth and survival and is thereby suitable as a potential target for hypothetical cancer therapies.

1.3.1 Targeting Ras Pathway in Cancer

There are several strategies that could be effective in inhibiting Ras signaling. First of all the expression of Ras genes theirself could be targeted. Two different approaches has been pursued to achieve the downregulation of Ras expression. First, the introduction of DNA encoding for antisense sequences designed to pair to and lead to the degradation of Ras mRNA was shown to effectively reduce the tumorigenic potential of Ras dependent transformed cell lines [Cen et al., 1993; Kawasaki et al., 1998]. As a second approach, siRNAs have been tested and have been proven to be at least as effective as antisense DNA, with the great benefit to allow a mutant

specific (es. Ras G12V) design of siRNA probes [Brummelkamp et al., 2002]. However both these approaches does not limit not to assure a fine tuned dosage of the inhibitors, mostly because of distribution or stability issues.

Another strategy that can be pursued to interfere with Ras signaling targets membrane localization, that is required by Ras to properly act as a transducer. As seen before, Ras subcellular localization is achieved mainly through post-translational modification carried out by enzymes such as Farnesyl-Transferase and Geranylgeranyl-Transferase. For this reason Farnesyl-Transferase and Geranylgeranyl-Transferase inhibitors have been developed (FTI and GGTI). At the moment, this kind of compounds are the most representative class of Ras inhibitors undergoing clinical trials. May of these molecules have been tested in advanced clinical trials (es. Tipifarnib) and have been shown useful in several cancer diseases, including leukemia [Karp et al., 2001] and breast cancer [Johnston et al., 2003], but less active, at the same time in other tumor kinds as in colon rectal [Rao et al., 2004] and pancreas [Van Cutsem et al., 2004] cancer. Better results have been achieved with a combination of these two classes of compounds [Sun et al., 1998; Lobell et al., 2002] with the predictable drawback of higher toxicity.

A third way to target Ras pathway resides in the inhibition of Ras activity itself. Interfering with Ras activation-deactivation cycle can lead to a decrease in its downstream signaling. To achieve this result two different approaches have been

employed. An immunological method is based on the administration of vaccines developed against Ras derived peptides (for example against peptides containing an important Ras mutation like G12V). By now these vaccines have been shown to be able to trigger an immunological response against the mutated Ras protein [Khleif et al., 1999; Gjertsen et al., 2001,Toubaji A et al, 2008]. A different method to achieve Ras activity inhibition is the use of small molecules designed and developed to interfere directly with the Ras GDP-GTP exchange reaction. The most interesting class of small molecules designed for this purpose comprises cyclic compounds derived from D-arabinose. The firsts molecules of such class were developed by Schering-Plough (es. SCH53870). Other sugar derived compounds have been sown to have Ras inhibiting activity even if derived from different sugars or containing different pharmacophores [Taveras et al., 1997; Peri et al., 2005; Palmioli et al., 2009].

Inhibiting Ras downstream effectors is another way to develop a treatment for Ras-dependent transformation. Even if, as we have seen before, Ras can regulate a great number of important pathways involved in cell growth, proliferation and motility, nowadays just some of these pathways have been targeted. First of all the MAPK phosphorylation cascade have been taken into account. The anti-cancer activity of Raf inhibitors have been proven either alone or in combination with other drugs (es. BAY 43-9006) [Wilhelm et al., 2004; Ratain et al., 2005; Strumberg et al., 2005; Hoeflich KP et al.

2009]. Similarly Mek inhibitors have been developed and tested in clinical trial. PD0325901 for example have been tested in several studies and is still under investigation especially for those kind of cancers harboring Ras or Raf mutations (www.clinicaltrials.gov). Finally mTOR (mammalian Target Of Rapamycin), another downstream effector of Ras pathway has been chosen as a interesting target. Many compounds structurally related to rapamycin have shown antitumor activity [Galanis et al., 2005; Vignot et al., 2005; Witzig et al., 2005].

One last strategy to inhibit Ras in a very specific way is represented by the use of dominant negative mutant of Ras specific GEFs like $Cdc25^{Mm}$ (also named RasGRF1). As a matter of fact, these mutant (harboring one single mutation like T1184E or W1056E) not only are incapable of Ras activation, but retains a high affinity to their target, leading to a strong Ras-sequestering property . These mutants, in fact, can actually bind to Ras and even compete and displace the wild-type protein, probably resulting in the formation of a stable binary complex with reduced affinity for the entering nucleotide [Vanoni et al., 1999]. Furthermore the expression of such mutants in *k-ras* dependent transformed murine fibroblast NIH3T3 can efficiently revert the malignant phenotype [Bossu et al., 2000]. Finally have been proven that the same Ras sequestering properties, although with a less magnitude, are retained by peptides derived from the protein sequences surrounding those mutations [Sacco et al., 2005].

1.4 Insulin-Like Growth Factor Signaling

The Insulin-Like Growth Factors (IGFs) were first identified in 1957. They were known by other names including sulfation factors, non-suppressible insulin-like activity, multiplication stimulating activity and somatomedins. These single-chain polypeptides are derived from insulin like pre-propeptides, but contain the C-peptide bridge between B- and A-chains that is cleaved in insulin. Thus, IGFs are highly similar in sequence to each other and to insulin.

In particular, IGF-I is a 70 amino acid peptide and IGF-II is a 67 amino-acid peptide and and encoded by a paternally imprinted gene. Loss of IGF-II imprinting has been described in several tumors. This loss of imprinting leads to overexpression of this GF. Both IGF-I and IGF-II have an heavy influence on cell growth and proliferation. As a matter of fact, IGF-IR KO mouse embryos are much smaller (50% in size) than wild type littermates. Moreover IGF-IR/IGF-IIR double KO mouse embryos are even smaller (30% in size than WT) suggesting that just about one third of embryos growth could occur in an IGFs independent manner and that a full half of it is accomplished, in a non redundant manner, through the IGF-IR (Fig 1.6). [Liu, Efstratiadis et al. 1993].

Fig 1.6: Size of single (R) or double (D) IGF-IR KO mouse embryos [Efstratiadis et al, 1993]. Heterozygous KO mice are nearly 50% in size and Homozygous KO mice are about 30% in size when compared to wild type littermates (W).

This striking role in total body growth, however, could only suggest the role of these growth factors in cell growth and proliferation. The first evidence of a direct role of IGFs in cell proliferation and cancer arose from the observation that mouse embryo fibroblasts (MEFs) lacking the IGF-IR genes, derived from the mice discussed above, could not be transformed by the Simian Virus 40 (SV40) T antigen (Sell et al 1993). The absence of IGF-IR genes alone was sufficient to prevent MEFs, that are usually highly prone to spontaneous transformation, to be transformed by a well characterized oncogene for mouse cells. This ability of such MEFs cells (named later R- cells for the lacking of the IGF-IR expression) to be resistant to malignant transformation was confirmed with a number of other oncogenes. However, they are not totally resistant to transformation as they can be transformed for example by the introduction of v-src, but they are certainly quite "reluctant" to transformation. This phenotype is clearly due to the absence of IGF-IR, since it's reintroduction can totally revert this behavior [for a review see Baserga et al. 2003].

Even more interesting, IGF-IR inhibition has been found to strongly inhibit anchorage independent growth (colony formation in soft agar or xenografts in mice), while having at the same time little to no effect on the same cells cultured as a monolayer [Baserga et al., 2003]. The ability to interfere severely with "abnormal" cell growth while exhibiting just a mild inhibition on "regular" proliferation makes the IGF-IR a

valuable target for cancer treatment, based on the hypothesys that this behaviour could lead to a strong selectivity against malignant cell growth.

The IGF-I signaling system is a very complex network, involving three different growth factors (IGF-I, IGF-II and Insulin) and their receptors (IGF-IR, IGF-IIR and Insulin Receptor) (Fig 1.7). Moreover each ligand is able to react with more than one receptor and different receptor subunits can combine and form hybrid receptors. Furthermore, the presence of several IGF Binding Proteins (IGF-BP) add even more complexity to the system, since they can contribute both to a positive regulation by increasing the growth factor stability and to a negative regulation by sequestering the growth factor and diminishing its availability to the receptor.

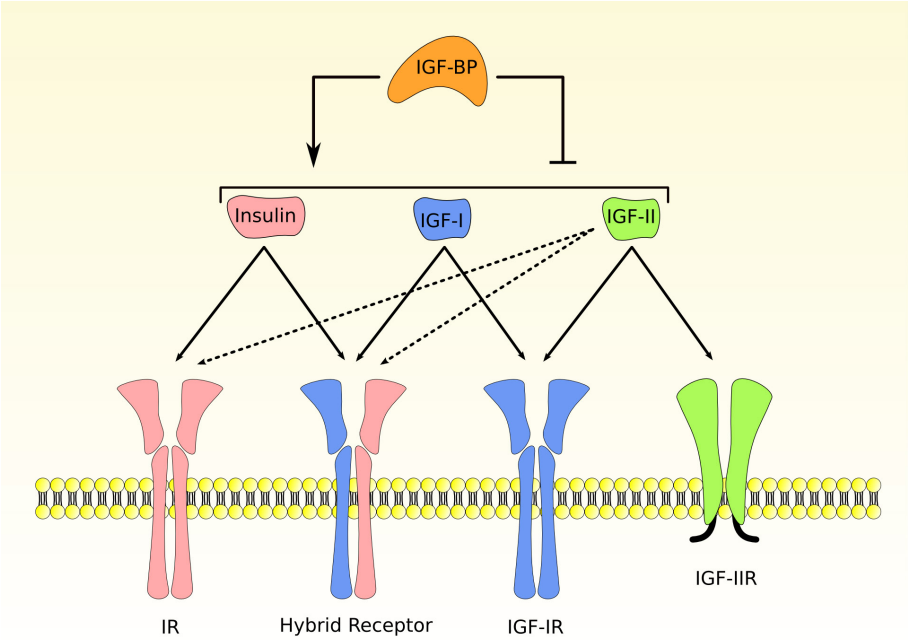

Fig 1.7: The IGF Receptor system

IGF-IR is an evolutionary conserved, ubiquitous transmembrane tyrosine kinase structurally similar to the insulin receptor (IR) (Ullrich et al., 1986). IGF-IR is composed of two extracellular alpha subunits and two intracellular beta subunits (Figure 1.8). The alpha subunits bind ligands (IGF-I, IGF-II, and insulin at supraphysiological doses), while beta subunits transmit ligand-induced signal. The beta subunits contain three major domains: the juxtamembrane domain, tyrosinekinase domain, and the C-terminus (Figure 1.8). The tyrosine kinase domain shares high (85%) sequence similarity

with its counterpart in IR, while the C-terminus just about 40% [Ullrich et al., 1986].

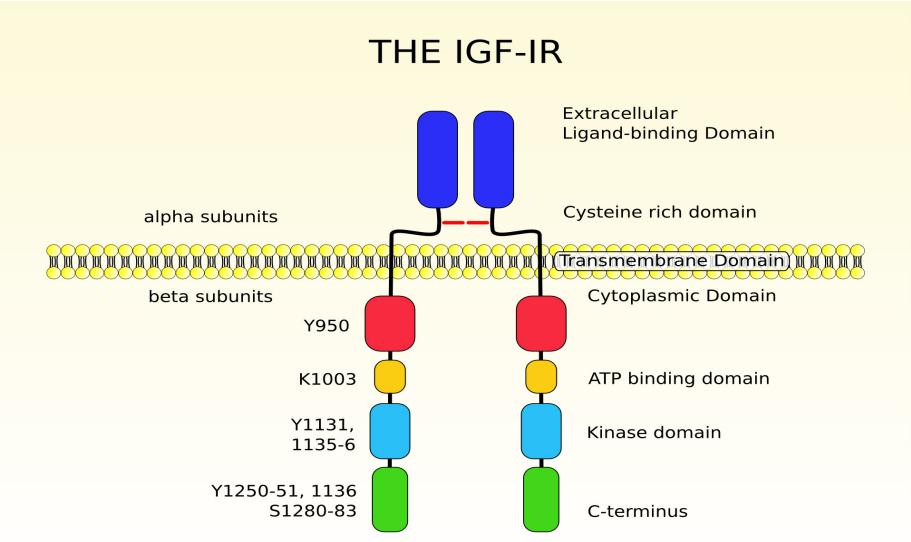

Fig 1.8: IGF-IR structure

Binding of ligands to IGF-IR induces its autophosphorylation and tyrosine phosphorylation of IGF-IR substrates, especially the IR substrate 1 (IRS-1) and SHC protein. Tyrosine-phosphorylated IRS-1 and SHC bind different effector proteins (enzymes and/or adapters) inducing multiple signaling cascades interconnecting pathways controlling cell survival

and proliferation [Shepherd et al., 1998; White, 1998, 2002; Adams et al., 2000; O'Connor et al., 2000; Surmacz, 2000].

The critical survival pathways activated by IGF-I starts from IRS-1. IRS-1 recruits and stimulates PI-3kinase (PI-3K), which then transmits signal to the serine/threonine kinase Akt (AKT). Activated AKT phosphorylates and blocks a variety of proapoptotic proteins, including BAD, caspase-9, forkhead transcription factors, and the GSK-3 beta kinase. In addition, IGF-IR can prevent cell death or induce proliferation via the SHC/Ras/ERK1/2 pathway [Peruzzi et al., 1999]. (Fig 1.9)

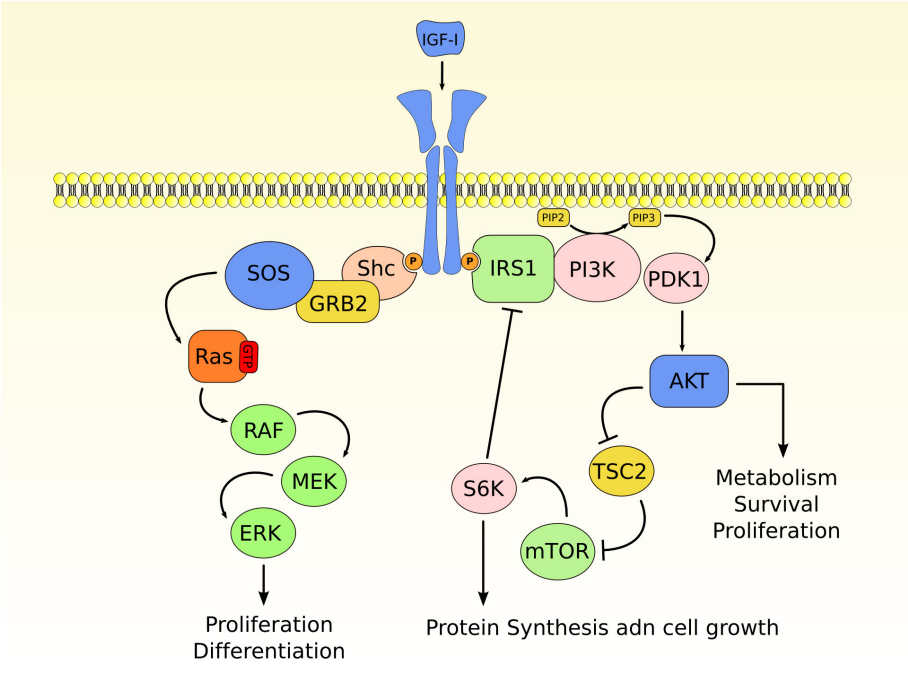

Fig 1.9: IGF-IR downstream signaling

1.4.1 Targeting IGF-IR Pathway in cancer

From what has been said above, the IGF-IR fulfills several criteria as an attractive pharmaceutical target. First of all it appears to be involved in neoplastic transformation. Moreover it can be easily detected and measured in clinical samples. Furthermore, being a tyrosine kinase receptor, several inhibitory strategies have already been developed. As a matter of fact, as seen before in the case of EGFR (Erb2) the first strategy involve the development of specific antibodies targeting the IGF-IR. The mouse MAb alpha-IR-3 raised against the alpha domain of IGF-IR inhibited IGF-IR activation and IGF-IR-dependent proliferation in several cell types in vitro [Jacobs et al., 1986], including breast carcinoma [Arteaga et al., 1989; Arteaga, 1992], rhabdomyosarcoma [Kalebic et al., 1994], Non-small cell lung carcinoma [Zia et al., 1996] and Ewing's sarcoma [Scotlandi et al., 1998]. However, in some cases alpha-IR-3 was ineffective in blocking IGF-I-sensitive tumors in animal models [Arteaga, 1992]. Furthermore, it has been reported that alpha-IR-3 may exhibit agonistic abilities too towards IGF-IR, depending on the cell line [De Leon et al., 1992; Kato et al., 1993].

Another mouse anti-IGF-IR antibody, MAb 391, inhibited IGF-IR autophosphorylation and signaling to Akt in several human cancer cell lines and chronic treatment with MAb 391 resulted in downregulation of receptors through a lysosome-dependent pathways [Hailey et al., 2002]. Later on, several other mouse

anti-IGF-IR MAbs were developed [Li et al., 1993, 2000]. One of them, MAb 1H7, which blocks IGF-IR/IGF-I binding and IGF-IR-dependent DNA synthesis, was used to engineer a single-chain humanized anti-IGF-IR scFv-Fc Ab that contains the Fc domain of human IgG1 fused to the Fv region of 1H7 [Li et al., 2000]. Treatment of MCF-7 breast cancer cells with scFv-Fc from 2 to 24h downregulates the levels of IGF-IR through the lysosomal/endocytic pathway, allowing the cells to be refractory to IGF-I stimulation [Sachdev et al., 2003]. Importantly, downregulation of IGF-IR by scFv-Fc occurs also in MCF-7 xenografts and is followed by reduced tumor growth [Sachdev et al., 2003]. These or similar humanized MAbs will likely become a model for future drug development once their specificity towards IGF-IR and lack of IR crossreactivity is demonstrated in vivo. One of the greatest challenge in targeting IGF- IR, in fact, is designing strategies that would specifically inhibit IGF-IR without blocking IR and producing diabetogenic effects.

Another strategy for targeting the IGF-IR involves the use of small molecules with antagonistic activity. High-throughput technology combined with computer modeling is currently used to identify low molecular weight compounds able to block the IGF-IR tyrosine kinase. The first described IGF-IR inhibitors, tyrphostins AG 538 and I-OMeAG, were modeled on the IR tyrosine kinase. The compounds inactivated the IGF-IR tyrosine kinase by blocking the substrate binding site;

however, crossreactivity with the IR tyrosine kinase was reported [Blum et al., 2000].

Recent advances in the characterization of the three dimensional structures of IGF-IR and IR greatly facilitated the design of specific IGF-IR inhibitors [De Meyts and Whittaker, 2002]. Most importantly, crystallographic studies revealed conformational differences in the phosphorylated forms of IGF-IR and IR kinases, this way allowing the development of selective therapeutic molecules [Favelyukis et al., 2001; Pautsch et al., 2001]. Several new compounds with enhanced specificity towards IGF-IR and low crossreactivity with IR entered into preclinical studies. The examples include derivatives of pyrimidine and podophyllotoxin, disclosed in patent applications WO 02/092599 and WO 02/102804, respectively. Specific small inhibitors of IGF- IR are likely candidates to become anti-IGF-IR drugs. The positive experience with similar therapeutics (Iressa, Gleevec), especially the possibility of oral delivery and low toxicity, makes this approach especially attractive.

One last approach includes the design of small peptides with an IGF-I related structure. A series of small IGF-I peptide analogues was designed by molecular modeling of the IGF-I protein [Pietrzkowski et al. 1993] to compete with IGF-IR ligands. The synthetic peptides were modeled on those domains containing the least similarity between IGF-I and insulin. One of the peptides, JB1 effectively inhibited IGF-I-dependent IGF-IR autophosphorylation and proliferation in

several tumor cell lines. The analogues used at nanomolar or micromolar concentrations exhibited good specificity for IGF-IR, and low toxicity for cells in cell culture [Pietrzkowski et al.,1993]. However, the efficacy of these compounds against experimental tumors in vivo has never been assessed.

Summarizing, we can say that IGF-IR is a promising target in cancer therapy because: (1) IGF-IR expression is easily measurable by conventional techniques; (2) tumor cells may be more sensitive to targeting IGF-IR than normal cells; and (3) IGF-IR is often required for the tumorigenic effects of other oncogenic agents. Thus targeting IGF-IR can be combined with other therapies. Unlike with HER2 and EGFR, the development of anti-IGF-IR pharmaceuticals is still in early discovery phases. Similar to HER2 and EGFR, however, the most advanced strategies are those involving small inhibitors of the IGF-IR tyrosine kinase and anti-IGF-IR antibodies. Other approaches, such as siRNA, antisense, and triple helix strategies are also promising, but they will require optimization of specificity in vivo and efficient and safe delivery systems.

1.4.2 Bladder Cancer and IGF-IR signaling

Bladder cancer is the second most common genitourinary malignant disease in the USA, with an expected 69.000 newly diagnosed cases in 2008, and 14.000 deaths. The incidence of bladder cancer rises with age, peaking between age 50 years and 70 years, and is three times more common in men than in women. [Jemal et al, 2008]. 95% of bladder cancers are Transitional Cell Carcinoma (TCC), and 80% of these are superficial (stage Ta or T1) at presentation. For patients with superficial tumors the initial treatment is transurethral resection of the bladder tumor (TURBT). After 5 years, half of these patients will have had no recurrence, a fifth will have only one recurrence and 30% will have had multiple recurrences [Fitzpatrick et al, 1986]. Of those patients who have a recurrence, 20–40% progress to muscle invasion [Abel et al, 1988]. The outlook for patients with invasive tumors is poor, with overall 5-year survival rates after radical therapy of ≈40% [Shelley et al 2004, May et al, 2004]. Of patients with metastatic disease, 45–50% respond to combination chemotherapy, with similar response rates to gemcitabine plus cisplatin compared with a four-drug combination (methotrexate, vinblastine, doxorubicin, cisplatin). The former is better tolerated, but the median survival after either regimen is only 15 months [von der Maase et al, 2000, Roberts et al, 2006]. Much work has therefore focused on identifying clinical and molecular factors that show potential as novel therapeutic

targets, aiming to reduce recurrence and progression in superficial tumors, and to improve the outlook for patients with advanced disease.

The epidermal growth factor receptor (EGFR) has been found to be over-expressed in bladder cancers, with higher expression in poorly differentiated and advanced-stage tumors [Neal et al 1985, Smith et al 1989]. Strategies to target this receptor in bladder cancer have proved successful in vitro and in vivo [Nutt et al, 2004, Dominguez-Escrig et al, 2004], and clinical trials of EGFR inhibitors are currently in progress. The IGF axis is known to be critically important in the biology of a range of tumors. The IGF-I receptor is required for cells to undergo transformation, and IGF-IR activation mediates tumor cell proliferation, motility and protection from apoptosis [Baserga et al, 1997, Bohula et al, 2003].

In 2007, Rochester et al., pointed out that the IGF-IR is up-regulated in bladder cancer compared with non-malignant bladder, and might contribute to a propensity for invasion. Of 15 samples of normal bladder, 14 showed negligible or light IGF1R immunostaining. By contrast moderate or heavy staining for IGF1R was detected in 89 (74%) of 120 samples of malignant urothelium. Moreover Q-RT-PCR showed significantly higher levels of steady-state IGF-IR mRNA in tumors than in normal bladder, indicating up-regulation at the transcriptional level. This difference was particularly evident when comparing normal urothelium with superficial or

invasive tumors, since only the latter showed significant IGF-IR over-expression at the RNA level.

2. Materials and Methods

2.1 Cell culture

Urothelial carcinoma-derived human 5637 and T24 cell lines were maintained in RPMI medium supplemented with 10 % fetal bovine serum (FBS). Mouse fibroblast NIH3T3 and NIH3T3 *kras* transformed were maintained in DMEM medium supplemented with 10 % newborn calf serum (NCS).
Serum-free medium (SFM) is DMEM supplemented with 0.1% bovine serum albumin and 50 μg/ml of transferrin (Sigma-Aldrich).

2.2 *Fos-luciferase* transactivation assay

Normal and kras-transformed NIH3T3 mouse fibroblasts were transfected with pcDNA3-derived plasmids and cotransfected with the ras-responsive *fos-luciferase* plasmid.
After transfection, cells were starved for 24h in serum-free medium supplemented with 4mg/ml transferrin and 0.346ng/ ml sodium selenite and collected. Luciferase activity was assayed using the luciferase assay system with reporter lysis buffer (Promega) and normalized to protein content determined with the DC Protein Assay (Bio-Rad).

2.3 Proliferation assay

Cells were plated in duplicate at a density of $3x10^4$ cells/35 mm2 plates in serum-supplemented medium. After 24 h, cells

were washed 3 times in DMEM and transferred to SFM or SFM supplemented with 50 ng/ml IGF-I (Calbiochem). Cells were counted after 48 and 72 h with a hemocytometer.

2.4 Migration and invasion assay

Boyden chambers 24 wells inserts with 8 um PET membrane (for migration) or BD BioCoat™ Matrigel™ Matrix inserts (for invasion) (Becton Dickinson) were saturated 2 h at room temperature with PBS-1% bovine serum albumin. After 24 h of starvation in SFM, cells were seeded in the upper chamber and incubated at 37°C for 24h (5637) or 8h (T24) in SFM or SFM supplemented with 50 ng/ml of IGF-I. Migration or invasion was blocked by fixing and staining the membranes in a blue coomassie solution. Cells on the upper surface of the filters were removed with cotton swabs. Cells that had invaded to the lower surface of the filter were counted under the microscope. Specific inhibitors for the Akt, LY294002, or ERK1/2, U0126, pathways (Calbiochem) were used at a concentration of 20 μg/ml and 10 μg/ml respectively. Tat-engineered peptides were used 500 nM.

2.5 Wound Healing

Cells were seeded onto 35 mm plates in serum-containing medium until sub-confluence and then transferred to SFM. After 24 h the plates were scratched with a thin disposable tip

to generate a wound in the cells monolayer and incubated in SFM or SFM supplemented with IGF-I. Cells were analyzed and photographed after 24 h with a Zeiss Axiovert 200M cell live microscope using the Metamorph Image Acquisition and Analysis software (Universal Imaging).

2.6 3D evasion assay

T24 cells (5000/drop) were included in Matrigel drops (6 mg/ ml, Becton Dickinson). Drops were incubated for 72 h in SFM or SFM supplemented with 50 ng/ml of IGF-I or complete medium as control. Cell motility was analyzed by microscopy. Images were collected using a digital camera (Canon) and cells outside each drop (five drops/cell line/experiment) were counted.

2.7 Ras–GTP pull down assay

Ras–GTP was assayed by a pull down assay using the Ras binding domain of Raf1 (RBD) bound to glutathione–sepharose with Ez-detect Active Ras Pull-Down and Detection Kit (Pierce). Ras–GTP was eluted with reducing Laemmli sample buffer, loaded on 12% SDS-PAGE and detected by anti-Ras antibodies (supplied with the Pierce kit) after western blotting. Ras–GTP levels were determined by densitometric scanning and quantitation with the ImageJ program (rsbweb.nih.gov/ij/)

2.8 Western Blot analysis

Cells were serum-starved for 24 h and then stimulated with IGF-I (50 ng/ml) for 10, 30 and 120 min. The activation of p90RSK, Akt, ERK1/2 and S6 Ribosomal Protein was analyzed by western immunoblot using the PathScan Multiplex Western Cocktail I (Cell Signaling Technology) which provides a mix of phospho-specific antibodies for different activated protein. ElF4E protein is the control to monitor the loading of the samples. For detection of paxillin phosphorylation, serum-starved cells were stimulated with IGF-I for 10 min or for 10, 30 and 120 min. Paxillin phosphorylation was detected by immunoblot either after immunoprecipitation with anti-paxillin monoclonal antibodies (BD Biosciences) or in cell lysates using various anti-phospho-paxillin antibodies: P-Y31 is from Genetex. P-S126 is from Biosource International. P-S178 is from Calbiochem. Densitometric analysis was performed using the ImageJ program (rsbweb.nih.gov/ij/). Pan anti-PY- HRP-conjugated monoclonal antibodies are from BD Transduction Laboratories.

2.9 siRNA-mediated Gene Silencing

To silence ERK1/2, Akt and paxillin we utilized RNA interference using small interfering RNA (siRNA). Subconfluent cells were transfected with vehicle (DEPC-treated water), control siRNA (scrambled), or siRNA specific oligos (200-400

pmol) using either TransIT-siQUEST or TransIT-siTKO reagents (Mirus Corporation) according to the manufacturer's instructions. Scramble and anti ERK2 (MAPK1) (AM51331), paxillin (NM002859) and Akt1 (4390824) SilencerTM siRNA oligos were from Ambion. One day after transfection, cells were serum-deprived for an additional day and then stimulated with IGF-I. After an additional day, the cells were processed and analyzed for migration and invasion as described above. The expression of ERK1/2 and paxillin proteins was detected by immunoblotting using anti-ERK1/2 and paxillin polyclonal antibodies (Santa Cruz Biotechnology) or monoclonal antibodies (BD Pharmingen). Akt was detected using an anti-Akt polyclonal antibody (Cell Signaling Technology).

2.10 Immunofluorescence studies of paxillin localization

Cells plated on cover slip were serum-deprived for 24 h and then stimulated for 30 min with IGF-I (50 ng/ml). Cells were fixed in 4% paraformaldehyde (PFA) at room temperature, permeabilized in PBS 0.1% Triton X- 100 and blocked in PBS 1% BSA. Incubation with anti-paxillin (BD Transduction Laboratories, 1:100) and anti-FAK (Invitrogen, 1:50) antibodies was performed for 1 h at RT (or overnight at 4°C) in PBS 1% BSA. Samples were then washed in PBS and incubated with Alexa Fluor 488 and 594 secondary antibodies (Molecular Probes, InVitrogen) for 1 h at RT. Cover slips were mounted in Vectashield and counterstained with Dapi (Vector

Laboratories). Images were analyzed a with LSM 510 Meta Confocal Microscope.

2.11 Immunofluorescence studies of peptides internalization

NIH3T3 cells (6000/cm^2) were plated on cover slip. 24H after plating, cells were treated with Tat-engineered peptides (5μM) for the indicated times. After treatments cells were fixed in 4% paraformaldehyde (PFA) at room temperature, permeabilized in PBS 0.1% Triton X- 100 and blocked in PBS 1% BSA. Incubation with anti-His antibody (Santa Cruz, 1:100) was performed for 1 h at RT in PBS 1% BSA. Samples were then washed in PBS and incubated with Alexa Fluor 488 secondary antibodies (Molecular Probes, InVitrogen) for 40'' at RT. Cover slips were mounted in DABCO-MOVIOL (Sigma) and counterstained with Dapi (Vector Laboratories).

3. Results

3.1 Aim

Aim of this thesis is the development of $Cdc25^{Mm}$ derivatives as anticancer drugs and their validation on bladder cancer cell lines. Since the mechanism of action of these peptides would be essentially based on their Ras sequestering properties (see 1.3.1), in order for this strategy to succeed a fundamental requirement was that the malignant phenotype of bladder cancer cell lines was dependent or at least affected by Ras activity. For such a reason and for better understand these cells's behaviours, first of all we assessed the role of IGF-I (a very important growth factor in bladder cancer, known to be linked to Ras activation in other cell lines and tissues) in bladder cancer cells.

3.2 IGF-IR Activation does not affect bladder cancer cell proliferation

In order to discern the role of ligand-activated IGF-IR signaling pathway in bladder cancer cells, we first determined the effect of IGF-I on two urothelial carcinoma-derived 5637 and T24 cells on cell proliferation. This was assessed comparing the growth kinetics of both cell lines, previously serum starved for at least 24h, in the presence or absence of 50ng/ml IGF-I.
As can be seen in figure 3.1 IGF-I did not induce a statistically-significant mitogenic response in both cell types

after either 48h or 72h of continuous exposure to the growth factor.

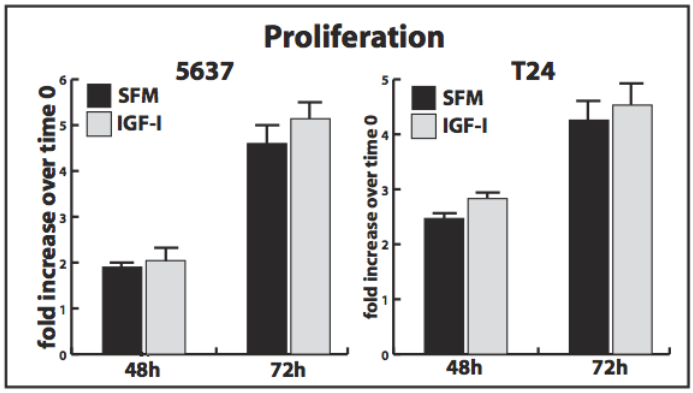

Fig 3.1 cell proliferation in SFM or SFM supplemented with 50 ng/ml of IGF-I in 5637 and T24 cells. Values represent the mean ±SD of four independent experiments run in triplicate

3.3 IGF-IR Activation strongly enhance bladder cancer cell motility

Since IGF-I had little or no effect on bladder cancer cell proliferation, we investigated its role in cell motility. Since cell motility is a very complex phenomenon, depending both on cell line and 2D and 3D environment, we used different approaches to evaluate cell motility.

First of all we performed a simple wound healing experiment on both cell lines. This method is based on observation of cell migration into a "wound" that is created on a cell monolayer. Although not an exact duplication of cell migration in vivo, this method mimics to some extent migration of cells during the closure of a real wound in human body. A scratch was made

with a sterile pipette tip on an a confluent monolayer of bladder cancer cells, previously serum starved for 24h. After the scratch, medium was replaced either with SFM or SFM supplemented with IGF-I. Cell migration was monitored with a standard microscope and pictures taken at different intervals. As clearly visible in fig 3.2, wound closure was stimulated by the presence of growth factor in both cell lines.

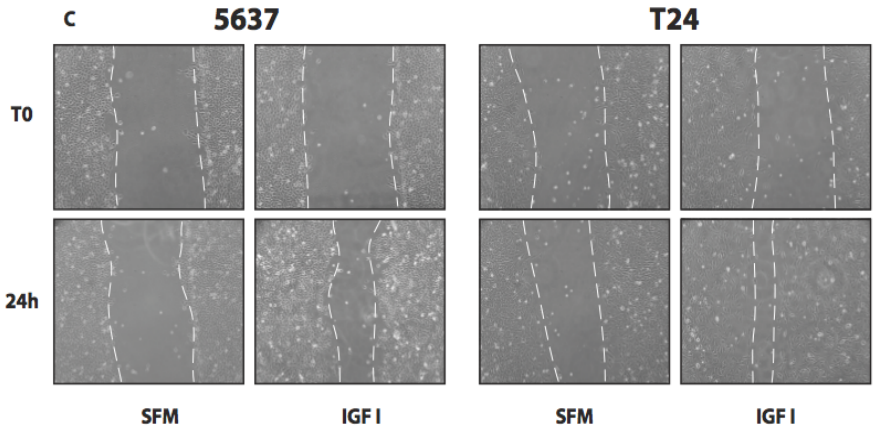

Fig 3.2 Wound healing experiments in the presence or absence of IGF-I (50 ng/ml) for the time indicated.. Cells were analyzed with live-cell microscopy using the Metamorph Image Acquisition and Analysis software (Universal Imaging).

We then tested both cell lines for their migration ability using a transwell (Boyden chambers) assay. Briefly, cells were plated into a transwell chamber having a porous membrane. IGF-I was used as a chemo-attractant in the lower chamber. After a cell line optimized incubation time, cells were fixed, stained and the number of cells migrated to the other side of the

membrane counted and expressed as fold change over cells migrated without any chemo attractant (Serum Free Medium). As visible in fig 3.3, both cell lines strongly respond to the IGF-I stimulus by increasing by 2 or 3 folds their ability to migrate across the porous membrane.

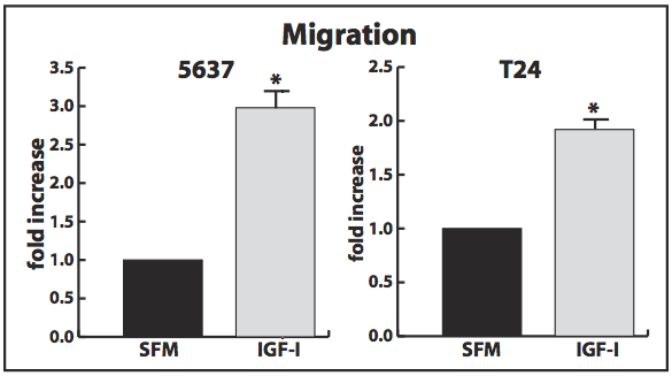

Fig 3.3 Cell migration assays in SFM or SFM supplemented with 50 ng/ml of IGF-I in 5637 and T24 cells. Values represent the mean ±SD of four independent experiments run in duplicate (*p<0.01).

The acquisition of an invasive phenotype is a critical step for tumor progression. To this end, we performed an assay similar to the Boyden Chambers migration assay, with the only difference of using Matrigel®-coated filters, a widely used strategy to examine invasive migration through a 3D extracellular matrix, since this material mimics in an effective way the the complex extracellular environment found in many tissues. The use of a growth factor reduced Matrigel® allows us to look at IGF-I effect on cell invasiveness in a very specific way.

As can be seen in fig 3.4 IGF-I strongly enhance bladder cancer cells ability to invade a tridimensional matrix. It's interesting to point out that the IGF-I influence is even more visible than in simple migration assays, suggesting a strong effect on cell invasiveness rather than on simple migration.

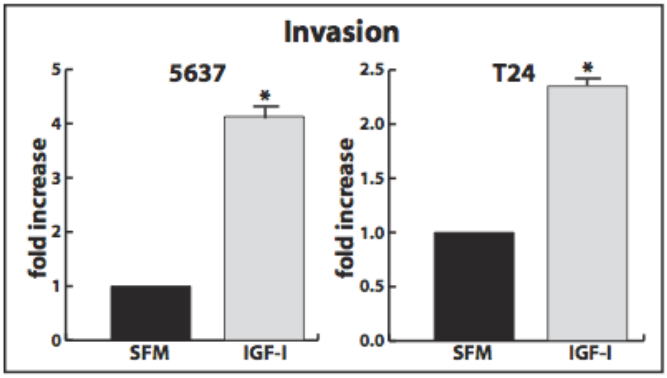

Fig 3.4 Cell invasion assays in SFM or SFM supplemented with 50 ng/ml of IGF-I in 5637 and T24 cells. Values represent the mean ±SD of four independent experiments run in duplicate (*p<0.01).

In order to fully characterize the enhanced invasiveness conferred to bladder cancer cells by IGF-I stimulation, an evasion assay was also performed. T24 cells was challenged to evade from a Matrigel® drop, in the presence of serum free medium or serum free medium supplemented with IGF-I. IGF-I stimulation significantly increased the number of T24 cells able to evade the 3D-matrix (Fig. 3.5 lower panel). Moreover, the migration distance covered by IGF-I- stimulated T24 cells was markedly increased ($p<0.05$) as compared to T24 cells incubated with SFM (Fig. 3.5 upper panel).

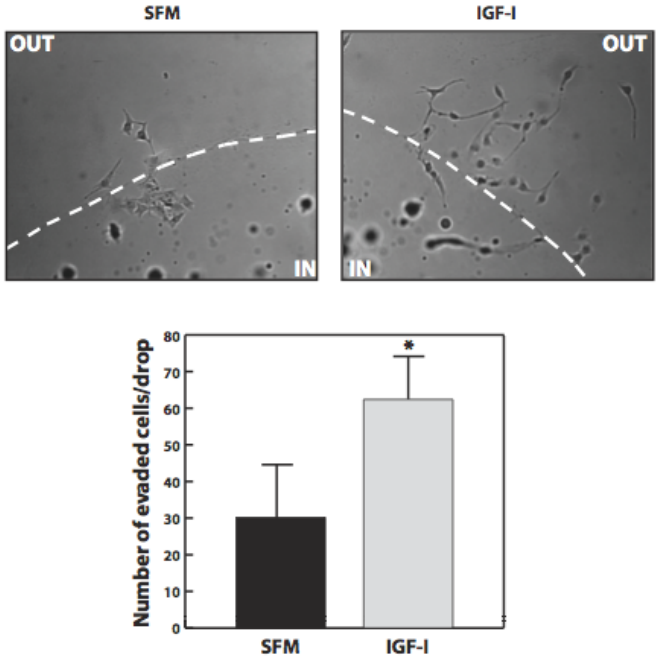

Fig 3.5 Matrigel evasion assays, T24 cells were included in a Matrigel drop and allowed to evade for 72 h. The dotted line indicates the edge of Matrigel drops. Values represent the mean ±SD of three independent experiments run in quintuplicate (*p<0.05).

3.4 IGF-I stimulation results in higher intracellular Ras•GTP levels

To investigate the mechanisms by which the IGF-IR promotes motility and invasion of bladder cancer cells, we first tested the Ras Pathway, a key components for growth factor mediated biological responses in many cellular systems. First of all we performed a Ras•GTP pull down assay, using a commercial kit (Pierce) able to selectively purify the active Ras sub-population

from a total protein extract. The amount of purified GTP bound Ras was then evaluated by western blot.

As can be noted in fig 3.6, after 5' IGF-I stimulation, in 5637 bladder cancer cells, intracellular active Ras content is dramatically higher, while the total Ras level is comparable.

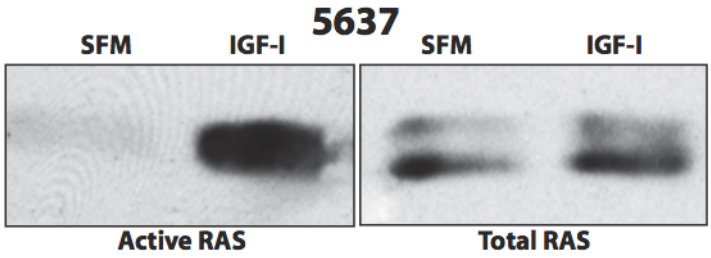

Fig 3.6 Ras•GTP pull down assay. 5637 bladder cancer cells were serum starved for 24h in SFM. After 5' of incubation with either SFM or SFM supplemented with 50ng/ml IGF-I, total protein extract was collected and the active Ras fraction purified using Ras EZ-Detect immunoblot assay and then analyzed by western blot. A sample of total extract was also analyzed to quantify total Ras content.

3.5 Increased levels of Ras•GTP correlates with activated MAPK and AKT pathways

Akt and MAPK pathways are key components for IGF-I-mediated biological responses in many cellular systems and they both can be activated, directly or indirectly, by upstream Ras signalling. Furthermore, the activation of the MAPK pathway plays a critical role in motility of epithelial cells, as has been recently shown for proepithelin-induced motility of bladder and prostate cancer cells [Lovat et al, 2009, Attached paper]. To this end, we used the PathScan Multiplex Western

Cocktail I (Cell Signaling Technology), which provides a mixture of phospho-specific antibodies directed toward different activated proteins. IGF-I induced a sustained activation of both Akt and ERK1/2 kinases, with a subsequent stronger activation of their downstream effectors, like S6 ribosomal protein and p90RSK (more evident in both cell lines after longer exposure of the film) respectively (Fig. 3.7). The level of Akt and ERK1/2 proteins was instead not affected by IGF-I stimulation, as determined by immunoblot with anti-Akt and ERK1 antibodies (not shown). Thus, IGF-I-stimulated activation of the Akt and MAPK pathways may be critical for IGF-IR- dependent biological responses in bladder cancer cells.

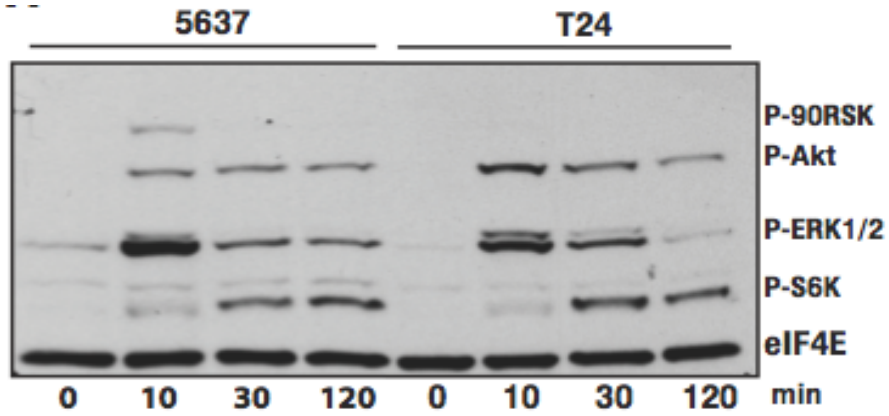

Fig 3.7 Time course activation of various signaling molecules evoked by IGF-I (50 ng/ml) in 5637 and T24 cells. The cells were serum- deprived for 24 h and then stimulated for 10, 30 and 120 min with IGF-I The activation of (top to bottom) p90RSK, Akt, ERK1/2 and S6 Ribosomal Protein was analyzed by immunoblotting using phospho-specific antibodies of the PathScan Multiplex western Cocktail I (Cell Signaling). ElF4E protein is the control to monitor the loading of the samples. The experiment shown is representative of three independent experiments.

3.6 MAPK and AKT pathways activation is needed for IGF-I induced increased motility

To corroborate the role of Akt and ERK1/2 activation in IGF-I-mediated responses in urothelial cancer cells we performed IGF-I-induced migration and invasion assays in the presence of specific inhibitors of either the Akt (LY294002) or MAPK (U0126) pathways. First, we verified that the concentrations of LY294002 and U0126 used for these experiments were effective in inhibiting the activation of Akt and ERK1/3. In 5637 cells, IGF-I- mediated activation of Akt and ERK1/2 was severely reduced in the presence of LY294002 (20 μM) or UO126 (10 μM) respectively, establishing the effectiveness of both inhibitors at the concentration used in subsequent migration and invasion assays (Fig. 3.8).

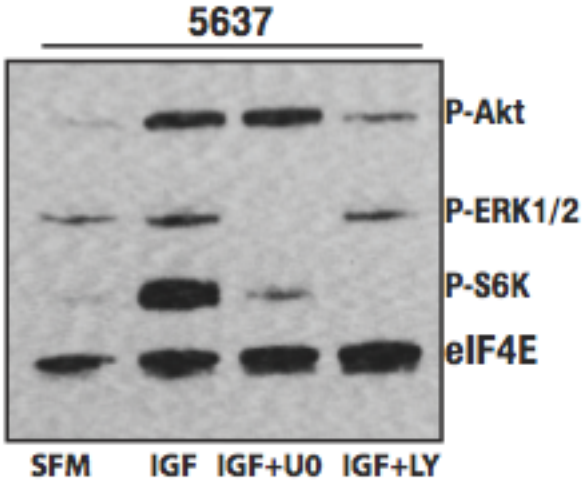

Fig 3.8 PathScan blot on 5637 cells stimulated with IGF-I for 10 min or IGF-I supplemented with specific inhibitors for the Akt, LY294002, or ERK1/2, U0126, pathways at a concentration of 20 μM and 10 μM, respectively. Blot is representative of two independent experiments.

Notably, incubation with LY294002 and U0126 reduced the ability of 5637 urothelial cells to migrate and invade in response to IGF-I stimulation (Fig. 3.9 upper panel), while T24 cells were less sensitive to MAPK inhibition in both migration and invasion. However, UO126 induced a statistically significant decrease in cell migration (Fig. 3.9 lower panel). In both cells, the combination of LY294002 and U0126 had additive effects and significantly inhibited migration.

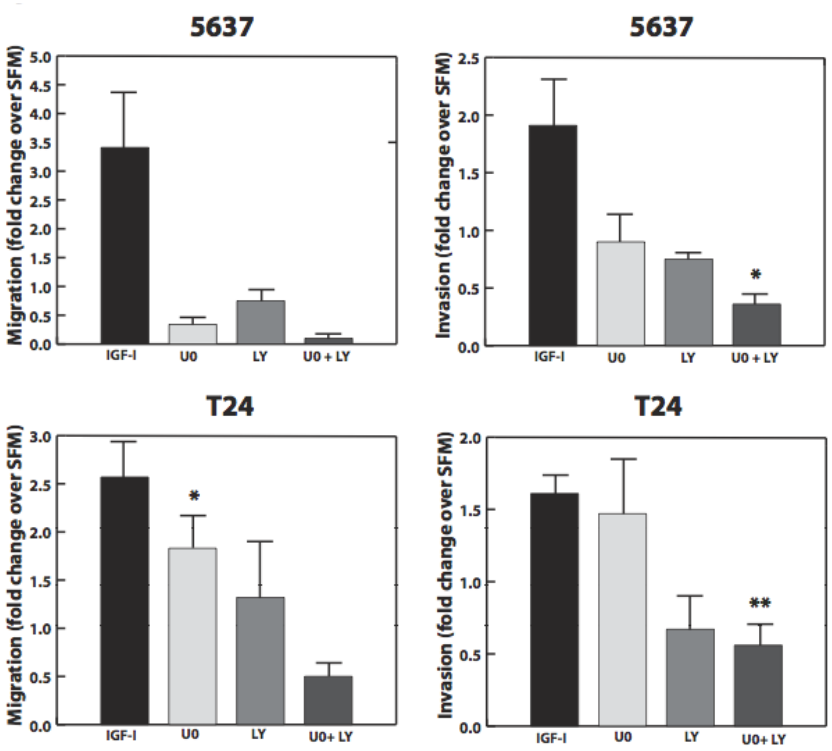

Fig 3.9 migration and invasion experiments in 5637 and T24 in the presence or absence of IGF-I or inhibitors as indicated . * $p < 0.05$ compared with LY294002 alone; ** $p < 0.05$ compared with IGF-I alone; *** $p > 0.05$ compared with LY294002 alone. Data are the average of three independent experiments ±SD run in duplicate

These results suggest that activation of MAPK and the Akt pathways is necessary for IGF-IR- mediated migration and invasion of urothelial carcinoma-derived cells.

Next, we investigated the role of MAPK and Akt signaling in IGF-I-promoted biological responses in urothelial cancer cells by targeting endogenous Akt and ERK1/2 proteins using siRNA strategies. Depletion of endogenous ERK2 and Akt1 (Fig. 3.10) significantly reduced the ability of 5637 cells to migrate and invade in response to IGF-I, confirming that the Akt and MAPK pathways are both required to fully sustain IGF-I-induced cell motility of bladder cancer cells. Confirming our previous results with the inhibitors, T24 cells are less sensitive then 5637 cells to ERK depletion, while endogenous Akt knock-down significantly reduced both migration and invasion in these cells (Data not shown).

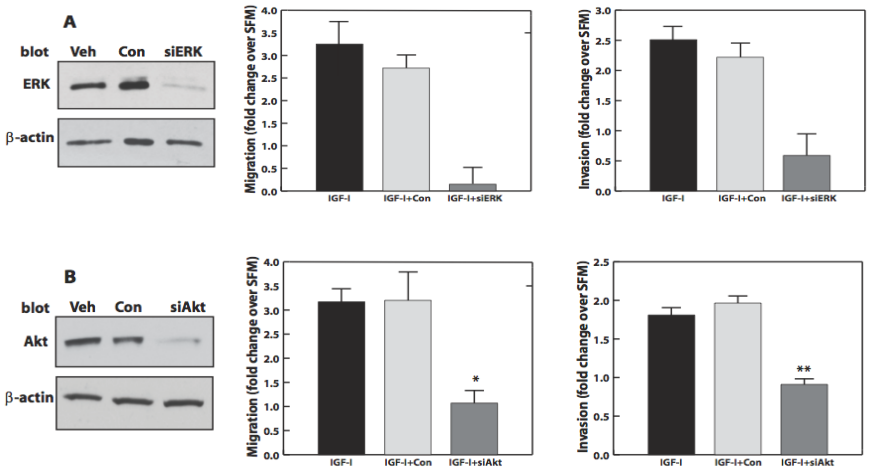

Fig 3.10 Gene knockdown for ERK2 and AKT1 in 5637 cells was achieved by siRNA. A, the level of endogenous ERK1/2 and B, Akt proteins was detected by immunoblot using anti-ERK1/2 (Santa Cruz Biotechnology) or anti-Akt polyclonal antibodies (Cell Signaling Technology). Protein loading was normalized using an anti-beta-actin polyclonal antibodies (Sigma-Aldrich). One representative blot of three independent experiments is shown. 24 h after transfection, cells were serum-starved for 24 h and then stimulated with 50 ng/ml of IGF-I. After 24 h cells were processed and analyzed for migration and invasion as indicated. Values are expressed as fold change over SFM ±SD. *, **: $p < 0.05$ compared with control oligo-treated cells (second columns).

3.7 IGF-IR induced Paxillin Activation

Paxillin, with FAK, plays a critical role in cellular motility at focal adhesions [Ishibe et al, 2004]. In addition, a recent work has pointed out a role of paxillin in cellular motility induced by the growth factor proepithelin in urothelial cancer cells [Monami et al, 2009].

To elucidate in more depth the mechanism by which the IGF-IR promotes motility and invasion of urothelial carcinoma-derived cells we tested whether IGF-IR activation of Akt and MAPK may promote phosphorylation of paxillin and whether paxillin would be necessary to promote migration and invasion in these cells. We discovered that IGF-I promoted a three-fold increase of tyrosine-phosphorylation of paxillin in 5637 cells as compared to unstimulated cells (Fig. 3.11).

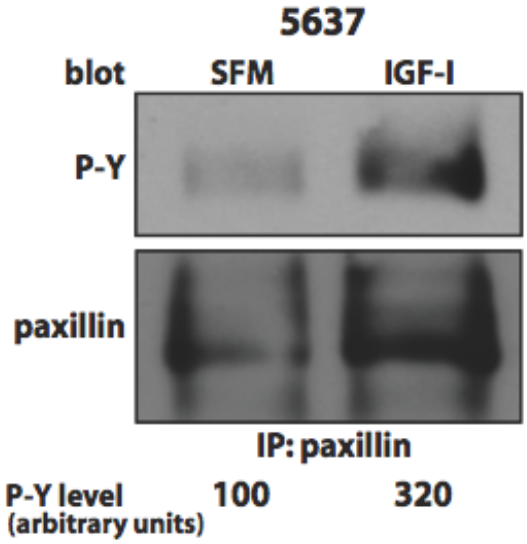

Fig 3.11 Lysates (600 µg) from unstimulated or IGF-I-stimulated (50 ng/ml for 10 min) 5637 cells were immunoprecipitated with anti paxillin monoclonal antibodies (BD Pharmingen). Tyrosine-phosphorylated paxillin was detected by immunoblot with anti-phospho-tyrosine-HRP-conjugated monoclonal antibodies (BD Pharmingen). Total paxillin was detected using anti-paxillin polyclonal antibodies (Millipore). The level of phospho-paxillin was normalized over total immunoprecipitated paxillin by densitometry and values were expressed in arbitrary units. The blot is representative of two independent experiments.

Specifically, IGF-I promoted an increase in phosphorylation of Tyr31 compared to unstimulated cells as we determined using pospho-Tyr-specific antibody and immunoblotting (Fig. x.x). We also detected paxillin phosphorylation on Tyr118 but this residue seemed to be constitutively phosphorylated and not modulated by IGF-I in both 5637 and T24 cells (data not shown). Significantly, prolonged IGF-I stimulation promoted paxillin phosphorylation on Ser126 and Ser178 (Fig. 3.12), two possible target sites for Akt and/or ERK-mediated phosphorylation on paxillin [Cai et al, 2006].

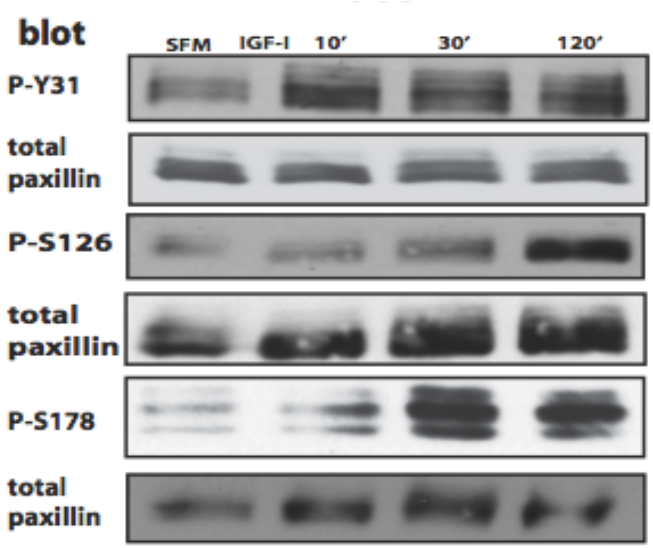

Fig 3.12 5637 cells were serum-starved for 24 h and then stimulated with IGF-I (50 ng/ml) for the indicated time. 20 μg of proteins were run on SDS-PAGE and then immunoblot was performed using phosphospecific-paxillin antibodies. Blots are representative of three independent experiments.

In addition, IGF-I stimulation induced the formation of a complex that includes phosphorylated paxillin and active ERK1/2, as determined by coimmunoprecipitation and immunoblot experiments (Data not shown).

3.8 Paxillin Activation requires both AKT and MAKP pathways

Next, we confirmed that paxillin phosphorylation was regulated by Akt and ERK1/2 by depleting 5637 cells of endogenous ERK2 and Akt using siRNA strategies (Fig. 3.13). ERK2 depletion almost completely inhibited paxillin phosphorylation of both Ser126 and Ser178, while Akt knock-down completely abolished Ser126 phosphorylation and reduced phosphorylation of Ser178.

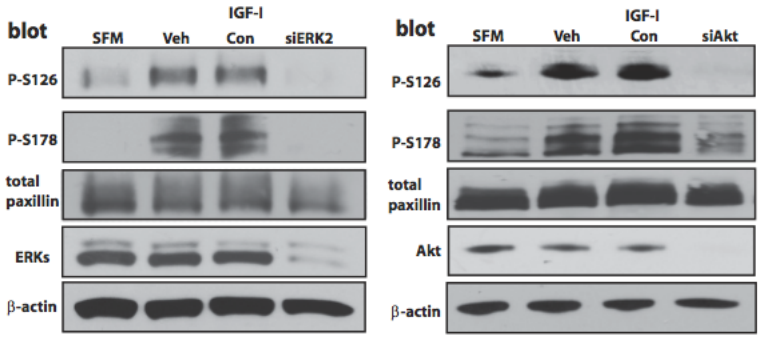

Fig 3.13 paxillin phosphorylation was assessed in ERK-depleted and Akt-depleted 5637 cells as described above. Depletion of endogenous ERK and Akt was performed as described in the motility assays.

Collectively, these results indicate that paxillin upon IGF-I stimulation may be a direct substrate of Akt and ERK-mediated phosphorylation in 5637 urothelial cancer cells. These results also suggest that paxillin may play an important role in mediating IGF-I-induced motility and invasion of bladder cancer cells.

3.9 Paxillin Colocalizes with FAK

The formation and disassembly of focal adhesions (adhesion turnover) at the cell front is a key process in the regulation of cellular migration [Webb et al, 2004]. Because paxillin and FAK are key constituents of focal adhesions, we determined whether IGF-I stimulation could induce a redistribution of paxillin at the cell edge of migrating cells. Following a 30-min stimulation with IGF-I, there was a significant redistribution of paxillin at the protrusive region of 5637 cells in focal adhesions where paxillin colocalized with FAK (Fig. 3.14 A). The same redistribution of focal adhesions and colocalization of paxillin with FAK in adhesions was detectable by confocal microscopy in T24 cells but with a slightly higher background in unstimulated cells (Fig. 3.14 B).

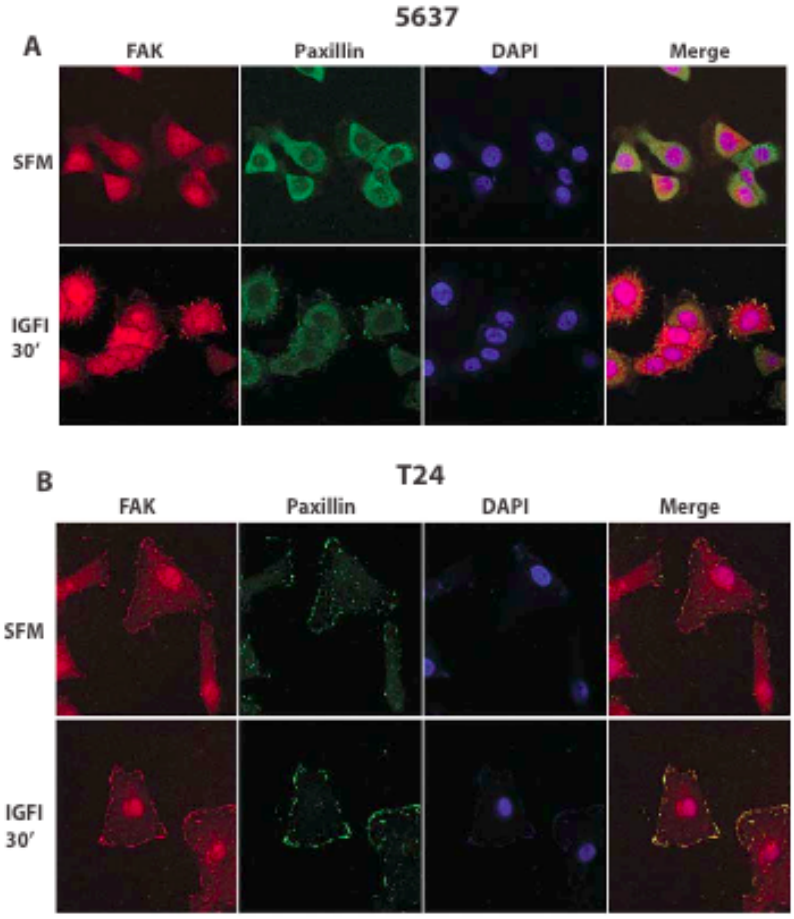

Fig 3.14 A, 5637 and B, T24 cells were plated onto cover slips, serum-starved for 24 h and then stimulated for 30 min with IGF-I (50 ng/ml). Colocalization was analyzed by immunofluorescence microscopy as described in Experimental Procedures. Cover slips were incubated with an anti-paxillin monoclonal antibody (BD Pharmingen) and then with a secondary antibody Alexa Fluor 488 (green) (Molecular Probes). FAK was detected using anti-FAK polyclonal antibodies (BD Pharmingen). The secondary antibody for FAK was Alexa Fluor 594 (red) (Molecular Probes). Images were analyzed at the Kimmel Cancer Center Bioimaging Core Facility with LSM 510 Meta Confocal Microscope. The images were merged using Photoshop CS4. The experiments are representative of three independent experiments

Taken together, these results suggest that paxillin may play an important role in IGF-I-induced motility of cancer urothelial cells by regulating adhesion turnover at the cell front, a process critical to cellular migration.

3.10 Paxillin depletion inhibits IGF-I-induced migration and invasion of bladder cancer cells

To confirm the role of paxillin in IGF-IR-induced motility of cancer urothelial cells, we utilized siRNA to target endogenous paxillin proteins in 5637 and T24 cells. Our approach yielded a ~70 % depletion of endogenous paxillin proteins in both 5637 and T24 cells as compared to cells treated with either vehicle or scrambled siRNA (Fig. 3.15 A) and a robust inhibition of IGF-I-mediated migration and invasion of 5637 (Fig. 3.15 B) and T24 (Fig. 3.15 C) cells.
Collectively, our findings reveal an essential role for paxillin in the IGF-IR functional regulation of tumor cell motility, a key property of the aggressive cancer phenotype.

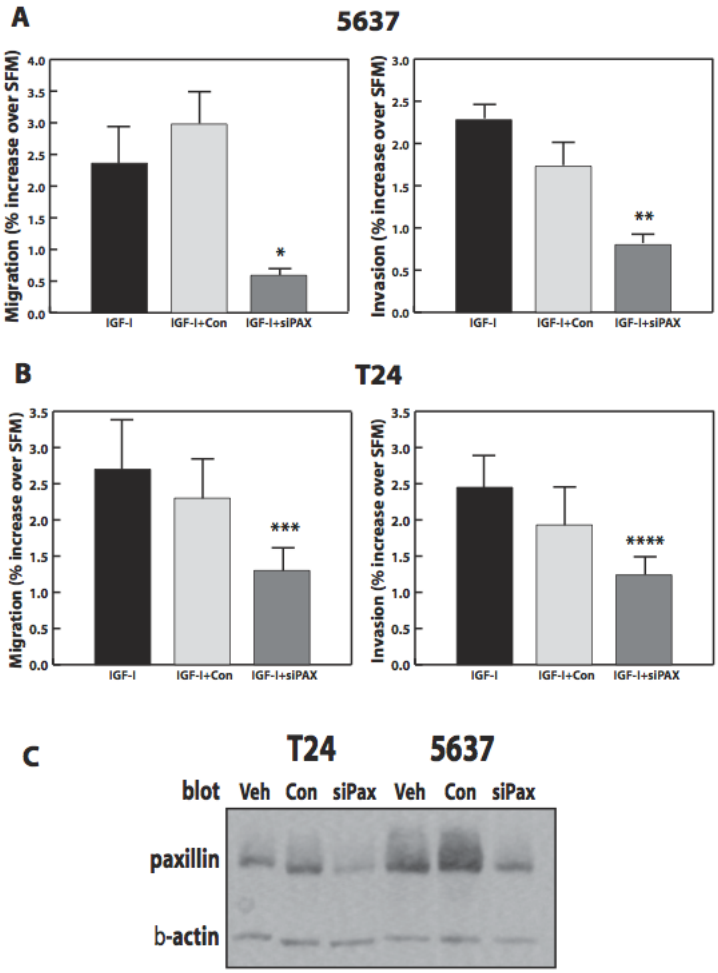

Fig 3.15 A, gene knockdown for paxillin was achieved by RNA interference using small interfering RNA (siRNA). Paxillin expression levels were analyzed by immunoblot using anti-paxillin polyclonal antibodies (Santa Cruz Biotechnology). Protein loading was monitored by immunoblot using ant-beta-actin polyclonal antibodies (Sigma-Aldrich). Blots are representatives of three independent experiments. B, 5637 and C, T24 cells were processed and analyzed for IGF-I-induced migration and invasion as described in Experimental Procedures. Values are expressed as % increase over SFM. B, *,**: P < 0.05 compared with control-oligo-transfected cells. C, ***,****: P > 0.05 compared with control-oligo-transfected cells. Data are the average of three independent experiments in duplicates ±SD.

3.11 Rational design of Cdc25Mm derived molecules with Ras sequestering properties

From the previous data it's quite clear how the enhanced motility induced in bladder cancer cells by IGF-I through MAPK and AKT pathways and Paxillin activation could be triggered by the single Ras activation event, as suggested by the higher Ras•GTP level found in IGF-I stimulated cells (see 3.3). This is reasonable, since Ras is a pivotal hub in signal transduction and could lead to the activation of both MAPK and AKT signaling pathways, either directly or through crosstalk with other molecules. For this reason a possible approach to interfere with the biological effects triggered by the exposure to this growth factor could be represented by the use of Ras specific inhibitors.

In our laboratory it has been shown that substitution of a single residue within αH (T1184E) or αAB (W1056E) can convert the GEF $Cdc25^{Mm}$ to a protein with dominant negative properties. Such mutants not only are no longer able to activate Ras, but can also efficiently compete with wild-type GEF for Ras binding [Vanoni et al, 1999]. The expression of the dominant negative proteins can also revert the phenotype of k-ras transformed NIH3T3 murine fibroblasts to normal [Bossù et al, 2000].

Moreover, these dominant negative properties are retained also in peptides (50-60aa) spanning mutation harbouring regions as shown previously by Sacco et al. in 2005.

A fundamental requirement for those peptides in order to be developed as therapeutic agents lays in the possibility to strongly improve their biodisponibility at the active site and their intracellular concentration, since their size could be a severe issue in pharmacokinetic. In order to address this goal, two different strategies has been chosen. First of all, two peptides sequences spanning the mutated regions (hairpins AB and HI of $Cdc25^{Mm}$) have been designed as fusion proteins with the Protein Transduction Domain (PTD) of the Tat protein. This domain, formed by a positive charged amino-acid stretch (YGRKKRRQRRR), is able to confere cell-penetrating properties to a wide range of proteins (even very large ones).
Once proved the effectiveness of such a fusion, the second step has been a further minimization of the inhibitory sequences, in order to reduce to the minimum the size of the peptides AB and HI.

3.12 The fusion of Tat PTD does not interfere with the inhibitory activity of the peptides

To be able to exploit PTD properties to drive the engineered peptides inside the cells, we first need to test the engineered peptides for the maintenance of the inhibitory properties even after the addition of such motif. In order to address this, the inhibitory effect of Tat-fused peptides was assessed through a *fos-luciferase* transactivation assay. In this assay the *luciferase* reporter gene is placed in a plasmid under the direct control of

a portion of the Ras responsive human promoter *fos*. Mammalian cells (in this case NIH3T3 mouse fibroblast, both normal or K-Ras transformed) are then transfected with this reporter construct and a plasmid encoding the inhibitory peptides. The peptides activity is then evaluated by dosing the luciferase activity in the protein extract of the trasnfectant cells, that will be proportional to the level of Ras pathway activation.

As can be seen in fig 3.16 both peptides still retain a dose dependent inhibitory activity, even in the presence of Tat PTD, proving how the addition of such domain does not interfere with their Ras sequestering properties.

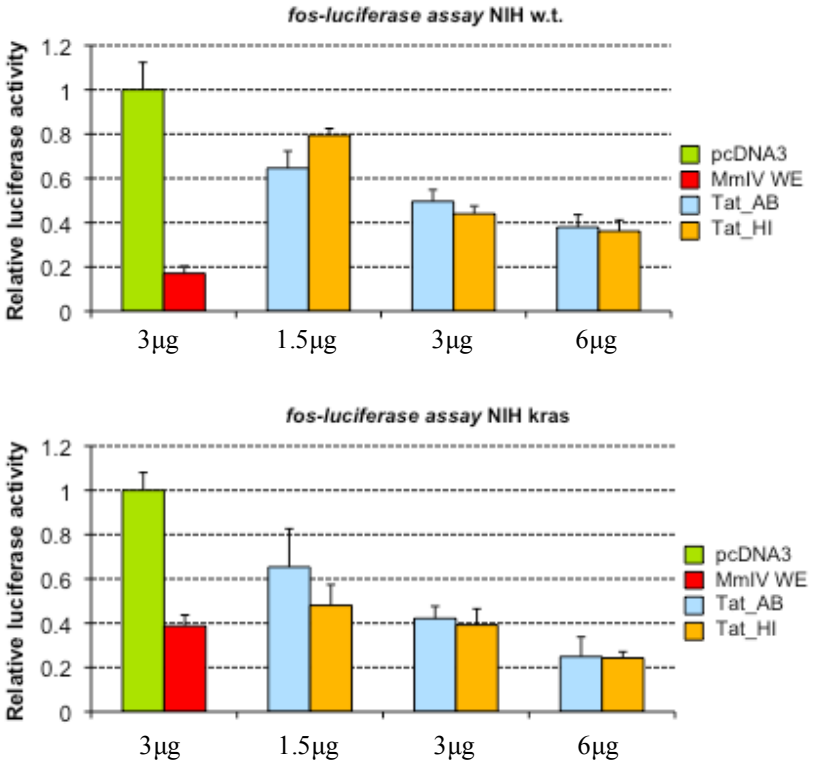

Fig 3.16 Transactivation *fos-luciferase* assay. Normal and *k-ras* transformed mouse fibroblast were co-trasnfected with the *fos-luciferase* reporter plasmid and with a plasmid encoding for the Tat-engineered peptides (total DNA amount as indicated). The relative luciferase activity is reported. The activity in cells trasnfected with just the reporter plasmid and an empty plasmid was set to 1 (pcDNA3, green bars). As a control, cells transfected with the reporter plasmind and a plasmid econding for the entire Dominant Negative $Cdc25^{Mm}$ were analyzed (MmIV WE, red bars)

3.13 Analysis of cell transduction ability of PTD engineered peptides

Since the addition of Tat PTD to the $Cdc25^{Mm}$ derived peptides did not interfere with their inhibitory activity, we next tested their ability to penetrate inside mammalian cells when given as recombinant protein in the cell growth medium.

The peptides were first expressed in a BL21 derived *E.coli* strain as an 6-Histidine fusion protein and then purified under denaturing conditions. The purified proteins were then added to the growth media and the internalization monitored at different time points as indicated in fig 3.17

The internalization of the peptide is visible through the increasing green fluorescent signal, obtained thanks to standard immunofluorescent techniques exploiting the same His tag used for the purification. The peptide entrance is clearly visible from 20' of exposure, with a maximum peak around 5h. Notably the peptide is still present inside the cells long time after its addition (24h).

Moreover the fluorescent signal from the peptides has a punctuated look. Probably this is due to a sub-optimal solubility that can, as a result, drive the peptides into small aggregates. This is in keeping with our need to purify the recombinant proteins under denaturing conditions.

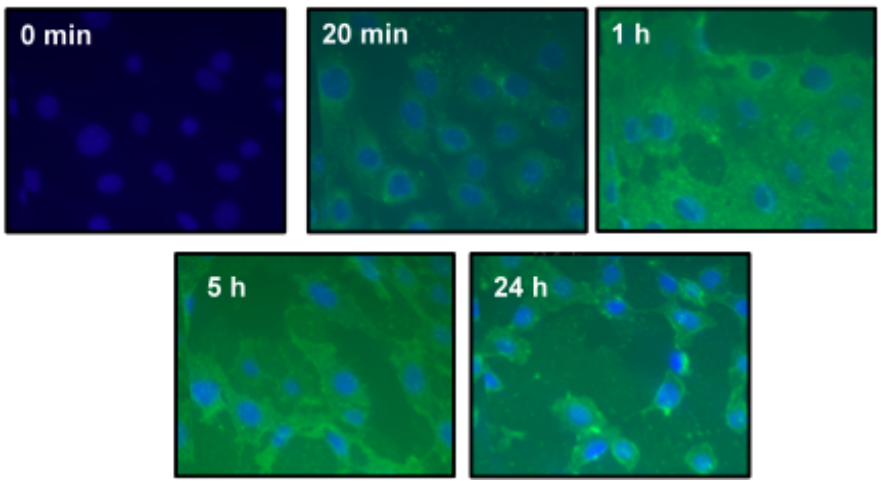

Fig. 3.17 Intake kinetic of Tat-engineered peptide. The peptide, at a concentration of 5μM, was supplied directly in the culture medium. At the indicated times, cells were washed, fixed processed to immunofluorescence against the His tagged peptide (green) and stained with DAPI for nuclei (blue)

3.14 Further downsizing of Cdc25Mm derived Ras inhibitors

In order to develop even better agents, possibly characterized with the same inhibitory properties of the peptides tested until now, but with better pharmacokinetic properties (and hopefully increased solubility), we performed a further downsizing of the peptide sequences. In a structure based manner several other peptides has been developed, by dissecting the AB and HI hairpins into smaller sequences, spanning each single helix or the loops that link them. All the candidates, fused in frame with the Tat PTD, were then tested

for their inhibitory activity, as seen before for the former Tat-engineered peptides, using a *fos-luciferase* assay.

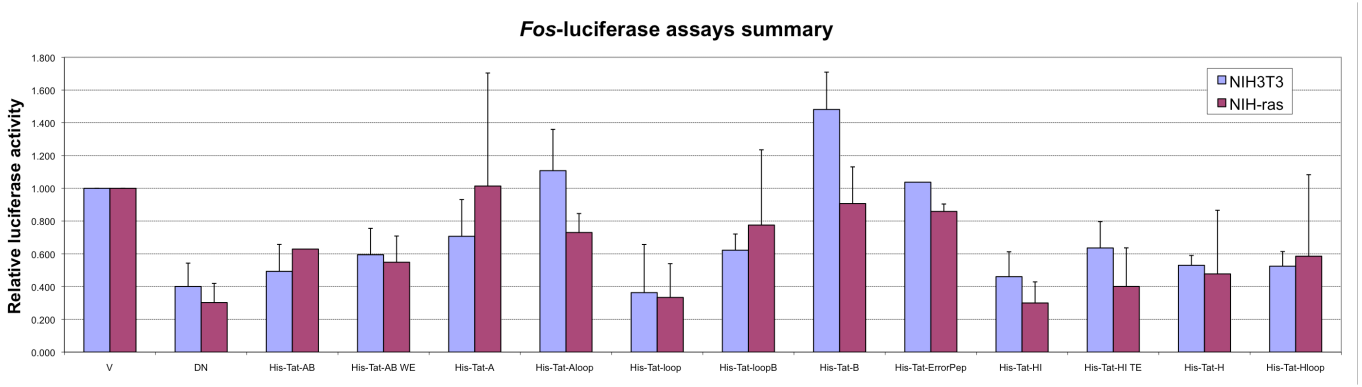

Fig 3.18 Transactivation *fos-luciferase* assay. Normal and *k-ras* transformed mouse fibroblast were cotrasnfected with the *fos-luciferase* reporter plasmid and with a plasmid encoding for the Tat-engineered peptides. The relative luciferase activity is reported.

As can be seen in fig 3.18, while some candidates still retain a suitable inhibitory activity, other loose almost completely their activity. In particular two major candidates (His-Tat-H and His-Tat-Loop) were chosen as "lead compounds". The first peptide comprises 20 residues coming from the H helix of $Cdc25^{Mm}$, while the second one 20 residues taken from the loop between helices A and B.

We tried then to obtain these two peptides as recombinant proteins (of course with Tat PTD and Histidine tag) in *E.coli*. Unluckily the protein expression in properly transformed *E.coli* strains was hardly detectable. However, since the total length of those peptides was just 33aa, we decided to have them produced by chemical synthesis (by Genescript®). Moreover, since one of those peptides comprises the turn loop between two helix, we designed, beside the plain sequence tested above in the *fos-luciferase* assay, a version with a disulfide bond able to constrain its tridimensional structure in an "U-shaped" loop.

3.15 The down scaled peptides are able to interfere with the enhanced motility induced by IGF-I in Bladder cancer cells.

Since the available amount of the peptides produced by chemical synthesis was limiting, we decided to test the agents directly for their biological activity. In particular all the peptides (at a concentration of 500nM) were tested for their ability to interfere with the enhanced motility induced by IGF-I

in Bladder cancer cells. As can be seen in fig 3.19 all the tested synthetic peptides are able to revert the enanched migration and invasion ability of 5637 cells in presence of IGF-I.

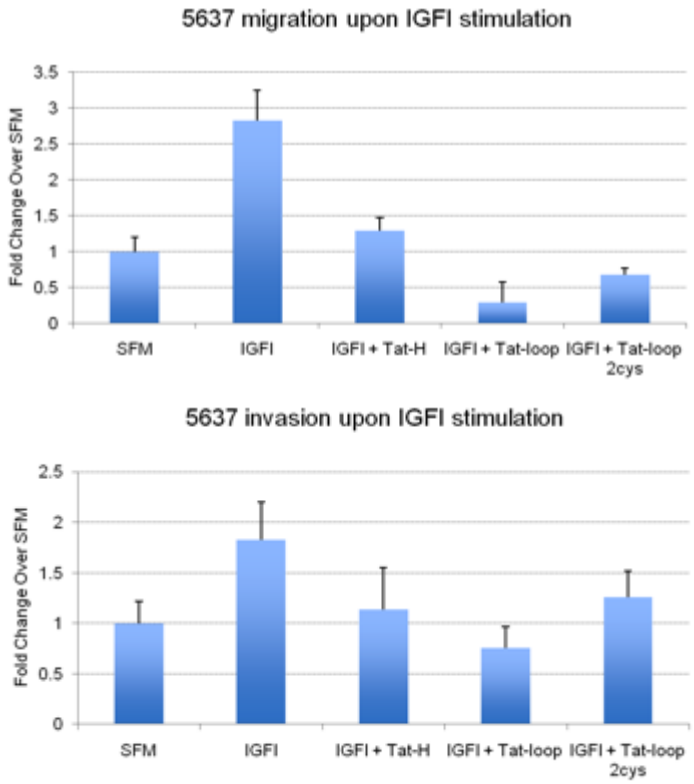

Fig 3.19 Effect of Tat-engineered peptides on IGF-I induced 5637 motility. Migration and invasion assays were performed as described previously. Ras inhibiting peptides were added directly in the medium during the assay at 500nM concentration in both the upper and lower chamber.

4. Discussion

4. Discussion

Ras proteins are a pivotal hub in signal transduction pathways regulating a number of biological responses like proliferation, differentiation and cell motility in all eukaryotic organisms. Mutations or alterations of the activity of such proteins have a very high influence in establishment and development of human tumors. For this reason, Ras proteins are a good target in anticancer therapy. However, despite the large amount of research, a Ras inhibitor with high specificity and low toxicity has not been developed yet. In our laboratory it has been shown that substitutions of a single residue within αH (T1184E) or αAB (W1056E) can convert the GEF $Cdc25^{Mm}$ to a protein with dominant negative properties. Such mutants not only are no longer able to activate Ras, but can also efficiently compete with wild-type GEF for Ras binding, hypothetically forming a stable binary complex with lower affinity for incoming nucleotide. The expression of the dominant negative proteins can also revert the phenotype of k-ras transformed NIH3T3 murine fibroblasts to normal. Moreover, all these dominant negative properties are retained also in very short peptides (50-60aa), designed around the two helix hairpins harbouring these mutations, as shown previously by Sacco et al, 2005.

Aim of this thesis was the development of $Cdc25^{Mm}$ derivatives as anticancer drugs and their validation on bladder cancer cell lines. In order to improve the therapeutic potential of $Cdc25^{Mm}$

derived peptides we performed a fusion with the Tat protein transduction domain. This addition not only did not interfere with their Ras inhibitory properties, but was shown to be able to drive peptide internalization into mammalian cells. Moreover we performed a further downsizing of those peptides, reaching candidates as small as 33aa that still retained the desired activity.

In order to test these compounds in the bladder cancer context, we had to previously better characterize the molecular abnormalities driving the malignant phenotype in such cells. As a matter of fact, although bladder cancer is one of the most common malignancies the molecular mechanisms that determine malignant transformation in the urothelia lining the bladder wall are still very poorly characterized. Most bladder cancers are frequently recurring and often progress into an invasive and metastatic phenotype. In particular we focused on the effect on bladder cancer cells of the growth factor IGF-I, since is suggested to be overexpressed in bladder cancer tissues and it's know to be strongly linked to Ras pathway at a molecular level.

Using urothelial carcinoma-derived human 5637 and T24 cells we were able to demonstrate that: (i) Activation of the IGF-IR by its ligand IGF-I promotes migration and induces wound healing in these cells without affecting cell proliferation; (ii) The IGF-IR stimulates the cells' ability to migrate through and from a complex 3D matrix such as Matrigel; (iii) IGF-I stimulation induces higher levels of Ras•GTP and the

subsequent activation of both the Akt and MAPK pathways, which are required for migration and invasion, as determined by pharmacological and genetic approaches; (iv) IGF-I stimulation induces Akt and MAPK-dependent paxillin phosphorylation; (v) Upon IGF-I stimulation, paxillin relocates at dynamic focal adhesion at the cell's edge where it colocalizes with FAK, and (vi) Depletion of endogenous paxillin proteins by siRNA strategies severely reduces motility and invasive ability of these cells.

All these data, taken together, strongly point out the IGF-IR pathway as a critical regulator of tumor cell motility and invasion of bladder cancer cells. For these reason it could represent a novel and important molecular target in bladder cancer. However these data also highlight a major role of MAPK and AKT pathway in the IGF-I induced biological effects. Moreover the activation of those pathways is probably driven by Ras, since IGF-I dramatically increases Ras•GTP levels. This scenario is thereby compatible with the use of Ras inhibitors in order to modulate the effects of IGF-I signaling in bladder cancers cells. Such approach would have the great advantage to allow the use of a single agent to block simultaneously two different pathways, MAPK and AKT, both crucial for the transformed phenotype of bladder cancer cells.

As a matter of fact, as has been clearly shown in the results, the exposure of bladder cancers cells to Tat-engineered $Cdc25^{Mm}$ derived Ras inhibitors (at a sub μM concentration) is able to completely revert all the biological response of such

cells to the growth factor, by limiting their enhanced motility and invasiveness.

These results strongly corroborate the use of $Cdc25^{Mm}$ dominant negative derivatives as Ras inhibitors and provide a validation of their possible application in human cancer. Moreover they prove that a Ras sequestering property can be retained almost perfectly by smaller and smaller peptides. Hypothetically even smaller molecules on whose scaffold would be attached the structural pharmacophores at the basis of Ras inhibition by Dominant Negative $Cdc25^{Mm}$ could be developed. Taken together, all these data open a way to the obtainment of highly active and highly specific Ras inhibitors, that could represent a powerful tool to modulate the activity of such important and pivotal hub in signal transduction, no matter what's the abnormal upstream stimulus (IGF-I, EGF or Ras itself) driving cell transformation.

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Riassunto

Le proteine Ras sono proteine G monomeriche, a basso peso molecolare e dotate di una bassa attività GTPasica intrinseca che svolgono un ruolo chiave nelle vie di trasduzione del segnale coinvolte in processi di crescita e differenziamento cellulare. Ras può funzionare come un vero e proprio interruttore molecolare, trovandosi alternativamente in due stati: uno attivo (legato a GTP) ed uno inattivo (legato a GDP). I passaggi dallo stato attivo a quello inattivo e viceversa possono avvenire spontaneamente, ma la velocità delle due reazioni in questo caso sarebbe molto bassa. Per questo motivo l'attività di Ras è regolata da due classi di proteine. Le proteine GAP (GTPase Activating Protein) sono in grado di catalizzare una più efficiente idrolisi del nucleotide da GTP a GDP, portando all'inattivazione di Ras. I GEF (Guanine nucleotide Exchange Factors), invece, sono in grado di favorire la dissociazione del nucleotide idrolizzato ed il conseguente scambio GDP/GTP, contribuendo alla modulazione positiva del pathway. Varianti mutate dei geni *ras* sono state identificate con alta incidenza in molteplici forme di patologie tumorali. Per tale ragione le proteine Ras sono considerate target molecolari d'eccellenza nella terapia di disordini proliferativi.

Nel nostro laboratorio è stato dimostrato come una singola sostituzione amminoacidica all'interno del GEF Ras-specifico $Cdc25^{Mm}$ sia in grado di convertire lo stesso in una proteina dotata di proprietà dominanti negative, capace di inibire specificamente l'attività di Ras in vitro e di attenuarne il

circuito di segnalazione in vivo, diminuendo il potenziale oncogenico di fibroblasti murini trasformati dall'oncogene *k-ras*. Inoltre, recentemente abbiamo dimostrato come anche singoli peptidi isolati dall'intero GEF (derivati dalle zone recanti le mutazioni puntiformi di cui sopra) mantengano, almeno parzialmente tali proprietà.

Scopo della presente tesi è lo sviluppo di peptidi derivati del GEF $Cdc25^{Mm}$ come agenti Ras inibitori e la loro validazione in linee cellulari di tumore alla vescica.

Considerato il meccanismo d'azione di tali peptidi, un requisito fondamentale per il loro funzionamento nei modelli cellulari d'interesse è la presenza di una correlazione tra il fenotipo trasformato di quest'ultimi e lo stato di attivazione di Ras. Dal momento che dati di letteratura suggeriscono un coinvolgimento del fattore di crescita IGF-I nelle patologie tumorali della vescica e dato il noto collegamento tra tale fattore di crescita ed il pathway di Ras, abbiamo inizialmente analizzato gli effetti della stimolazione da IGF-I su due linee cellulari derivate da carcinoma alla vescica (5636 e T24).

Sorprendentemente tale fattore di crescita non ha evidenziato grandi effetti sul potenziale proliferativo delle linee oggetto di studio, rivelandosi tuttavia estremamente efficace nello stimolare una aumentata motilità cellulare, come evidenziato da saggi di migrazione, invasione e wound-healing. A livello molecolare tali effetti sono accompagnati da un forte innalzamento dei livelli di Ras•GTP e dalla successiva attivazione delle vie di segnalazione a valle di Ras, come MAPK

e AKT. La stimolazione da IGF-I è inoltre in grado di determinare la fosforilazione e l'attivazione di Paxillina (una proteina adattatrice nota per il suo importante ruolo nei fenomeni di migrazione cellulare) e la sua colocalizzazione con FAK (Focal Adhesion Kinase) a livello dei punti di adesione focale. Infine, tale attivazione di Paxillina, così come l'aumentata motilità cellulare indotta da IGF-I, risulta essere dipendente, in modo diretto o indiretto, dall'attività di MAPK e AKT. L'utilizzo di inibitori chimici di suddette vie o di siRNA specifici per le suddette chinasi in grado di regolarne negativamente l'espressione è in grado di attenuare infatti gli effetti indotti da IGF-I sia a livello biochimico sia a livello fenotipico.

Nel complesso, questi dati evidenziano come il pathway dell'IGF-I sia di fondamentale importanza nella regolazione della motilità in cellule tumorali di vescica, fenomeno strettamente collegato al potenziale metastatico. Questi stessi dati suggeriscono inoltre l'importanza per questi stessi fenomeni di due vie di trasduzione parallele (MAPK e AKT), la cui attivazione potrebbe tuttavia essere guidata da un unico segnale a monte, ovvero l'innalzamento dei livelli di Ras•GTP. Per questo motivo lo sviluppo di molecole Ras inibitorie altamente specifiche, in grado di attenuare il circuito di segnalazione a monte di entrambe le suddette vie, potrebbe risultare di enorme interesse per il trattamento della patologia in esame.

Per questo motivo abbiamo esplorato la possibilità di migliorare le proprietà farmacocinetiche e farmacodinamiche dei peptidi derivati da $Cdc25^{Mm}$ e dotati di proprietà Ras sequestranti. Allo scopo di ottenere una migliore veicolabilità in cellula, ad esempio, tali peptidi sono stati ingegnerizzati tramite l'aggiunta della sequenza responsabile delle proprietà auto-penetranti della proteina Tat. Tale sequenza (PTD, Protein-Transduction-Domain, aa 47-57 YGRKKRRQRRR) è costituita prevalentemente da aminoacidi carichi positivamente ed è in grado di veicolare l'internalizzazione di molteplici proteine, fungendo da carrier.

Abbiamo innanzitutto dimostrato, tramite saggi di transattivaizone *fos-luciferasici*, come l'aggiunta di tale dominio non alteri in maniera significativa le proprietà inibitorie dei peptidi di partenza e come tali peptidi siano in grado di essere effettivamente internalizzati da cellule di mammifero (fibroblasti murini NIH3T3) qualora somministrati nel mezzo di coltura.

Successivamente la ricerca è proseguita con l'ottenimento di varianti più piccole dei peptidi in oggetto, presumibilmente dotate di migliori proprietà farmacocinetiche e facilmente sintetizzabili a livello industriale. È stato quindi effettuato un design struttura-guidato, sulla base degli elementi di struttura primaria e secondaria maggiormente coinvolti nell'interazione Ras•GEF. Le varianti minimizzate sono state quindi selezionate sulla base delle proprietà Ras inibitorie misurate tramite saggi

di transattivazione *fos-luciferasici* ed i candidati più promettenti ottenuti tramite sintesi chimica.

Dato il ruolo cruciale emerso a carico delle vie di trasduzione a valle di Ras nei fenomeni di migrazione ed invasioni stimolati dalla presenza di IGF-I in linee cellulari di vescica, tali peptidi sono stati testati in saggi di migrazione ed invasione nei modelli cellulari caratterizzati precedentemente. Tutti i peptidi Ras inibenti, anche se con intensità diversa, si sono rivelati estremamente attivi nel ridurre l'aumentata motilità indotta da IGF-I delle cellule in esame a concentrazioni sub-μM.