From DEPARTMENT OF ONCOLOGY PATHOLOGY
Karolinska Institutet, Stockholm, Sweden

# IGF-1R: STUDIES ON THE EXPRESSION AND ROLE IN TRANSFORMATION

Natalia Natalishvili


Karolinska Institutet

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***To my Mum***

*Somewhere something incredible is waiting to be known.*
Carl Sagan

*Science may set limits to knowledge, but should not set limit to imagination.*
Bertrand Russel

# ABSTRACT

Transformation is a complex nonlinear multistep process during which normal cell becomes cancerous. Main characteristics of transformed cells include uncontrolled growth, invasion and metastasis. Extensive research over recent decades on mechanisms behind transformation has established the tyrosine kinase receptor, Insulin-like Growth Factor 1 Receptor (IGF-1R) as the major factor involved. Consequently, IGF-1R has gained ever increasing attention as a promising target in cancer therapy. The current opinion is that inhibition of IGF-1R activity is not enough to cause massive apoptosis and tumor regression. To obtain these responses the receptor must be downregulated.

The small molecule picropodophyllin (PPP), discovered by our group, inhibits IGF-1R signaling. PPP induces massive apoptosis in tumor cells and causes tumor regression in various animal models. PPP is well tolerated *in vivo*.

This thesis focuses on mechanisms of IGF-1R expression and their role in transformation. Paper I shows that IGF-1R knockout cells (R-) cultured over long time express the kinase subunit (β-subunit) of the receptor. This aberrant receptor is demonstrated to be important for survival of these cells. Paper II shows that the aberrant IGF-1R β-subunit in R- cells represents not only a beneficial factor for cell survival but is crucial for transformation of these cells. Knockdown by siRNA targeting IGF-1R abrogates oncogenic transformation by H-RasV12 and/or polyoma middle T-antigen. The β-subunit in R- cells is shown to be intracellular and does not interfere with ERK and Akt phosphorylation. These findings may suggest involvement of a non canonical pathway of signaling in the IGF-1R dependent transformation. Paper III shows that PPP induces partial downregulation of the IGF-1R and that this action may be important for its apoptotic effect in tumor cells. β-arrestin1, adaptor protein involved in IGF-1R signaling seems to be important for transduction of this effect. Paper IV reveals that β-arrestin1 mediated IGF-1R signaling is important for Ras induced transformation. Signal transduction of activated Ras is impaired in the absence of β-arrestin1. Incomplete Akt and ERK activation after IGF-1 stimulation cannot sustain growth and proliferation under anchorage independent conditions.

In conclusion, this thesis suggests that the holo-IGF-1 receptor is not necessary for transformation, but action of the intracellular β-subunit is sufficient. This finding may have patho-physiological relevance since the occurrence of intracellular IGF-1R in malignant cells has been widely reported. PPP-induced IGF-1R downregulation may contribute to its strong anti-tumor effect. Finally, β-arrestin appears to be important for Ras-induced transformation.

# LIST OF PUBLICATIONS

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# CONTENTS


1 Introduction ..... 1
1.1 Cancer ..... 1
1.1.1 Etiology ..... 1
1.1.2 Characteristics of transformation ..... 1
1.1.3 Cancer as a complex disease ..... 1
1.1.4 Models of transformation in the laboratory ..... 2
1.1.5 Cancer in humans and mice ..... 4
1.2 Oncogene induced transformation ..... 5
1.2.1 Ras ..... 5
1.2.2 Polyomavirus oncogenes ..... 10
1.2.3 Src ..... 13
1.3 IGF family ..... 15
1.3.1 Insulin-like Growth Peptides I and II ..... 15
1.3.2 Insulin-like Growth Factor-1 receptor ..... 16
1.3.3 Insulin like Growth Factor-2 receptor ..... 16
1.3.4 System regulation - binding proteins and proteases ..... 17
1.4 IGF-1 receptor ..... 18
1.4.1 Structure ..... 18
1.4.2 Receptor activation and signaling ..... 19
1.4.3 Signal termination and role of modifications ..... 20
1.4.4 Relation to insulin receptor ..... 21
1.4.5 Interaction with other signaling pathways ..... 22
1.4.6 IGF-1R and cancer ..... 24
1.4.7 Targeting the IGF-I receptor in cancer ..... 25
1.5 Arrestin protein family ..... 26
1.5.1 β-arrestins ..... 26
1.5.2 Isoform differences ..... 27
1.5.3 IGF-1R and β-arrestin ..... 28
1.5.4 GPCR, RTK and cancer ..... 29
2 Methodological considerations ..... 30
2.1 Induction of transformation ..... 30
2.1.1 Oncogenes ..... 30
2.1.2 Cell lines ..... 30
2.1.3 Plasmids and transfection procedures ..... 31
2.2 Analysis of transformed phenotype ..... 32
2.2.1 Cell survival and proliferation ..... 32
2.2.2 Anchorage-independent growth ..... 32
2.2.3 Tumorigenic properties *in vivo* ..... 33
2.3 DNA, RNA and protein analysis ..... 33
2.3.1 PCR ..... 33
2.3.2 DNA/RNA sequencing ..... 33
2.3.3 SDS/PAGE and Western Blotting ..... 33
2.3.4 Confocal microscopy ..... 34
2.4 IGF-1R and β-arrestin1 targeting ..... 34
2.4.1 PPP ..... 34

2.4.2 Gene silencing –small interfering and hairpin RNA .. 34
2.5 Statistical methods ..... 34
3 Aims of the study ..... 36
4 Results and discussion ..... 37
4.1 Paper I ..... 37
4.2 Paper II ..... 37
4.3 Paper III ..... 39
4.4 Paper IV ..... 40
5 Conclusions and future perspectives ..... 43
6 Acknowledgements ..... 44
7 References ..... 46

# LIST OF ABBREVIATIONS

| | |
|---|---|
| EF, MEF | Embryonic fibroblast, mouse embryonic fibroblast |
| EGFR | Epidermal growth factor receptor |
| ER | Estrogen receptor |
| FAK | Focal adhesion kinase |
| GEF | Guanine nucleotide exchange factors |
| GPCR | G protein coupled receptor |
| GRB2 | Growth factor receptor binding protein 2 |
| IGF, IGF-1R | Insulin-like growth factor, IGF-1 receptor |
| IGFBP | IGF binding proteins |
| IR | Insulin receptor |
| IRS | Insulin receptor substrate |
| KO | β-arrestin1 knock-out cells, *β-arr1-/-* MEF |
| MAPK | Mitogen activated protein kinase |
| MDM2 | Murine double minute 2 |
| MEF | Mouse embryonic fibroblast |
| MPR | Mannose 6-phosphate receptor |
| mTOR | Mammalian target of rapamycin |
| PCR | Polymerase chain reaction |
| PDGF | Platelet derived growth factor |
| PI3K | Phosphatidylinositol 3 kinase |
| PIP2 | Phosphatidylinositol 4,5 bisphosphate |
| PIP3 | Phosphatidylinositol 3,4,5 trisphosphate |
| PP2A | Protein phosphatase 2A |
| PPP | Picropodophyllin |
| PPT | Podophyllotoxin |
| PY | Mouse polyoma virus |
| PyMT | Polyoma middle T antigene |
| Rb | Retinoblastoma protein |
| RTK | Receptor tyrosine kinase |
| SCID | Severe combined immuno deficiency |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| shRNA | Small hairpin RNA |
| siRNA | Small interfering RNA |
| SV40 | Simian virus 40 |
| TKR | Tyrosine kinase receptor |
| VEGF | Vsascular endothelial growth factor |
| v-Src, c-Src | Viral Src, cellular Src |
| WB | Western blotting |

Genes *italics (IGF-1R)*; human proteins, capital letters (IGF-1R); mouse and vertebrate proteins, initial capital letter (Igf-1r)

# 1 INTRODUCTION

## 1.1 CANCER

Cancer is a class of diverse clinical diseases characterized by a defect in tissue growth control. Its main characteristics include uncontrolled growth and ability to invade and metastasize within normal tissue.

### 1.1.1 Etiology

Despite the complexity of the cancer phenotype, early studies indicated that cancer might be the result of very few changes in the genome (Gross 1970). Later on however it became evident that genetic and epigenetic abnormalities accumulate in the premalignant cell over the time before it is eventually transformed. Increased mutation rates are traditionally viewed as the driving force of the malignant transformation. Primarily cancer occurs due to various chemical carcinogens, ionizing radiation, viral or bacterial infection, hormonal or immune imbalance. A very large variety of genetic and epigenetic changes occurs all the time that favor cancer development. It is a Darwinian process, based on variation and selection of the fittest. In the context of cancer, the fittest means an ever-increasing potential for autonomous growth. Accumulation and further progress of these changes results in escape of the cells from internal and external control mechanisms and eventually macroscopic tumor growth.
Instability found in cancer affects two general classes of genes. Cancer-promoting oncogenes are activated ensuring increased growth and proliferation rates of the cancer cells and the ability to form tumors in diverse tissue environments. Tumor suppressor genes are then inactivated in cancer cells, resulting in the loss of normal cell functions, such as precise DNA replication, control over the cell cycle, orientation and adhesion within tissues, interaction with protective cells of the immune system.

### 1.1.2 Characteristics of transformation

Malignant phenotype includes a broad spectrum of characteristics that together define cancer as an entity. The most prominent features of malignant cells were listed by Hanahan and Weinberg (Hanahan and Weinberg 2000) and include: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis. While these are important phenotypic markers, not all tumor cells acquire all of these properties and the sequence of these events may vary.

### 1.1.3 Cancer as a complex disease

Cancer evolves as a result of genetic and epigenetic changes and has characteristic phenotype. However it represents an even more complex system since tumors need to continually interact with their host environment to be able to propagate. Two important concepts here that are also widely

discussed in the literature during the last years are cancer stem cells and the role of intercellular communication (James E. Trosko 2004; Eyler and Rich 2008). Cancer stem cells, possessing properties of the normal stem cells have been implicated in the tumor self-renewal and establishment of new tumors, such as relapse and metastasis.
Another mechanism important for tumor development is an intercellular communication (Glick and Yuspa 2005). One example is based on the interaction between tumor cells and their host environment. Another is the fact that normal cells can inhibit the growth of tumor cells *in situ* (Butcher, Alliston et al. 2009). Long-lasting presence of disseminated tumor cells, followed by sudden awakening many years or decades later, may also be due to this control, rather than from immune reactions as usually assumed.
Another dimension in the tumor-host interaction is represented by numerous safeguard mechanisms that have been evolved during evolution to ensure tight control over the individual cells. In the body, surveillance against tumor development is performed primarily by the immune system and intracellular mechanisms (Klein 2007).
Among immune systems components the effector cells and particularly the sentinel tumor draining lymph nodes are the most powerful promises against disease. Other types of immune response have less importance, apart from a few virus-induced human malignancies most tumors are immunologically recognizable (Marits, Karlsson et al. 2006).
Intracellular control refers to the powerful safeguards against tumor development that operate within every cell. At the level of DNA these are presented by repair and epigenetic maintenance of imprinting and chromatin structure. Further recognition of the signaling of illegitimately activated oncogenes can trigger one or several pathways of apoptosis. The p53 together with its upstream regulators and downstream effectors constitutes one of the most important apoptotic pathways (Levine 1997). The Rb and its crosstalk pathways are amongst the most important tumor growth antagonizing pathways (Sellers and Kaelin 1997). Here, one of the few general rules that seem to be mandatory is inactivation of both pathways in all malignant human tumors, at one point or another. Additionally, suppressed autophagy is frequently associated with the transformed phenotype. Recent implication of tumor suppressors such as Beclin 1, DAP-kinase and PTEN in autophagic pathways indicates a causative role for autophagy deficiencies in cancer formation (Gozuacik and Kimchi 2004).

### 1.1.4 Models of transformation in the laboratory

Tumors are complex tissues continuously interacting with the surroundings. The ideal system to study cellular transformation would be the one reproducing all components of this environment, including vessel, immune or stroma cells in an authentic manner. Therefore, *in vivo* models of cancer represent today the closest and best possible solution.
Rodents, especially genetically engineered mice are currently central tools for the investigation of the molecular and cellular basics of tumor growth and

translational applications in cancer research. However conventional knockout mice models of human tumors often display lesion spectra different from that in humans (Anisimov, Ukraintseva et al. 2005). Therefore techniques creating murine cells that increasingly approach their human counterparts have been introduced (Herzig and Christofori 2002). These methods modify mouse germ line cells by replacing mouse genetic elements with human homologues, thereby "humanizing" the cellular regulatory circuitry (Rangarajan and Weinberg 2003). Other recent approaches such as conditional on/off knockouts, cell - specific targeted mutations and *in vivo* gene-array experiments are also opening new possibilities for intelligent remodeling of the mouse genome to ensure its resemblance to human orthologues (Van Dyke and Jacks 2002).

The next line of research is represented by animals with transplanted human tumors or other tissues that are called xenograft models. This approach allows us to study pathological processes such as invasion and metastasis that are not mimicked by the genetically engineered mouse models and cannot be reproduced by use of *in vitro* studies. However, interspecies incompatibilities in receptor–ligand interaction between engrafted human tumor cells and the surrounding mouse tissue can contribute to the failure of tumor establishment and growth. In addition, xenograft establishment is time and resource consuming since they require the sacrifice of large cohorts of animals at different time points to track tumors and metastatic spread. Therefore some modifications, such as the hollow fiber assay (Hollingshead, Alley et al. 1995) used by US National Cancer Institute have been proposed. Modern imaging technologies, for example preclinical MRI, CT, PET, fluorescence and bioluminescence, are another solution to this problem and allow the non-invasive, real-time high resolution data be acquired using fewer animals with greater speed than traditional methods.

The multistep process of carcinogenesis can also in part be studied by the use of cell culture models. Different stages of the neoplastic transformation have been described for various cell lines. Among them mouse, rat or Syrian hamster embryo fibroblasts and human embryonic fibroblast (Barrett 1985) have global recognition.

Several well established *in vitro* systems for malignant transformation are known. Three dimensional cell culture models (Pickl and Ries 2008) are often used to improve tissue architecture. Such systems can be used for anchorage independent growth, invasion, metastasis, angiogenesis assays. In most of these models cells are cultured in different matrix which allows investigation of the separate aspects of cell–cell, cell–matrix interaction and loss of attachment to substratum in cancer development. Many genes required for the escape of culture-induced growth arrest and senescence, oncogene-induced proliferation, and anchorage-independent growth have been first identified in these models *in vitro*.

Finally, computer programs and algorithms have been developed to qualitatively and quantitatively evaluate tumor growth rates, possible pathways and protein modifications involved (Sanga, Frieboes et al. 2007; Hall and Baracos 2008; Poh-Kuan Chong, Lee et al. 2008). Ability to predict tumor

growth curves permits a more precise evaluation of therapeutic effects than can be obtained with conventional methods (O'Rourke, McAneney et al. 2009).

### 1.1.5 Cancer in humans and mice

The spectrum of spontaneous tumors that develop in humans and in rodents has little similarity (Anisimov, Ukraintseva et al. 2005). Yet genetically engineered mouse models are major contributors into identifying tumor genes and understanding particular mechanisms of transformation. Just a few examples are tumor suppressor p53, Arf knockout mice and RIP-Tag mice (Zender, Zuber et al. 2007). Another example is the use of mice for identification of carcinogens that can provide many positive findings relevant to humans (Rangarajan and Weinberg 2003). However results obtained in rodents often cannot be directly extrapolated into the humans.

The developmental course of mice and humans is markedly different. The first obvious difference is organism size. Consequently, the cellular targets for oncogenic transformation are substantially fewer and differ in nature in mouse tissues from their human counterparts. The much shorter lifespan of mice means that the cancers which appear in these animals have an accelerated progression compared with human malignancies, which can take 20 or more years to progress. Another distinctive property of human cells is immortalization as a prerequisite for the transformation to occur.

Human and rodent cells differ in at least two molecular mechanisms leading to immortalization. In humans, at least four to six mutations are required to escape mechanisms controlling cell proliferation whereas much fewer are required in mice (Hahn and Weinberg 2002). Telomere length in normal human cell lines is successively shortened during proliferation. In cancer cells derived from patients, telomeric DNA sequences are maintained at stable lengths throughout repeated cell-division cycles (Counter, Avilion et al. 1992). In most cases these cells express telomerase, a reverse transcriptase that extends telomeric ends (Counter, Hirte et al. 1994; Kim, Piatyszek et al. 1994; Shay and Bacchetti 1997). In contrast, a mouse cell's life span is not controlled by this mechanism (Blasco, Lee et al. 1997) due to a number of reasons including telomeres 3–10 times longer than in human cells and telomerase expression in most normal mice tissues (Prowse and Greider 1995).

The second crucial mechanism that differs in human and mice are tumor suppressor pathways p53 and Rb (Levine 1997; Sellers and Kaelin 1997). In humans two alternatively spliced proteins, p16/INK4A and p14/Arf from the locus *CDKN2A* regulate activities of the Rb and p53 and both have to be disrupted in most cancers (Ouelle, Zindy et al. 1995). In mice however disruption of single p53/Arf pathway is enough for the cells to escape senescence (Harvey and Levine 1991).

In conclusion, as a result of more complicated control over the cell proliferation capacity, single carcinogenic factor-induced transformation of human cells is an extremely rare event, whereas rodent cells can be relatively easily transformed. Nonetheless, rodents still remain the best and most accepted

long-term carcinogeneous and transformation models we know today, until something more rapid, accurate and less expensive can be found.

## 1.2 ONCOGENE INDUCED TRANSFORMATION

Oncogenes are proteins which interfere with signal transduction pathways and ultimately cause changes in the pattern of gene expression. Expression of the oncogenes results in alterations of the morphology and growth properties of the affected cells. Introduction of a cloned oncogene into a mammalian cell will often provoke senescence or apoptosis. This can be bypassed by elimination of p53 function alone in mouse cells, but requires disruption of both the Rb and p53 pathways in human cells (Serrano, Lin et al. 1997).
Interesting is also the response of tumors induced by oncogenes to subsequent oncogene deprivation. Partial or completely sustained regression, apoptosis or massive differentiation can all be observed. In some cases tumor growth resumes independent of the oncogene, or by reactivating the oncogene in cells which have become dormant (Jonkers and Berns 2004).

### 1.2.1 Ras

The Ras proteins are low-molecular-weight G proteins that possess the ability to hydrolyze GTP to GDP and phosphate. Cycling between their inactive, GDP bound state and active, GTP bound form is catalyzed by guanine nucleotide exchange factors (GEFs). At the same time slow intrinsic GTP hydrolysis is stimulated by GTPase-activating proteins.
Two regions, important for Ras enzymatic activity have been identified. One of them, switch I (aa 30–38), forms part of the $Mg^{2+}$ binding site. The other one, called switch II (aa 59–67), contacts the γ-phosphate of GTP. Conformational changes of both switch regions are implicated in the binding to downstream effector proteins (Marshall 1996).
First discovered as oncogenes capable of inducing malignant transformation, Ras proteins represent a well established critical link between receptor tyrosine kinases (RTK) and downstream signaling implicated in the control of cellular growth and proliferation. The Ras oncogenes are mutated in over a quarter of all human cancers and can induce malignant transformation in a wide variety of cell cultures. However, Ras-induced signaling often controls multiple functions within the same cell and is therefore tightly regulated by positive and negative feedback loops. Importance of this mechanisms is illustrated by the fact that transfection of wild-type Ras in transformed cells reverses the malignant phenotype suggesting that wild-type Ras has tumor suppressive properties. Indeed, expression of wild-type *Ras* genes in several human malignancies is associated with good prognosis (Spandidos, Sourvinos et al. 2002).

#### *1.2.1.1 Ras modifications and activation*

Following activation by upstream receptor tyrosine kinases, guanine nucleotide exchange factors stimulate GDP dissociation and its rapid replacement by GTP, whose intracellular concentration is higher than that of GDP while GTPase activating proteins stimulate intrinsic GTPase activity of Ras up to 10000-fold (Scheffzek and Ahmadian 2005). To date four known subfamilies of GEF molecules include ubiquitously expressed SOS; RasGRF, localized predominantly in the central nervous system; RasGRP characteristic for haematopoietic cells and CNRasGEF (Buday and Downward 2008).

The best-characterized route of Ras activation occurs at the plasma membrane. Ras proteins lack signal sequences and trans-membrane regions and therefore are not intrinsic membrane proteins. They are synthesized in the cytosol and require post-translational modifications targeting them to cellular membranes. All Ras proteins undergo farnesylation, a process depending on a conserved CAAX motif (C - cysteine, A - aliphatic amino acids, X - any amino acid) at the C-terminus. This is followed by cleavage of the final three amino acids and methylation of cystein at the C-terminus at the endoplasmic reticulum. In addition to these modifications of the C-terminus of Ras proteins, H-Ras, N-Ras, and K-Ras4A, but not K-Ras4B, are further modified on other cysteine residues near the C-terminus by addition of one or two palmitic acids upstream of the site of farnesylation.

Ubiquitination has recently been found to be another post-translation modification that distinguishes the Ras proteins and contributes to their intracellular localization (Jura, Scotto-Lavino et al. 2006). The selective mono- and di-ubiquitination of H-Ras and N-Ras, but not K-Ras, is determined by their HVRs and requires farnesylation and palmitoylation. Ubiquitination of these Ras proteins stabilizes their association with endosomes and modulates their ability to interact with downstream cascades.

According to the current model activation of Ras is mediated by the SOS guanine nucleotide exchange factor interacting with Grb2 in two steps. This process is based on that GDP-bound Ras is required for the lower level of SOS GEF activity, whereas binding of Ras-GTP to the allosteric site induces maximal catalytic activity. First, in response to the activated receptor tyrosine kinase, Shc or one of the membrane-localized docking proteins (Quilliam, Khosravi-Far et al. 1995), SOS is translocated to the plasma membrane through at least two independent sites: C-terminal Grb2-binding site and its lipid binding PH domain. The SOS PH domain can recognize PIP2 in the membrane. Contact of the PH domain with phospholipids induces conformational changes allowing binding of Ras-GDP and low-level activity of SOS. Next, Ras-GTP, generated in the course of low-level activity, binds to the same site leading to the maximal activity and further stabilization of the membrane localization of SOS.

However, isolation of active signaling complexes from endosomes that contain tyrosine-phosphorylated EGFR, Grb2/Sos, Ras and Raf provided evidence that Ras might also signal from other intracellular sites.

Recently it was revealed that activated Ras proteins can be detected on various intracellular membranes, including the Golgi and endoplasmic reticulum as well as endosomes. Additionally, the kinetics of Ras activation differ at various intracellular sites; whereas Golgi-associated Ras induces phosphorylation of Erk and PI3K with a potency equal to membrane bound Ras, the Jnk pathway is poorly activated. Conversely, ER-tethered Ras is a potent activator of Jnk but a relatively poor activator of Erk and PI3K (Chiu, Bivona et al. 2002). This suggests that compartmentalization plays an important role in determining the ultimate biological consequences for the cell (McKay and Morrison).

#### *1.2.1.2 Ras signaling*

Today, more than ten different functional classes of downstream effector proteins have been implicated as interacting with Ras. Among them are Raf kinases, phosphatidylinositol-3 kinase (PI3K), Ral-guanine nucleotide exchange factors (Ral-GEFs), the Rac exchange factor Tiam1, and phospholipase C (Downward 2003). However, Ras transformation of rodent fibroblasts was shown to be mediated primarily through three main classes of effector proteins, Rafs, PI3K, and RalGEFs, with Raf generally being the most potent at transforming murine cells (Fig. 1).

The Raf/MEK/ERK pathway is critical to Ras transformation. Constitutively activated mutants of Raf or MEK cause tumorigenic transformation while dominant-negative mutants and pharmacologic inhibitors of MEK effectively block Ras induced tumorigenic growth *in vivo* (Bonner, Kerby et al. 1985; Stanton, Nichols et al. 1989; Kolch, Heidecker et al. 1991; Schaap, van der Wal et al. 1993; Cowley, Paterson et al. 1994; Leevers, Paterson et al. 1994; Stokoe, Macdonald et al. 1994; Westwick, Cox et al. 1994; Khosravi-Far, Solski et al. 1995; Qiu, Chen et al. 1995; Monia, Johnston et al. 1996; Sebolt-Leopold, Dudley et al. 1999). There are three known Raf isoforms (A-Raf, B-Raf and C-Raf) all existing in an inactive state in the cytosol. Activation of these kinases is a complex process requiring several cycles of phosphorylation and dephosphorylation and membrane lipid interactions resulting in Ras binding to the Raf kinase domain and stabilizing active conformation of the protein (Wellbrock, Karasarides et al. 2004). Activated Raf then colocalizes with and activates MEK which in turn phosphorylates ERK. Active ERK then dissociates from MEK and phosphorylates more than 150 substrates throughout the cell. Here, scaffold proteins play an important role in bringing specific components of this signaling cascade together and targeting assembled complexes to various intracellular compartments. Examples of such proteins include kinase suppressor of Ras 1, MEK-partner 1, Sef and Paxillin. Recently, several lines of evidence implicated also β-arrestin in the transmission of signals from Raf to MEK and Erk on endosomes. Raf overexpression increased MEK and Erk binding to β-arrestin (Luttrell, Roudabush et al. 2001) and a dominant-negative form of β-arrestin blocked Erk activation downstream of a GPCR (Daaka, Luttrell et al. 1998; DeFea, Zalevsky et al. 2000).

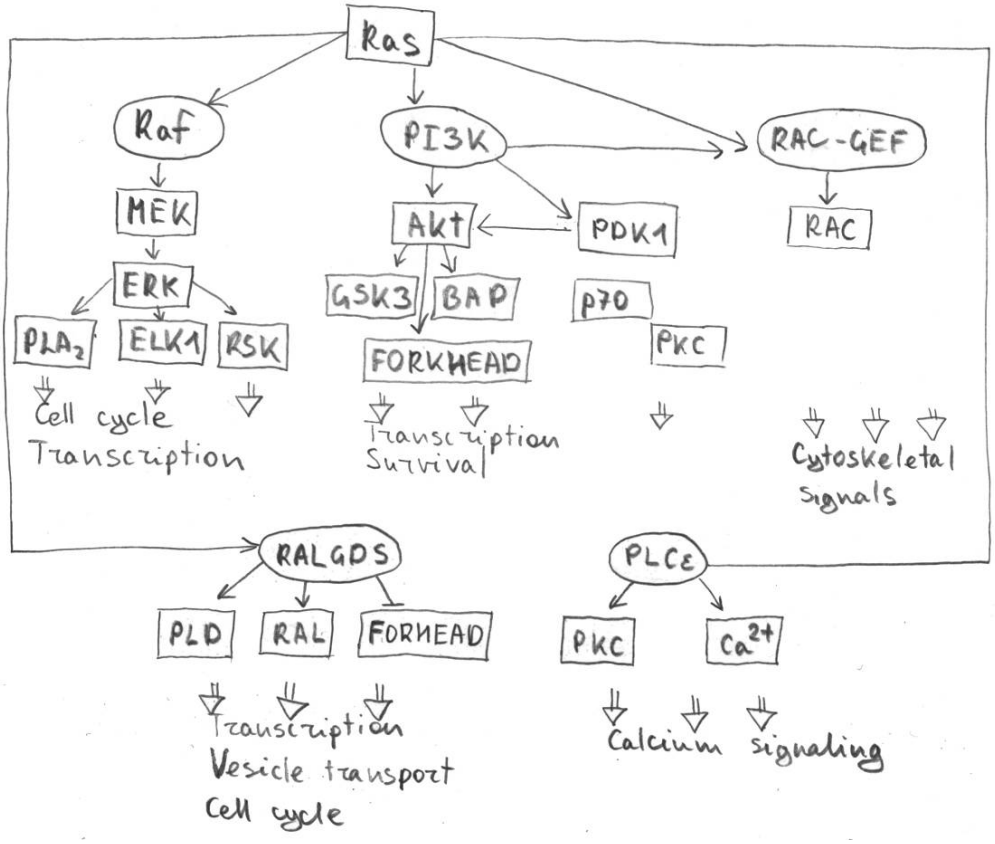
Activated PI3K, a lipid kinase, facilitates the conversion of phosphatidylinositol 4,5 bisphosphate (PIP2) to phosphatidylinositol

3,4,5-trisphosphate (PIP3). PIP3 levels are elevated in Ras-transformed cells and dominant-negative mutants of PI3-kinase can effectively block Ras transformation. Direct interaction between Ras and PI3 kinase was confirmed in a mouse model *in vivo* and provides one of mechanisms through which RTK signal to PI3K (Ramjaun and Downward 2007). Finally, whereas activated PI3-kinase alone cannot cause transformation of NIH3T3 cells, activated variants of PI3K can cooperate with activated Raf to cause synergistic transforming activity (Rodriguez-Viciana, Warne et al. 1997).

The third class of effectors with a role in Ras transformation are guanine nucleotide exchange factors (GEFs) for the Ral small GTPases (Wolthuis and Bos 1999). Inhibition of these RalGEFs, by expression of a dominant-negative Ral protein, blocks Ras dependent focus formation and metastatic growth of specific cell lines. Furthermore, whereas constitutively activated variants of RalGEFs are not transforming, their co-expression with activated Raf induces synergistic focus formation (Urano, Emkey et al. 1996; White, Vale et al. 1996; Lu, Hornia et al. 2000; Ward, Wang et al. 2001).

However, high levels of activated Ras in many cell lines induce cellular senescence acting as a barrier to cell transformation. Recently, Sun et al (Sun, Yoshizuka et al. 2007) identified one of key component of a Ras induced senescence pathway that prevents tumorigenesis in a mouse model. They show that the p38-regulated/activated protein kinase (PRAK) induces senescence

**Figure 1. Main downstream pathways mediated by Ras protein.**



downstream of oncogenic Ras by directly phosphorylating and activating the tumor-suppressor protein p53 (Yaswen and Campisi 2007). In contrast to the mice cells, inactivation of both the Rb and p53 pathways is needed for human cells to tolerate high levels of Ras activity. This additional requirement for inactivation of the Rb pathway exists despite evidence that Ras signaling leads to induction of cyclin D1 expression, which alone might be expected to inactivate Rb. Another difference in response of human and mice cells to high levels of activated H-Ras is upregulation of Arf in MEF but not in human fibroblasts (Hahn and Weinberg 2002).

#### *1.2.1.3 Ras isoforms*

There are three *Ras* genes known giving four protein products: N-Ras, H-Ras and two alternatively spliced K-Ras (4A and 4B isoforms). All members are very closely related sharing 85% amino acid sequence identity and are widely expressed, with K-Ras being ubiquitous in almost all cell types. However, although they function in a very similar way, some subtle differences between three isoforms have recently come to light. Knockout studies for example have shown that H-Ras and N-Ras, either alone or in combination, are not required for normal development in the mouse, whereas K-Ras is essential (Johnson, Greenbaum et al. 1997).

The distinct pattern of interaction with various downstream effectors is often unique to different Ras isoforms and depends to the great part on the intracellular localization of the protein. Processed K-Ras is confined primarily to the plasma membrane; however, when the polybasic region is phosphorylated or targeted by Ca2+/calmodulin, K-Ras relocalizes to other endomembrane compartments, including the Golgi, endoplasmic reticulum and mitochondria (Fivaz and Meyer 2005; Bivona, Quatela et al. 2006). In contrast, H-Ras and N-Ras constantly shuttle between the Golgi and plasma membrane as a result of a constitutive depalmitoylation/repalmitoylation cycle (Goodwin, Drake et al. 2005).

Mutations in Ras genes that render protein "locked", the GTP-bound constitutively active state have strong transforming potential. The first such mutation described was in a hot-spot codon 12 of H-Ras in urinary bladder carcinoma were guanosine was exchanged to thymidine in the middle position (Cerutti, Hussain et al. 1994). Later, other activating mutations of individual Ras isoforms and as well as preferential activation in particular malignancies proveded even more evidence on the non-interchangeable roles of Ras protein isoforms. Almost 90% of pancreatic and 50% of colon cancers have mutated K-Ras. This isoform mutations are also found preferentially in adenocarcinomas, for example cholangiocarcinomas or adenocarcinoma of the lung. Acute leukemia and the myelodysplastic syndromes often possess activated N-Ras (Bos 1989). Furthermore, N-Ras mutations occur in approximately 20% of human melanomas. H-Ras mutations in turn can be found in cutaneous squamous cell carcinomas and in squamous head and neck tumors.

### 1.2.2 Polyomavirus oncogenes

Polyomavirus oncogenes are among the first oncogenes discovered (Dawe and Law 1959). Studies on SV40 became basis for our knowledge in gene structure, transcription control and cell signaling in transformation. However most of these studies were done in rodents.

Simian virus 40 (SV40) and mouse polyoma virus (PY) are a DNA tumor viruses in the polyoma virus family. Both viruses are very similar with respect to size (5.2 kbp), genome organization, and DNA sequence. The circular genome of these viruses contains two regions of approximately equal size known as the early and late transcription units.

The early region of viruses encodes several proteins; large T and small t antigenes produced through alternative splicing and acting together in transformation by SV40; and middle T antigen that has the most prominent role in the polyoma virus infection. In the host cells the virus DNA is integrated at random positions with respect to the cellular chromosomes. The exact integration site also appears to be random with respect to viral DNA. However, integration in all transformed cell lines is such that the early promoter and T antigen coding sequences are intact, thus ensuring continuous T antigen expression.

Rodent cells are non permissive for SV40 productive infection, thus no progeny virions are produced when cells are infected. However, viral attachment, penetration and uncoating proceed normally in these cells and the early viral proteins are expressed. Infection is blocked because viral DNA replication and late gene expression do not occur. However, a few days after infection the entire cell population acquires transformed morphology. This effect is termed abortive transformation, because the phenotype lasts for a few days before cells resume a normal untransformed appearance (Yaniv 2009). Stable transformants, about one cell in every thousand emerge from this population and are easily detected because they overgrow the monolayer and form dense foci.

#### *1.2.2.1 Large T antigen*

The Large T antigen is a 708 residue protein with four structural domains: a J domain at the amino-terminus, a sequence specific DNA binding domain (OBD), a zinc binding domain, and an ATPase domain (Li, Zhao et al. 2003). LT transforms cells and induces tumors in animals by altering the functions of tumor suppressors (pRb and p53) and other key cellular proteins. LT is also a molecular machine that melts the replication origin of the viral genome and unwinds duplex DNA.

The current model is that T antigen must block the growth-suppressive functions of all three Rb proteins, pRb, p107 and p130 in order to induce transformation. LT interacts with Rb family proteins through its conserved LXCXE motif that lies downstream of the J-domain (Dyson, Bernards et al. 1990). The next region involved in Rb suppression and essential for transformation is the J amino-terminal domain of LT that it shares with small t antigen. This region recruits cellular hsc70 chaperone protein so that energy

derived from hsc70-mediated ATP hydrolysis can be used to disrupt the association of Rb proteins with E2F transcription factors (Sullivan, Gilbert et al. 2001).
In many cell-types, stimulation of S phase after Rb protein inactivation results in the activation of the p53 pathway. This is prevented by direct interaction of the ATPase domain of LT with p53 and block of DNA binding by p53, so that it cannot associate with target promoters (Gjoerup, Chao et al. 2000). As a consequence of its interaction with T antigen, p53 is stabilized and SV40-transformed cells contain large amounts of T antigen–p53 complexes. Apparently T antigen also can block p53-dependent transcription and growth-arrest by mechanisms that are independent of direct association (Pipas and Levine 2001).
However while the association of T antigen with p53 and Rb is necessary for transformation, it is not sufficient in human cells. Recently interactions with CBP/p300, TEF-1, Cul7, and Bub1 have been also implicated in transformation (Pipas 2009).

#### *1.2.2.2 Small t antigen*

Next product of the early region, 174 amino acid long small t antigen (ST) is also an important player in the transformation of human cells. Its J-domain at amino-terminus which is similar to large T antigen, is followed by a uniquely structured C-terminal domain that interacts with the cellular protein phosphatase 2A, (PP2A). Observations indicate that in some circumstances N-terminal along with C-terminal sequences are both required to transform cells (Colby and Shenk 1982). J-domain of ST protein also cooperates in inactivation of Rb and p53 in human cells through mechanisms that are distinct from those made by LT. When complemented with human adenovirus E1A it can alone become transforming (Yu, Boyapati et al. 2001; Hahn, Dessain et al. 2002; Hahn and Weinberg 2002). However main tumorigenic activity of ST is dependent on its interaction with PP2A (Chen and Hahn 2003).
PP2A is a member of the family of heterotrimeric enzymes accounting for the majority of total Ser/Thr phosphatase activity in cells. PP2A is composed of a structural A subunit, a catalytic C subunit and regulatory B subunit. It has been determined that the ST binds to the same region of structural subunit A as the regulatory subunit B, displacing it and interfering with its function (Cho, Morrone et al. 2007).

#### *1.2.2.3 Middle T antigen*

Polyoma middle T antigen (MT), a 421 amino acids protein is transcribed from an extra reading frame in the early region of the virus. Since all T antigens are produced by differential splicing of common early transcripts, MT shares 79 amino acids that represent a J-domain with both LT and ST, as well as an additional 112 amino acids with ST. A stretch of 22 hydrophobic residues represents a membrane anchor sequence at the C-terminus.
MT readily transforms many established cells to a fully tumorigenic phenotype. However unlike LT and ST it cannot overcome the senescent

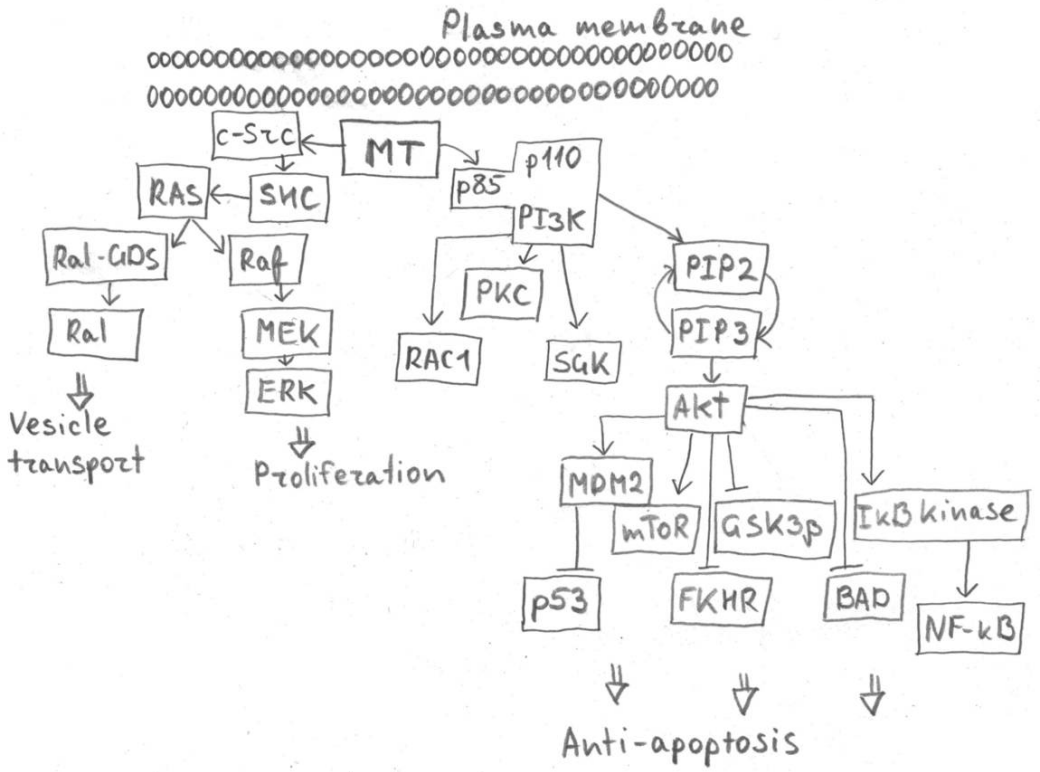



**Figure 2. Polyoma middle T antigen assembles large signaling complexes at the plasma membrane and resembles activated tyrosine kinase receptor.**

properties of primary cells (Treisman, Novak et al. 1981). The ability of MT to transform depends on its association with membranes (Carmichael, Schaffhausen et al. 1982) where it interacts with a number of the proteins used by receptor tyrosine kinases to stimulate mitogenesis. Therefore MT can be considered as a permanently active analogue of a receptor.

MT has no known catalytic activity, but functions as a scaffold for signaling proteins that assemble on it and become activated (Fig. 2). It binds the A and C subunits of PP2A and, using both a motif on MT and a portion of the PP2A further binds protein tyrosine kinases of the Src family (Src, Yes, Fyn). In this complex, MT is phosphorylated on three major tyrosine residues each representing a connection to a signal generator: 315 - to PI3K and one or more additional interacting proteins; 250 - to Shc and further Grb2 and SOS; and 322 - to PLCγ1. However, additional minor tyrosine phosphorylation sites may also contribute to MT function. Finally, serine phosphorylation at 257, which controls association with the 14-3-3 family, affects tumorigenic ability of MT.

In transformation signaling induced by MT there is a large degree of cross talk between the ShcA/MAPK and the PI3K pathways. Both pathways appear to be initiated as separate entities but interact further downstream. Interestingly, sites for binding both ShcA and PI3K have to be on the same molecule to function (Ichaso and Dilworth 2001).

### 1.2.3 Src

The Src family kinases consist of at least nine approximately 60 kDa proteins with similar structure and functions - c-Src, Fyn, c-Yes, Fgr, Lyn, Hck, Lck, Blk, and Yrk. Some of them, such as Src, Fyn and Yes are ubiquitous while others are tissue specific.
Src is either over expressed or activated in many human cancers including breast, colorectal, and hepatocellular cancers where it contributes to anchorage-independent survival by mediating the mitogenic effects of tyrosine kinase receptors. It also modulates focal adhesion components through tyrosine phosphorylation of focal adhesion substrates, especially focal adhesion kinase (FAK). It is known that Src directly forms a signaling complex with FAK, thus activating downstream MAPK or PI3K signaling (Johnson, Agochiya et al. 2000)

#### *1.2.3.1 Cellular Src*

Cellular Src (c-Src) is a non-receptor tyrosine kinase. It is ubiquitously expressed but especially abundant in platelets, neural tissue, and osteoclasts where it is implicated in multiple pathways that regulate cell growth, migration, and survival. Its activity increases in response to a number of signals, especially downstream to tyrosine kinase growth factor receptors, G-protein coupled receptors, integrin receptors for adhesion molecules, and cytokine receptors (Rucci, Susa et al. 2008).
The main functional entities of c-Src include the SH4 domain which allows binding of c-Src to the plasma membrane; the SH3 domain mediating protein-protein interactions by binding to proline-rich sequences; the SH2 domain, involved in phosphotyrosine-mediated interactions; catalytic SH1 domain, the most conserved domain in all tyrosine kinases, which contains the ATP-binding pocket; and COOH-terminal tail that upon phosphorylation can bind c-Srcs SH2 domain (Roskoski 2004).
Two major tyrosine phosphorylation sites regulate activity of c-Src: Tyr(416/419), a negative regulatory site and Tyr(527/530), positive regulator necessary for the full c-Src kinase activity.
In the cells c-Src is normally maintained in an inactive or "closed" conformation where the SH2 domain is engaged with the phosphorylated Tyr527/530. In the active state phosphorylated Tyr416/419 is displaced from the substrate-binding pocket, giving the kinase access to substrates. Two tyrosine kinases, named c-Src kinase (CSK) and CSK homologous kinase negatively regulate c-Src activity by phosphorylating Tyr527/530.
c-Src plays an important role in the genesis and progression of human cancers, including carcinomas of the breast, colon, prostate, lung and ovary, and in myeloproliferative disorders. It acts through downstream cascades to support proliferation and migration. In colorectal carcinoma c-Src activity can predict poor prognosis (Aligayer, Boyd et al. 2002). Also, formation of the complex of prostaglandin E/β-arrestin1/c-Src is a crucial step in PGE2-mediated transactivation of the epidermal growth factor receptor (EGFR) and metastatic spread of colorectal carcinoma *in vivo* (Buchanan, Gorden et al. 2006).

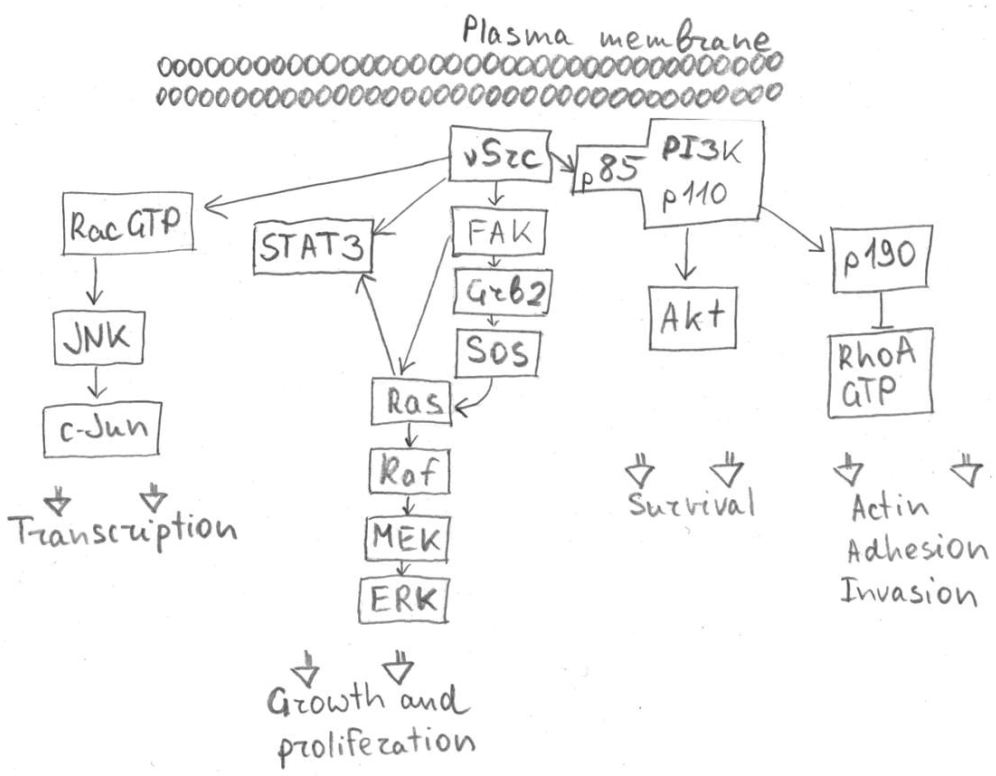



**Figure 3. Major v-Src pathways that control cellular transformation.**

*1.2.3.2 Viral Src*

The oncogene responsible for the transforming activity of Rous sarcoma virus, v-Src is a viral counterpart of the cellular Src and differs from it by substitution of sequences at the C-terminus, which results in loss of amino acids that normally bind to the SH domains and stabilize the "closed" conformation of the molecule (Frame 2002). Other alterations, such as those in the SH3 domain, also contribute to activity of this oncogene.

v-Src induces many in vitro and in vivo parameters of oncogenic growth including loss of contact-inhibition, increased growth factor- and anchorage-independence, decreased anoikis, and the ability to induce tumors in animal models. This v-Src-induced transformation is typically characterized by reorganization of the actin cytoskeleton, loss of F-actin stress fibers and typical focal adhesion complexes, decreased adhesion to ECM and in cell–cell interactions, and an increase in cell motility.

Intracellular signals required for transformation by v-Src are mediated by Raf-1 (Fig 3). Expression of the Raf-1 mutant blocked v-Src-induced transformation, as determined by reversion to a flat non-transformed morphology and the inability to form colonies in soft agar into BALB/c 3T3 cells (Qureshi, Joseph et al. 1993). More recently Src phosphorylation of Akt also has been shown to be required for Akt activation (Chen, Kim et al. 2001; Conus, Hannan et al. 2002; Jiang and Qiu 2003).

v-Src-transformed fibroblasts may also display constitutively altered cell-cycle control, with elevated expression of cyclin D, cyclin A and cyclin-dependent kinase 2, hyperphosphorylation of the pRb and stimulation G1/S-phase progression (Riley, Carragher et al. 2001). v-Src is critical to the cells balance between proliferation and death, particularly when serum survival factors are limiting. It can both induce apoptosis by a 53-independent mechanism and protect against it under low-serum conditions (Frame 2002).
Based on studies using dominant-interfering or constitutively active alleles, or selectively inhibitory drugs it is known that activation of three major signaling pathways contributes to v-Src induced oncogenic transformation, Ras-Raf-MEK-MAPK, PI3K, and STAT3.

## 1.3 IGF FAMILY

The Insulin-like Growth Factor (IGF) family includes three ligands, three cell membrane receptors, six binding proteins, five known adaptors and a number of associated proteins like insulin-receptor related receptor IRR, IGFBP-related proteins and IGFBP proteases.
Two isoforms of insulin receptor, IR-A and IR-B are structurally closely related to the IGF-1R. They control cell metabolism and have also been implicated in cancer cell growth. In addition, hybrid receptors (Pandini, Vigneri et al. 1999) assembled with one chain of IGF-1R and one chain of insulin receptor also exist. Here we discuss only the main components and their activity regulation in the body.

### 1.3.1 Insulin-like Growth Peptides I and II

Insulin-like growth factors (IGFs) are an important growth factors. Signaling primarily through the IGF-1R they are involved in development and cancer. IGFs are produced by the liver and their expression is stimulated by growth hormone (GH). Other organs can also produce IGFs in an autocrine and paracrine manner. It is interesting that the circulating concentration of IGF-I is considerably lower than that of IGF-II (20 and 90 nmol/l respectively).
The mature IGF-I and IGF-II peptides consist of B and A domains that are homologous to the B and A chains of insulin. The IGF-I and IGF-II pro-hormones contain also a C-terminal E peptide that is cleaved in the Golgi apparatus during secretion. Mature IGF-I and IGF-II are linked by C domains and contain short D domain (Daughaday and Rotwein 1989). IGF-I shares over 75% sequence identity with IGF-II and they are structurally very similar. However, IGF-I is unable to bind to the IGF-2R.
Mature IGF-I consists of 70 amino acid residues (7.65 kDa). It is a major mediator of growth hormone effects but is also known for its role in oncogenic cellular signaling. Most IGF-I knockout mice die after birth, although some animals surviving until adulthood. Their phenotype is associated with infertility, delayed ossification and reduced muscle development.
In humans mutations of the IGFs have been described and lead to growth retardation, microcephaly and neurodevelopmental delay (Miller and Yee

2005). In patients with prostate cancer the 19-CA-repeat allele of IGF-I was found to be more frequent than in controls and might be a novel predictor in prostate cancer patients with bone metastasis (Tsuchiya, Wang et al. 2005). Another two single nucleotide polymorphisms have been shown to have significant associations with prostate cancer risk, and haplotype analysis has demonstrated an association between certain haplotypes and the risk of developing cancer (Cheng, Stram et al. 2006).
Mature IGF-II is a single-chain polypeptide consisting of 67 aminoacids (7.47 kDa). It is synthesized as a 180 amino acid pre-hormone with a 24 amino acid signal peptide at the N-terminal end and a C-terminal extension of 89 aminoacids called the E-domain. During intracellular processing the signal peptide and the E-domain are cleaved in several steps from the precursor protein, resulting in mature IGF-II (Duguay, Jin et al. 1998). Due to incomplete processing of pro-IGF-II, larger forms of IGF-II can be formed that still contain 21 amino acids of the E domain. These forms of incompletely processed IGF-II are called "big"-IGF-II and constitute <10% of total human serum IGF-II.
The gene encoding IGF-II is maternally imprinted. However, some malignancies especially sarcomas, secrete large amounts of "big"-IGF-II, resulting in high circulating levels of this protein with associated hypoglycemia. Loss of imprinting here might represent mechanism leading to overexpression in neoplastic tissue (Kaneda, Wang et al. 2007). The idea that IGF-II represents growth and survival advantages and is selected during neoplastic progression is further confirmed by evidence that IGF-II is the single most overexpressed gene in colorectal neoplasia (Zhang, Zhou et al. 1997) relative to normal colorectal mucosa.

### 1.3.2 Insulin-like Growth Factor-1 receptor

Insulin-like Growth Factor Receptor type 1 (IGF-1R) is a transmembrane tyrosine kinase receptor. It is a tetramer composed of two α and two β covalently linked polypeptide chains. Activated IGF-1R recruits and phosphorylates adaptor proteins which then serve as docking sites for other signaling molecules. This results in the activation of the downstream pathways such as phosphatidylinositol 3-kinase, mitogen-activated protein kinase (MAPK) and 14-3-3 mediated pathways.
Knockdown of the *IGF-1R* gene causes a 50% reduction in the size of mouse embryos. Both the size and the number of cells are reduced. Thus, it seems reasonable to conclude that the IGF-1R controls about 50% of cell and body growth (Renato Baserga 2003). In addition IGF-1R mediates proliferation and apoptosis protection in cancer cells.

### 1.3.3 Insulin like Growth Factor-2 receptor

Insulin-like Growth Factor Receptor type 2 (IGF-2R) or the cation-independent mannose-6-phosphate is structurally unrelated to IGF-1R. It is a single-chain polypeptide approximately 300 kDa with a repetitive extracellular domain (15 units) and a short intracellular tail. IGF-2R transports ligands from the Golgi to the pre-lysosomal compartment and thereafter to and from the cell surface.

Receptor binds IGF-II but lacks tyrosine kinase activity and does not transduce signals. In frogs and chicken it is exclusively mannose 6-phosphate receptor (CI-MPR) which is involved in the trafficking of lysosomal enzymes, while in mammals it also mediates turnover of IGF-II via receptor-mediated endocytosis. IGF-2R regulates growth, placental development, tumor suppression and signaling.
Loss of functional IGF-2R allows for enhanced interaction of IGF-II with IGF-1R. Mouse mutants inheriting maternally a targeted disruption of the imprinted *IGF-2R* gene, which is normally expressed only from the maternal allele, have increased serum and tissue levels of IGF-II and exhibit overgrowth (135% of normal birth weight) and generalized organomegaly, postaxial polydactyly, heart abnormalities, and edema. These mutants usually die perinatally, but a small minority can survive depending on genetic background. Overstimulation can be avoided in the double knockout animals (*IGF-1R/IGF-2R* double mutants) in which embryonic development is normal and animals differ from wild-type siblings only in the pattern of postnatal growth (Kim and Accili 2002). Loss of heterozygosity of IGF-2R has been shown in breast cancer.

### 1.3.4 System regulation - binding proteins and proteases

IGFs differ from many other similar regulatory peptides in that they regulate physiology at both the whole organism level and at the cellular level. They have properties of tissue growth factors, but also have additional well-recognized functions as hormones that regulate growth and energy metabolism at the whole organism level. Two sources of the IGFs in tissues including malignancy are known: they can be produced in the liver under dominant control of growth hormone and delivered through the circulation; or they can also be produced locally in tissues acting in autocrine and paracrine manners.
In humans, the effect of IGFs is also controlled by the amount of free peptides available for interaction with receptor. Normally about 99% of IGFs is bound to the IGF-binding proteins (IFGBPs) 1-6 which have high affinity for both IGF-I and IGF-II and less than 1 % is free. In addition, IGFBP regulate cell activity in various ways. By sequestering IGFs away from the IGF-1 receptor, they may inhibit IGF-stimulated events. Alternatively, IGFBP interaction with cell or matrix components may concentrate IGFs near their receptor, enhancing IGF activity. The tumor suppressor p53, as well as many growth inhibitors including vitamin D, anti-oestrogens, retinoids, and transforming growth factor-β reduce IGF bioactivity by increasing the secretion of IGFBP (Pollak 2008). IGFBP proteolysis can reverse this inhibition or generate IGFBP fragments with novel bioactivity.
IGF receptor independent actions of IGFBP are also increasingly recognized. IGFBP-1 interacts with αVβ1 integrin, influencing cell adhesion and migration. IGFBPs 2-6 have heparin-binding domains and bind glycosaminoglycans. IGFBP-3 and -5 have carboxyl-terminal basic motifs incorporating heparin-binding and additional basic residues that interact with the cell surface and

matrix, the nuclear transporter importin-β, and other proteins. IGFBP-3 binds and modulates the retinoid X receptor-α, interacts with TGF signaling through Smad proteins, and influences other signaling pathways (Firth and Baxter 2002).

## 1.4 IGF-1 RECEPTOR

### 1.4.1 Structure

IGF-1R is a transmembrane receptor protein composed of two extracellular α-subunits and two transmembrane β-subunits. Complete primary structure of the human IGF-1R from cloned cDNA was determined more then twenty years ago (Ullrich, Gray et al. 1986). The sequence includes single 1367 amino acid receptor precursor and a 30-residue signal peptide, which is removed during translocation of the nascent polypeptide chain. The receptor coding sequence is located within single *IGF-1R* gene and contains 21 exons (Abbott, Bueno et al. 1992).

The IGF-1R (180 000 $M_r$) modified polypeptide precursor is cleaved at position 707 (the Arg-Lys-Arg-Arg sequence, coded within exon 11) and generates one α- (glycosylated - 135000, unmodified - 80423 $M_r$) and one β- subunit (glycosylated - 90000, unmodified - 70866 $M_r$). IGF-1R is then transported to the membrane fully assembled in the dimeric form where it is organized in heterotetramer.

The ligand binding pockets of the IGF-1R are formed by extracellular α subunit and possibly some extracellular portions of the β-subunit. Receptor complex spans plasma membrane via two β-subunit membrane spanning domains leaving 195 amino acid portions of the β-subunit protruding from the cell surface to which extracellular α subunits are attached by disulfide bonds. β-subunit of the IGF-1R possess tyrosine kinase activity.

Only few reports on mutations in IGF-1R in humans (Ellen W. Roback 1991), often associated with growth inhibition are known: Arg108Gln/Lys115Asn substitution located in the leucine-rich repeats 1 (L1) region of the IGF-1R, causes changes in the amino acid charge and leads to reduced ligand-receptor binding (Abuzzahab, Schneider et al. 2003). Arg709Gln mutation at the cleavage site of the IGF-1R precursor affects the processing of pro-receptor (Kawashima, Kanzaki et al. 2005). Arginine481 substituted by glutamine, Arg481Gln (Inagaki, Tiulpakov et al. 2007) and Glu1050Lys substitution in the intracellular kinase domain (Walenkamp, van der Kamp et al. 2006) both cause reduction of receptor phosphorylation and downstream signaling. Arg59stop codon causes a truncated IGF-1R (Raile, Klammt et al. 2006).

Several mutations in the β-subunit of IGF-1R have been developed in the laboratory providing significant information on the different domains and single aminoacid's function. The tyrosine Y1136 is important for stabilization of kinase activity (Favelyukis, Till et al. 2001), since a point mutation at this site (Y1136F) leads to decreased kinase activity. The lysine K1003 point mutation restricts the ATP binding at this site and leads to disruption of autophosphorylation (Sperandio, Poksay et al. 2004). Constructs with a single

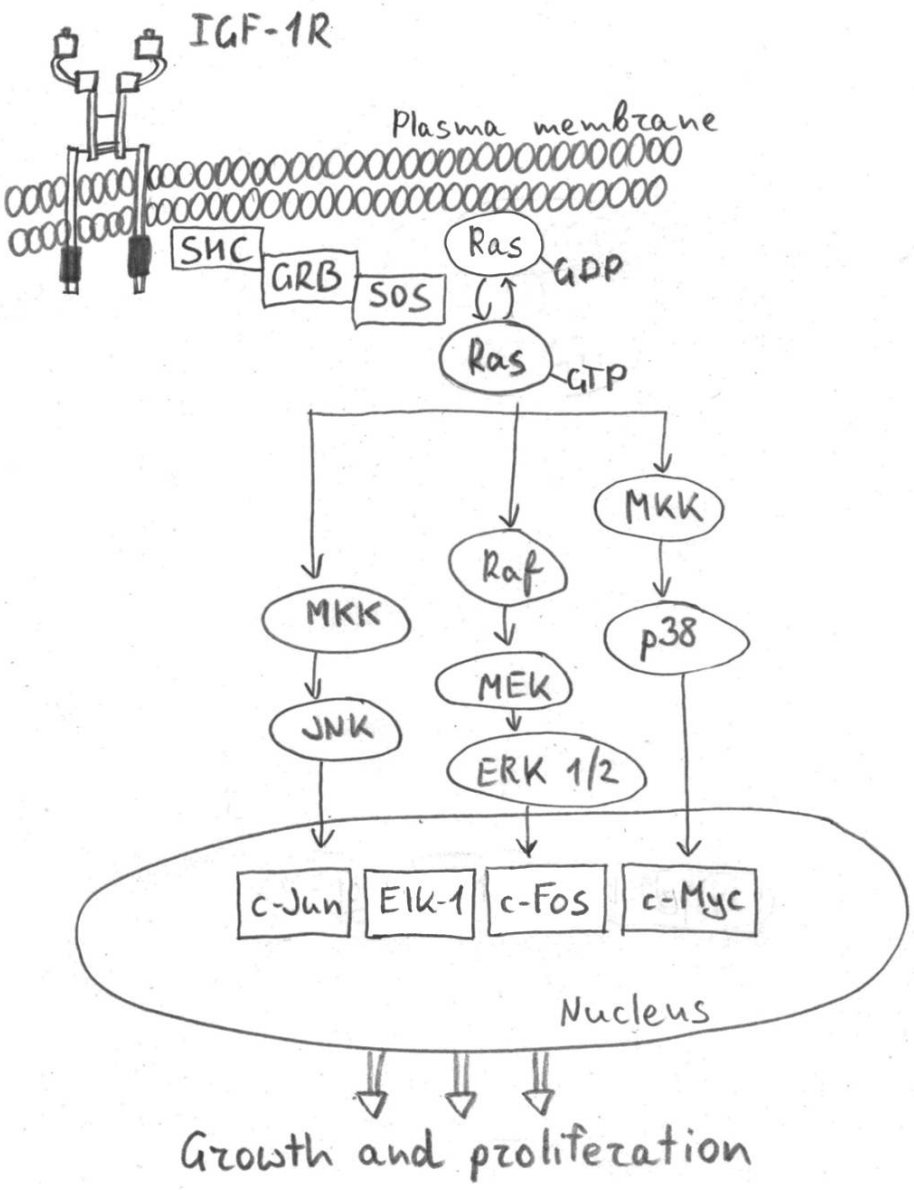



**Figure 4. IGF-1R induced mitogen-activated protein kinase cascade mediated through Shc**

mutation at Y950 site (Y950F) leads to impaired signaling since Y950 is important for binding of IRS-1 and Shc (Yu, Watanabe et al. 2003). The C-tail truncated IGF-1R without the last 92 amino acids (Δ1245) is also significant for phosphorylation and signaling (Furlanetto, Dey et al. 1997; Chow, Condorelli et al. 1998). Finally the Y950F+Δ1245 construct lacks both the C-terminal domain and also has impaired IRS-1/Shc binding.

### 1.4.2 Receptor activation and signaling

IGF-I or IGF-II ligand binding to α-subunit of the IGF-1R results in a conformational change leading to transphosphorylation of the activation loop of Tyr1131, Tyr1135 and Tyr1136 of one β-subunit by the other. As a result of

this phosphorylation conformational changes occur in the β-subunit allowing access
of the substrate and ATP to the tyrosine kinase site (Favelyukis, Till et al. 2001). Activation of IGF-1R results in phosphorylation of adaptor proteins and initiation of the receptor downstream cascade. There are several main pathways in IGF-1R downstream signaling; however most of them can contribute to each other at various levels and crosstalk to other pathways in the cell. Major known immediate downstream signaling adaptors for the IGF-1R are IRS1-4 and Shc.
Engagement and activation of the mitogen-activated protein kinase (MAPK) cascade through Shc is essential for receptor mediated signaling (Fig. 4). Activation of extracellular signal–regulated kinase (ERK1/2) of the MAPK cascade via the growth factor receptor binding protein 2 (Grb2)/Sos/Ras/Raf/MEK pathway. Activation of ERK, p38, and JNK, results in the transcription of genes that drive proliferation. This pathway can also mediate differentiation, in the context of unopposed Shc binding to the IGF-1R in cells lacking IRS-1.
Following phosphorylation by the activated IGF-1R, IRS proteins bind to the p110 subunit of PI3K, leading to the generation of PIP3 and phosphorylation of Akt by phosphoinositide dependent kinase. Phosphorylation of Akt leads to subsequent activation of mammalian target of rapamycin (mTOR), eukaryotic translation initiation factor 4E, and p70S6 kinase. Activation of these downstream signaling pathways leads to enhanced proliferation, survival, and metastasis in cancer cells (Sachdev and Yee 2007). The activated Akt induces also inhibitory phosphorylation of pro-apoptotic factors like Bcl-2 family member Bad, members of the forkhead transcription factor family and caspase 9 (Baserga, Hongo et al. 1997). In addition, AKT activation leads to increased expression of anti-apoptotic proteins, including Bcl-2, Bcl-x and NF-κB (Brazil, Yang et al. 2004).
Alternative pathway for protection from apoptosis is represented by 14-3-3 (Peruzzi, Prisco et al. 1999). This protein is activated by IRS-1 or independently, by direct interaction with IGF-1R at serine residues located in the C terminus, between positions 1272 and 1284. This later interaction depends on the phosphorylation of the appropriate serines. This pathway induces activation of Raf-1 and its translocation to the mitochondria, and also phosphorylation and inhibition of Bad.

### 1.4.3 Signal termination and role of modifications

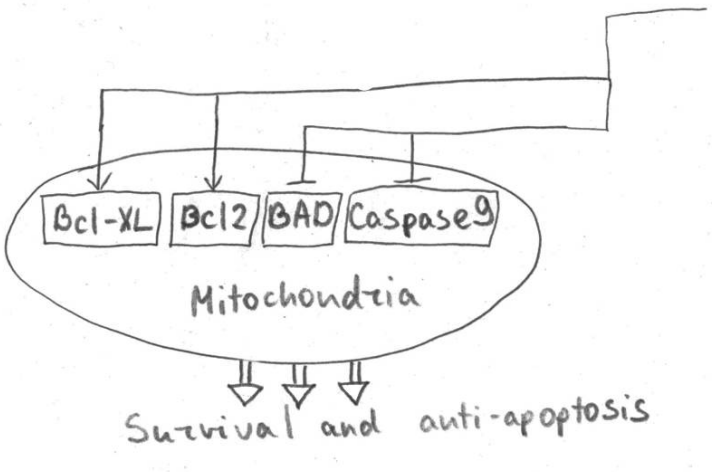
For many years, it was thought that signaling from receptor occurs only at the plasma membrane and is mediated by a simple, linear pathway (McKay and Morrison). However, it is now known that receptor downregulation via internalization not only terminates its signaling but is an important part in regulation of the downstream cascades. For example MAPK/ERK signaling trough Ras can be activated at various intracellular compartments and IGF-1R can modulate it from these sites. Moreover, ERK scaffolding proteins and

signaling modulators have been identified that play critical roles in determining the strength, duration and location of this ERK signaling.
Recent data suggest that ubiquitination is crucial in mediating both IGF-1R signaling and degradation. β-arrestin1, adaptor protein for GPCR has been shown to bring MDM2, ubiqitin E3 ligase to activated receptor. Work from our group demonstrates that receptor stimulation also leads to ubiquitination of β-arrestin1, which regulates further receptor vesicular trafficking and activation of ERK1/2. In the absence of β-arrestin1, ligand-stimulated IGF-IR activation of ERK is eliminated. Interestingly, this β-arrestin1-dependent ERK activity can occur even when the classical tyrosine kinase signaling is impaired (Girnita, Shenoy et al. 2007). Both IGF-1R autophosphorylation and C-terminal domains are required for ubiquitination and ERK activation and are completely abolished in C-terminal truncated mutants (Sehat, Andersson et al. 2007). At the same time kinase impaired IGF-1R can both be ubiquitinated and induce ERK phosphorylation but fails to activate Akt. Further, receptor destruction is also dependent on signaling context. Mutants with impaired PI3K/Akt signaling are degraded mainly by the proteasomes, while C-terminal truncated receptor is recycled through the lysosomal pathway.
Additionally, a novel E3 ligase, c-Cbl is found for IGF-1R. Depending on the IGF-1 dose used to stimulate the receptor, both Mdm2 (low ligand concentration 5 ng/mL) and c-Cbl (high ligand concentrations 50–100 ng/mL) mediate receptor polyubiquitination (Sehat, Andersson et al. 2008). Mdm2-ubiquitinated IGF-1R is then internalized through the clathrin endocytic pathway whereas c-Cbl-ubiquitinated receptors are endocytosed via the caveolin route. Taken together, these results show that different adaptor proteins and ligases complement each other in fine-tuned control of the IGF-1R signaling initiation and termination in response to ligand stimulation.

### 1.4.4 Relation to insulin receptor

IGF-1R and insulin receptor (IR) are both tyrosine kinase receptors. They share similar structure (84% in the intracellular β-chain and 47-67% homology in the extracellular α-chain) and some of the downstream signaling effectors but their physiological roles are different (Werner, Weinstein et al. 2008). Insulin receptor is mainly involved in metabolism signaling whereas IGF-1R controls cell growth, proliferation or differentiation depending on cell context.

Two isoforms of IR are generated by alternative splicing of exon 11, giving rise to the B-isoform (IR-B) and A-isoform (IR-A), which lacks the 12 amino acids due to exclusion of exon 11. The two isoforms are expressed in a developmentally specific manner with IR-A highly expressed in fetal tissue and IR-B found mostly in an adult tissues. IGF-II binds IR-A with high affinity whereas IGF-I does not (Frasca, Pandini et al. 1999). Both IGF-I and IGF-II can bind to IR-B at high concentrations; however insulin is the main ligand for IR and IGF-I for IGF-1R.
Similar to IGF-1R activation of IR leads to phosphorylation of IRS adaptor proteins and activation of downstream PI3K and MAPK. Evidence from gene deletion studies suggests that the functions of IR and IGF-1R, although




physiologically distinct, are partially overlapped. Depending on context, IR can stimulate growth (Ludwig, Eggenschwiler et al. 1996) and IGF-1R is able to regulate a metabolic response (Cola, Cool et al. 1997). It is believed that, in addition to intrinsic signaling differences, tissue distribution, relative number of cell-surface receptors and developmental regulation also determine the specificity of signaling via IR compared tp IGF-1R (Kim and Accili 2002).

### 1.4.5 Interaction with other signaling pathways

Besides insulin receptor, IGF-1R can interact with several other receptor systems. Epidermal Growth Factor Receptor (EGFR) appears to be induced by activation of the IGF-1R. *Vice versa*, IGF-1R requires EGFR activity for downstream activation of ERK. Receptors can interact indirectly by releasing EGFR ligand or directly through dimerization of EGFR and IGF-1R subunits (Kuribayashi, Kataoka et al. 2004).
Synergy between estrogen and IGF-I has been seen in both normal and cancerous breast and uterus. In breast cancer cells, IGF-I can increase transcriptional activity of the estrogen receptor (ER) through the expression of progesterone receptor or directly in the absence of estradiol. Estrogen in turn can activate the growth stimulatory properties of the IGF pathway. Blockade of ER function can inhibit IGF-mediated mitogenesis and blocking IGF action can

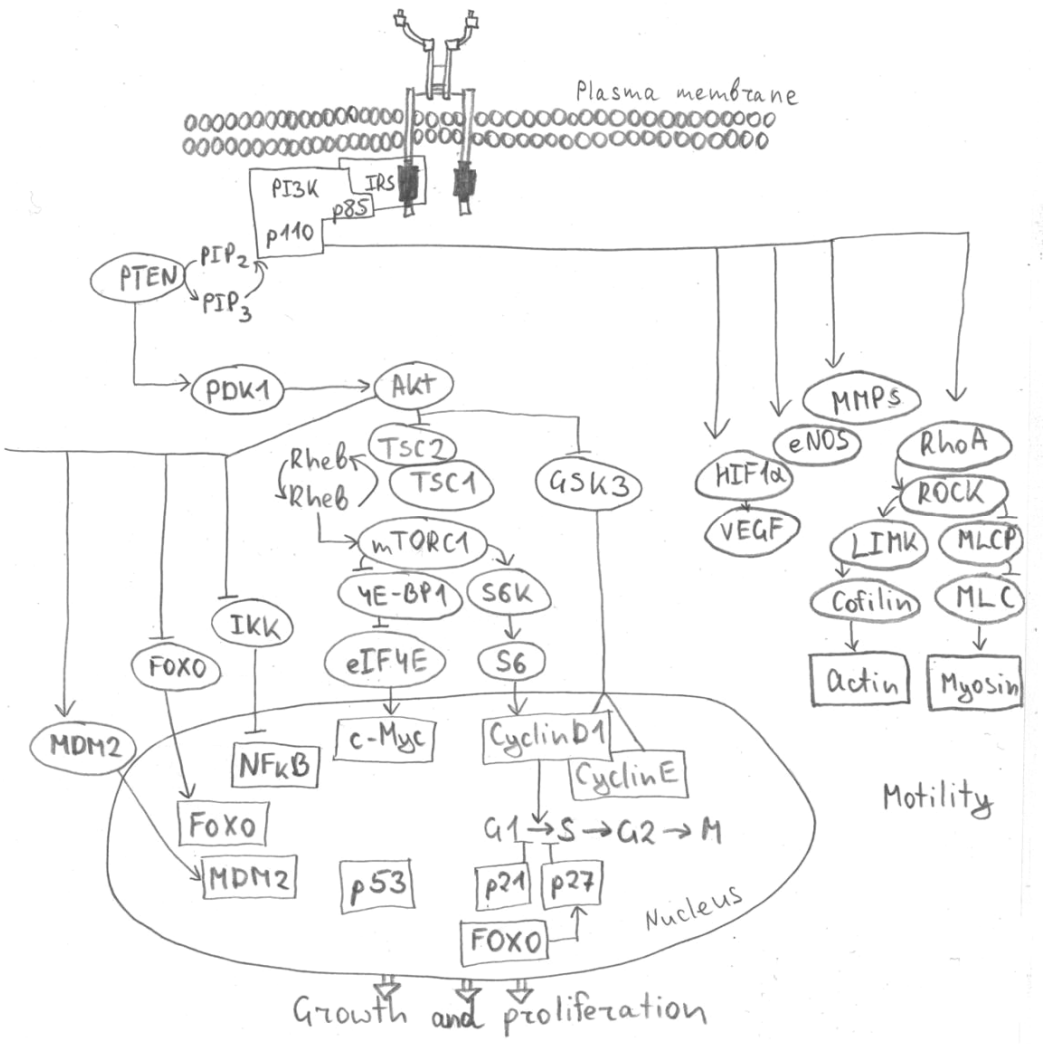



**Figure 5.** IGF-1R induced PI3K pathway mediated through IRS

inhibit estrogen stimulation of breast cancer cells (Fagan and Yee 2008). IGF-1R has also been shown to alter androgen receptor activity in prostate cancer (Wu, Haugk et al. 2006).

It is well established that the interaction between IGF-1R and HER2/neu. In human breast cancer models increased level of IGF-1R signaling interferes with the action of Trastuzumab (Herceptin), an anti-HER2/neu receptor monoclonal antibody. Addition of IGF-binding protein-3 decreases receptor signaling and restores trastuzumab-induced growth inhibition (Lu, Zi et al. 2001).

Another system that IGF-1R interacts with is the vascular endothelial growth factor (VEGF). VEGF secretion is regulated by IGF-I directly and through downstream ERK and Akt cascades that both increase expression of VEGF (Slomiany, Black et al. 2006). Interaction between IGF-1R and platelet derived growth factor was also seen in human diploid fibroblasts were PDGF was demonstrated to increase the number of IGF-I binding sites (Carlberg and Larsson 1996).

In RACK1 over expressing cells, while IGF-I-induced activation of IRS-1 other signaling pathways including IRS-1, Shc, PI3K, and MAPK are unaffected, IGF-I-inducible β1 integrin-associated kinase activity and association of Crk with p130CAS are significantly inhibited. Further, delayed cell cycle progression in G1 or G1/S are correlated with Rb hypophosphorylation, increased levels of p21Cip1/WAF1 and p27Kip1, and reduced IGF-I-inducible Cdk2 activity (Hermanto, Zong et al. 2002).

### 1.4.6 IGF-1R and cancer

It has been mentioned that large body size and high height are both associated with an increased risk of breast cancer in postmenopausal women. Taller people also have an increased risk of colon cancer (Micozzi 1993; Monson and Wals 1996).

Carcinogenic effect of tall stature in humans is hypothetically related to an increased exposure of certain individuals to growth factors, an excess of which is considered to have a role in cancer development (Giovannucci 2001). In accordance with this hypothesis is also the assumption that growth hormone deficiency and suppression of peripheral IGF-I levels have key functions in the significantly lower risk for neoplasia in Ames dwarf mice (Ikeno, Bronson et al. 2003). In humans increased circulating levels of insulin-like growth factor I are associated with increased risk of breast, colon, and prostate cancers (Sachdev and Yee 2007). The IGF-1R has been implicated in several different cancers including breast, prostate, colon, liver, pancreatic cancer, melanoma, multiple myeloma, mesothelioma, glioblastoma, and childhood malignancies (Tao, Pinzi et al. 2007). Cellular transformation and progression of several types of sarcoma, including rhabdomyosarcoma, synovial sarcoma, leiomyosarcoma, Ewing's sarcoma and osteosarcoma are influenced by IGF-1R (Rikhof, de Jong et al. 2009). In Ewing's sarcoma humanized monoclonal antibodies directed against IGF-1 receptor induced objective tumor responses.

In contrast to many other receptors involved in transformation, gene amplification associated with substantial overexpression and ligand-independent activation is uncommon for the IGF-1R. However there are numerous reports of genetic polymorphisms in genes encoding either IGF1 or IGFBP-3 proteins (Tao, Pinzi et al. 2007).

Early *in vitro* experiments demonstrated importance of IGF-1R in breast cancer cells and dose-dependent increases in neoplastic cell proliferation (Myal, Shiu et al. 1984). Later on transforming action of many oncogenes was discovered to require IGF-1R signaling. Simian virus 40 large T antigen (Sell, Rubini et al. 1993) and/or activated Ha-Ras expressed from a stably transfected plasmid (Sell, Dumenil et al. 1994), bovine papillomavirus E5 (Morrione, DeAngelis et al. 1995), EGFR, IR or PDFGR were unable to transform cells lacking IGF-1R (Baserga, Hongo et al. 1997). The only two proteins are known so far to sustain oncogenic properties in the absence of IGF-1R - v-Src (Valentinis, Morrione et al. 1997) and constitutively active GTPase-deficient mutant $G_{\alpha 13}$ (Liu, Blakesley et al. 1997). However, even in these later cases

co-expression of the IGF-1R has a synergistic effect on cell growth and transformation.
Recently several downstream effector proteins for IGF-1R have also been implicated in malignancy. IRS-1's role in cell transformation is quite prominent. The literature strongly suggests that IRS-1 should be considered a biomaker for cancers susceptible to IGF-1R targeting. In addition, IRS-1 may have a more general role in cancer, and could be considered as a protein having the opposite effect of tumor suppressors (Baserga 2009).

### 1.4.7 Targeting the IGF-I receptor in cancer

IGF-1R signaling can be targeted at multiple levels. However among different strategies three main approaches can be identified: interaction with the production or availability of ligand, inhibition of receptor function using receptor-specific antibodies and small-molecule tyrosine kinase inhibitors.
First group includes ligand-targeting strategies to reduce circulating IGF-I levels. Among them attempts using somatostatin analogues were unsuccessful since the desired suppression of ligand levels was not achieved (Pollak 2008). Pegvisomant, growth hormone receptor antagonist has been shown to cause regression of MCF-7 xenografts (Divisova, Kuiatse et al. 2006) and meningiomas (McCutcheon, Flyvbjerg et al. 2001). Neutralising antibodies to IGFs have also been developed and successfully used in experimental models; the IGF-1 neutralising antibody, KMI1468 dose-dependently suppressing prostate cancer cells in animals.
However, at present time most research is focusing on the second group - interference with IGF-1R and its downstream signaling. Receptor-specific antibodies at this point are the best evaluated treatment with many of them being in phase II clinical trials for oncological indications in various combinations with approved agents. Extensive clinical experience has been reported with antibody CP-751871 (Pfizer) which caused most prominent improvement in squamous cancers (Lacy, Alsina et al. 2008). Other IGF-1R-specific antibodies showing *in vitro* and *in vivo* anti-tumor activity include AMG479 (Amgen), AVE1642 (Sanofi-Aventis), A12 (Imclone), MK0646 (Merck) and R1507 (Roche) (Haluska, Shaw et al. 2007). Many of these antibodies showed also additive effect with cytotoxic chemotherapy. Di-diabody, an antibody with dual specificity targeting both IGF-1R and EGFR has also been used to prevent association between the receptors and trigger IGF-1R internalization.
The next logical step in targeting IGF-1R is represented by the third group, small molecule tyrosine kinase inhibitors. Since IR tyrosine kinase domain is highly homologous to IGF-I receptor most development efforts have been applied here on increasing selectivity and avoiding possible cross-inhibition of the IR. Several small molecules have been described so far with good results with PQIP among them (Ji, Mulvihill et al. 2007). Another, 3-(Benzimidazol-2-yl)-pyridine-2-one-based ATP competitive inhibitors of IGF-1R are optimized for reduced Cyp3A4 inhibition and improved oral exposure (Zimmermann, Wittman et al. 2008). BMS-554417 (Haluska, Carboni et al. 2006) is the dual

Insulin-Like Growth Factor-1/Insulin Receptor Inhibitor with good effect *in vivo* and *in vitro*.
Cyclolignan picropodophyllin (PPP) was discovered by our group some time ago (Girnita, Girnita et al. 2004). It was demonstrated that it inhibits phosphorylation of the IGF-1R and pAkt activation and does not interfere with insulin receptor. PPP has shown good effects *in vitro, in vivo* and is currently in phase I clinical trial (Girnita, All-Ericsson et al. 2006; Menu, Jernberg-Wiklund et al. 2006; Stromberg, Ekman et al. 2006; Vasilcanu, Weng et al. 2006; Economou, Andersson et al. 2008). Recently, PPP has been used in a mouse model of mammary invasive carcinomas induced by oncogenic mutant K-RasG12D and exhibiting up-regulation of the *IGF-1R* gene. In this model, treatment with PPP resulted in a dramatic decrease in tumor mass of the main forms of basal-like carcinomas. PPP also was effective against xenografts of the human basal-like cancer (Klinakis, Szabolcs et al. 2009). Mechanism of action of PPP is not known in detail but it is shown that it can act as partial agonist-antagonist, inducing Erk activation and inhibiting Akt phosphorylation upon ligand stimulation.
In early clinical trials, therapy targeting IGF-1R is well tolerated. Predicted side effects of treatment directed against IGF-1R system include growth alteration in children, neuro- and cardiac toxicity (Hewish, Chau et al. 2009). In patients, fatigue, loss of appetite, mild skin rashes were reported. A compensatory increase in the circulating concentrations of growth hormone and IGF-I occurs on administration of IGF-1R specific antibodies. Also modest treatment-induced hyperinsulinaemia and hyperglycaemia (20% of treated patients) encounters during same treatment and probably reflects the insulin resistance that is induced by the high levels of growth hormone. However, because IGF-I, IGF-II and insulin signal also via the insulin receptor to stimulate the growth of cancer cells, inhibition of IR might be advantageous and even necessary to totally disrupt the action of IGFs and their receptors (Sachdev and Yee 2007).

## 1.5 ARRESTIN PROTEIN FAMILY

In mammals four arrestins have been isolated and described. Two of them, v-arrestin/arrestin1 and c-arrestin/arrestin4 are visual isoforms and regulate signaling in retinal rods and cones. Other two, β-arrestin1/arrestin2 and β-arrestin2/arrestin3 are ubiquitously expressed (Kingsmore, Peppel et al. 1995).

### 1.5.1 β-arrestins

The best described function of β-arrestins is formation of clathrin coated pits by interacting with the components of the clathrin coat, the adaptor protein 2 and clathrin (Breann L. Wolfe 2007). β-arrestins are phosphorylated under basal conditions and may form homo- and heterodimers at high concentrations. They are cytosolic proteins that form complexes with seven-transmembrane receptors. After agonist stimulation and receptor phosphorylation by the G protein-coupled receptor (GPCR) kinases arrestins are recruited to the

activated receptor (Claing, Laporte et al. 2002). Many GPCRs are then desensitized and removed from the plasma membrane via clathrin-mediated endocytosis (Krupnick and Benovic 1998).
In addition to receptor desensitization, β-arrestins also function as scaffolding agents that recruit a variety of cytosolic proteins to their sites of action at the plasma membrane. Tandem mass spectrometry approach found 71 proteins interacting with β-arrestin1, 164 - with β-arrestin2, and 102 interacting with both β-arrestins. Some of these proteins were bound only after agonist stimulation, whereas others dissociated. (Xiao, McClatchy et al. 2007)
Arrestins also function as mitogen-activated protein kinase scaffolds, bringing together three components of MAPK signaling modules, JNK3, MKK4, and ASK1 (Song, Coffa et al. 2009).

### 1.5.2 Isoform differences

β-arrestin1 and β-arrestin2 are structurally highly homologous sharing 78% amino acid identity (Attramadal, Arriza et al. 1992) with most differences found in the C-terminus. Studies based on knockout mice suggest that arrestins can partially substitute each other's functions since mouse model for double knockout is lethal at early embryonic stages whereas mice knockout for β-arrestin1 or β-arrestin2 have been obtained.
However some of β-arrestin-mediated functions are not redundant. β-arrestin1 absence in the mice (Conner, Mathier et al. 1997) results in altered cardiac response to β-adrenergic stimulation whereas β-arrestin2 knockout mice (Bohn, Lefkowitz et al. 1999) have been described to develop enhanced morphine antinociception, disrupted morphine tolerance, reduced locomotor activity, disrupted dopamine-mediated behaviors, deficient lymphocyte chemotaxis, altered susceptibility to endotoxic shock and expression of proinflammatory cytokines, altered CXCR2-mediated neutrophil chemotaxis, altered asthmatic response to allergens, decreased bone mass and altered bone architecture.
Internalization of some GPCR is mediated primarily by one isoform, as in the case of the β2AR via β-arrestin2. For the protease-activated receptor 1, only β-arrestin1 can desensitize phosphoinositide turnover (DeWire, Ahn et al. 2007). At the same time, for the angiotensin II type 1A receptor internalization, both isoforms are equally capable. For PAR-2 β-arrestin1 colocalizes with a lysosomal marker and mediated early, while β-arrestin-2 mediates delayed receptor internalization and membrane-associated ERK1/2 activation (Kumar, Lau et al. 2007).
In case of β-arrestin1, translocation to the nucleus was demonstrated with protein being selectively enriched at promoters of p27 and c-fos, where it facilitates transcription of these genes (Kang, Shi et al. 2005). β-arrestin1 is also involved in the Src signaling. Agonist occupied receptor leads c-Src binding independent of Src C-terminus phosphorylation status. It was shown c-Src SH3 domain and hydrophobic domains within aminoacids 1 to 185 from the NH2-terminus of β-arrestin1 contribute to the binding of the two intact proteins. β-

arrestin1-dependent Src recruitment is important for Erk activation by β2AR but not for EGF-induced EGFR activation (Luttrell, Ferguson et al. 1999)
As for β-arrestin2, a carboxyl-terminal sequence (RRSLHL) responsible for the interaction and enhancement of phosphorylation of the JNK3 was identified (Conner, Mathier et al. 1997), (Miller, McDonald et al. 2001)

### 1.5.3 IGF-1R and β-arrestin

At the moment, ever increasing evidence demonstrates interaction between the β-arrestins and IGF-I receptor. Yet not all the mechanisms underlying this event have been determined. It is shown that both arrestins associate with the IGF-1R in a ligand dependent manner (Lin, Daaka et al. 1998; Dalle, Ricketts et al. 2001).
As a clathrin adapter, β-arrestin mediates IGF-1R endocytosis. On the other hand receptor-induced Shc phosphorylation and MAPK activation are dependent on the endocytosis of the IGF-1R and therefore could be coordinated by β-arrestin. Indeed, expression of a dominant-negative mutant of β-arrestin1 (S412D) impairs IGF-1R internalization and MAPK signaling after IGF-I treatment, presumably by reducing β-arrestin1 mediated targeting of the IGF-1R to clathrin-coated membrane pits. Over expression of wild-type β-arrestin1 or β-arrestin2 increases IGF-1R internalization, as does over expression of a constitutively active form of β-arrestin1 (S412A) (Lin, Daaka et al. 1998). In another series of experiments performed in adipocytes, microinjection of β-arrestin1 targeting antibody inhibited the transcriptional activity of ERK reporter gene construct (Dalle, Ricketts et al. 2001). Taken together, these data suggest that β-arrestin mediated, clathrin-dependent IGF-1R internalization is a critical pathway to ERK activation by IGF-I.
In addition to its role as mitogenic MAPK pathway activator, the IGF-I receptor mediates activation of phosphatidylinositol-3 kinase, subsequent Akt activation and anti-apoptosis. The pathway fails in mouse embryo fibroblasts lacking both β-arrestins and is restored by stable transfection of β-arrestin1 (Povsic, Kohout et al. 2003). The mechanism of β-arrestin1 dependent PI3K activation remains unclear. However it is suggested that β-arrestin may scaffold PI3K to the IGF-1R, near its lipid substrates at the plasma membrane, bypassing the need for tyrosine-phosphorylated sites on the receptor or on IRS-1 even though direct interaction between arrestins and PI3K has not been demonstrated (Hupfeld and Olefsky 2007).
In our group it was shown that the E3 ligase MDM2 can ubiquitinate the IGF-1R in vitro and cells lacking MDM2 have reduced IGF-1R ubiquitination (Girnita, Girnita et al. 2003). Further studies show that β-arrestin1 is acting as a scaffold protein for the recruitment of MDM2 to the activated IGF-1R (Girnita, Shenoy et al. 2005). Co-immunoprecipitation studies discover IGF-1R, β-arrestin and MDM2 complex associated upon IGF-I treatment and addition of β-arrestin1 *in vitro* to IGF-1R, E1, E2, and MDM2 greatly enhances IGF-1R ubiquitination. In mouse P6 cells overexpressing the IGF-1R, the absence of β-arrestin1 inhibits receptor ubiquitination.

### 1.5.4 GPCR, RTK and cancer

Receptor crosstalk is of prime importance in the cell for coordination of multiple extracellular signals. G-protein coupled receptors and receptor tyrosine kinases represent two major groups of receptors involved in cancer signaling whose action is modulated by β-arrestins. While the role of the RTK as a major transforming and prosurvival factor in tumor growth is well established (Amit, Wides et al. 2007; Frank, Trevor et al. 2007; Ghoreschi, Laurence et al. 2009), the importance of the GPCR has only recently been highlighted.

The oncogenic potential of GPCRs is in first place the result of a complex interplay among downstream heterotrimeric G-proteins. Gα12/13 proteins seem to be the most potent oncogenes, because they comprise the only family for which over expression of wild type proteins has been found to be transforming in several in vitro model systems (Dhanasekaran and Dermott 1996; Radhika and Dhanasekaran 2001). Other examples include Mas, G2A, and the PAR-1 thrombin receptor that transform cells via activation of Rho family small GTPases. (Whitehead, Zohn et al. 2001).

Further evidence comes from recent examination of publicly available gene expression data. A variety of types of GPCRs such as neuropeptide receptors, adenosine A2B receptor, P2Y purinoceptor, calcium-sensing receptor and glutamate receptors were expressed at a significantly higher level in some cancer tissue. Analysis of cancer samples in different disease stages also suggests that some GPCRs, such as endothelin receptor A, may be involved in early tumor progression and others, such as CXCR4, may play a critical role in tumor invasion and metastasis (Li, Huang et al. 2005).

Crosstalk between GPCR and RTK has been illustrated recently. The availability of endogenous EGFR ligands has been reported to be regulated indirectly by the activation of several G protein-coupled receptors in many cancer cells. This EGFR transactivation mechanism required the initial activation of a GPCR that in turn induced the cleavage of membrane-bound EGFR ligands precursors (Paolillo and Schinelli 2008). G protein-coupled receptors can also directly activate the juxtamembrane tyrosine kinase domain of EGFR. The progression of colon, lung, breast, head and neck, prostate and ovarian cancers have all been reported to be mediated, at least in part, by GPCR-EGFR crosstalk (Bhola and Grandis 2008).

The discovery of novel nuclear roles for heterotrimeric G-proteins expands the direct impact of G-protein signaling on processes fundamental to the pathology of cancer (Spiegelberg and Hamm 2007). Increasing evidence suggests that GPCR are involved in tumorigenesis and metastatic progression of melanoma (Hwa Jin Lee 2008) and prostate cancer (Marinissen and Gutkind 2001).

# 2 METHODOLOGICAL CONSIDERATIONS

## 2.1 INDUCTION OF TRANSFORMATION

Transformation of the cultured cells can occur spontaneously (Daniel CW 1975; Rubin and Ellison 1991), be induced by applying carcinogenic agents (McCormick JJ 1988; McCormick JJ 1989) or after introduction of the oncogenes (Boylan, Jackson et al. 1990; Naoyoshi Maeda 2008; Pipas 2009; Yaniv 2009). In this study oncogene induced transformation of the established cell lines with manipulated protein expression was assessed.

### 2.1.1 Oncogenes

Three well known oncogenes, **H-RasV12**, **PyMT** and **vSrc** were chosen here primarily due to their widely known role in the IGF-1R mediated transformation. R- cells, lacking IGF-1R were shown to be refractory to transformation by activated Ha-Ras (Sell, Dumenil et al. 1994), simian virus large 40 T antigen (Sell, Rubini et al. 1993; DeAngelis, Chen et al. 2005) and activated cellular Src527 but could be transformed by viral Src (Valentinis, Morrione et al. 1997). We therefore hypothesized that same or closely related oncogenes would be able to pinpoint the IGF-I receptor's relevance for transformation in our system.

### 2.1.2 Cell lines

Embryonic fibroblasts (EF) represent a classical tool in the study of transformation and cell division controlling mechanisms (McCormick JJ 1989; Naoyoshi Maeda 2008). It has been established for long time that primary mouse embryonic fibroblasts (MEF) when cultured for several generations can overcome cellular senescence and spontaneously immortalize (Todaro and Green 1963; Barrett 1985; McCormick JJ 1989). Once immortalized cells are having limitless growth capacity and can be transformed by single oncogene (Sun and Taneja 2007). In the same time it is known that different lines can acquire different properties, depending on the culture conditions and chromosomal changes employed (Todaro and Green 1963; Barrett 1985; Rubin and Ellison 1991; Eyden 2004). Therefore to obtain uniform cell population and reproducible results in our study we chose to focus on existing cell lines from other groups. Several lines established according to 3T3 protocol by Todaro and Green (Todaro and Green 1963) for culturing primary MEFs in which cells are split every 3 d with fixed seeding density of $3 \times 10^5$/6-cm cell culture dish were used in this study. Following immortalized mouse embryonic fibroblast (MEF) cell cultures are having central part in *Paper I, Paper II* and *Paper IV*:

**R- cells** are MEFs from manipulated mice with disrupted *IGF-1R* gene expression (Sell, Rubini et al. 1993; Sell, Dumenil et al. 1994). In these cells *Igf1r* gene fragment containing 240 bp of the 3' terminal portion of exon 3, coding for tyrosine kinase domain of the receptor and 17 bp of the downstream intron sequence were replaced with the neo cassette positioned in the same transcriptional orientation as the endogenous *Igf1r* (Liu, Baker et al. 1993). R-

mouse embryo fibroblasts were established from 18-day embryos after animal being genotyped by Southern analysis, using DNA prepared from their tails. Wild-type and homozygous *Igflr(-/-)* mutant littermates were used to establish primary cultures. Primary cultures underwent crisis after 2-4 weeks in culture. When characterizing R- cultures authors mentioned that they had relatively slow doubling rate and entered crisis later than the wild-type cells (Sell, Rubini et al. 1993; Sell, Dumenil et al. 1994).
**KO cells**, *β-arrestin1* knockout MEF. Cells were prepared from day 10.5 to day 13.5 embryos derived from crosses between *β-arrestin1(+/−)* mice in which *β-arrestin1* gene was disrupted by homologous recombination with 11-kb EcoRV fragment inserted into a Bluescript SK II (+)–based plasmid (Stratagene Inc) containing the neomycin resistance gene under control of the phosphoglycerate kinase promoter (Conner, Mathier et al. 1997). Both β-arrestin1 knockout and wild type littermate embryos were used to establish respective cell line. None of later two cell lines was described to differ in their ability to spontaneously transform or to become established cell lines (Kohout, Lin et al. 2001). **MEF from wild-type** animals established as described above were used as a control in all our experiments.
In *Paper III* we used malignant cell lines shown to express and depend on IGF-1R: human melanoma cells **DFB** and **BE** (Girnita, Shenoy et al. 2005), human uveal melanoma **OCM1** and **OCM3** (Girnita, All-Ericsson et al. 2006) and mouse fibroblast cell lines overexpressing IGF-1R - **P6** (Yoshinouchi, Miura et al. 1993) or both IGF-I and IGF-1R - **P12** (Baserga 1992). Other cell lines used include porcine aortic endothelial cells - **PAC**, human glioblastoma cell line **U343MG** and human breast adenocarcinoma cell line **MCF7**.

### 2.1.3 Plasmids and transfection procedures

Activated RasV12 expression vector **dsRed-Ha-RasG12V** (Rubio and Wetzker 2000; Augsten, Pusch et al. 2006) was constructed by PCR cloning of GTPase protein in fusion with amino-terminally located DsRed1 vector (Fischer, Hekman et al. 2007).
Polyoma middle T antigene sequence (PyMT) (Martens, Nilsson et al. 1988) was from **pMT1** plasmid (Zhu, Veldman et al. 1984) which is variant of p85.3.SP lacking the introns of the middle T and was shown to efficiently induce transformation in the cultured cells (Treisman, Novak et al. 1981; Martens, Ramqvist et al. 1990). PyMT was clonedinto and expressed using pSVneo vector confirming neomycin resistance.
Plasmid **pMvsrc** (Valentinis, Morrione et al. 1997) was constructed by standard recombinant DNA techniques. 3.1-kb Schmidt Ruppin A v-src fragment (Cross and Hanafusa 1983) from plasmid pN4 (Iba, Takeya et al. 1984) was inserted into the pEVX (Kriegler, Perez et al. 1984). This fragment contains 276 bp of pBR322 DNA from the pBR322 followed by 2.8 kb of Rous sarcoma virus (RSV) DNA 750 bp upstream of the env termination codon and down to about 90 bp downstream of the v-src termination codon.
Transfection protocols using Lipofectamine2000 in all cases of oncogene transfection were optimized to ensure maximal transfection rates (Hawley-

Nelson and Ciccarone 2003; Dalby, Cates et al. 2004). Oncogene expression in form of protein or mRNA expression was always controlled by SDS/WB or PCR. Transient transfection was used in case of R- cells were cells were subject to rapid subsequent seeding into the soft agar or other analysis. In case of KO cells stable transfection was achieved by co-transfection of the puromycin resistance encoding vector at rate 1:5 rate to ensure the same level of the oncogene expression during study.

## 2.2 ANALYSIS OF TRANSFORMED PHENOTYPE

There are several phenotypic markers that have found wide acceptance throughout the world in assessing malignant potential of the cell (Hanahan and Weinberg 2000). These include: self-sufficiency in growth signals; insensitivity to growth-inhibitory signals; evasion of apoptosis; limitless replicative potential; sustained angiogenesis; and tissue invasion and metastasis. While some of these cell properties can easily be tested in *in vitro* conditions others requires rigorous experiments and/or *in vivo* models. In our study we chose three methods to complement each other to measure full transformation and to cover maximally cancerous cells' properties.

### 2.2.1 Cell survival and proliferation

Analyses of cell turnover can provide useful information about tumor's doubling time, malignancy level and prognosis. Wide variety of possible methods for detection of the cell proliferation rate or acquired independence from external growth signals include measuring of DNA content or apoptotic markers by radioactive, colorimetric, luminescent or fluorescent assays. In our experiments however we chose direct viable cell count after staining with trypan blue as a cumulative measure of equilibrium between survival and proliferation in cells grown under diverse conditions such as serum deprivation, stimulation with growth factors or anchorage-independent culturing conditions. Cell counts were obtained using automated cell counter Countess or cell count chamber and provided reliable, easily obtained and highly reproducible results.

### 2.2.2 Anchorage-independent growth

To assess anchorage independent phenotype we used soft agar colony formation test (Macpherson and Montagnier 1964) as one of the most widely used and reliable *in vitro* tests for cancer phenotype. Good correlation between growth in soft agar and tumor formation in an animal host has been widely reported (Montesano R, Drevon C et al. 1977; Colburn, Bruegge et al. 1978; Salmon SE, Hamburger AW et al. 1978). Although frequently associated with malignant cells, soft agar colony formation phenotypes has never been demonstrated to be sufficient criteria for tumorigenicity *in vivo* (Dodson, Slota et al. 1981).

Another system used in our study is PolyHEMA, a hydrogel widely used as a substrate in cell culturing (Lombello, Malmonge et al. 2000; Velzenberger, Kirat et al. 2009) and in medicine (Lipson and Musch 2007). PolyHEMA represents a solid material formed by cross linked hydrophilic polymer. It resembles cartilage matrix by physicochemical characteristics and is known to prevent cell attachment and spreading.

### 2.2.3 Tumorigenic properties *in vivo*

Tumor formation *in vivo* is the ultimate criteria for transformed cultured cell lines. While wild type mice would be the adequate host for the cancerous mouse fibroblasts we chose immuno-compromised athymic "nude" animals (Rygaard and Polvsen 2007) to avoid immune reaction against expressed oncogene.

## 2.3 DNA, RNA AND PROTEIN ANALYSIS

### 2.3.1 PCR

In *Paper I* three pairs of primers were used to check for the mouse IGF-1R expression. First pair (5′-GGCCAAACTCAACCGTCT-AA-3′ and 5′-GCTGAA-ATACTCGGGGTTCA-3′) was designed to target receptor β-subunit's juxtamembranous and kinase domain corresponding to aa 764–856, second pair (5′-TGGGAGGTAGCTCGAGAGAA-3′ and 5′-CACTCTGGTTTCGGG-TTCAT-3′) - kinase domain, aa 866–904 and third one (5′-ATGAACCCGAAA-CCAGAGTG-3′ and 5′-CCAGCCATCTGGATCATCTT-3′) kinase domain at the level of aa 898–990.
In *Paper II* PCR was performed in cell lines transfected with oncogenes to confirm mRNA presence. Primers for H-RasV12 were: 5′-CCAGCTGATCCA-GAACCATT-3′ and 5′-ATGGCAAACACACAAGGAA-3′; for polyoma middle T antigen: 5′-CTGCTACTGCACCCAGACAAAGGTG-3′ and 5′-GCA-GGTAAGAGGCATTCTGCAGAACC-3′.
For genotyping of R- cells previously described (Spence, Shaffer et al. 2006) primers flanking exon 3 of the mouse *IGF-1R* gene (5′-ATCATCCTTACCACC-CTCT-3′ and 5′-GGCACCCTCAAAGTTTAG-3′) were used.
For the amplification of IGF-1R in *Paper III* following the primers corresponding to the human IGF-I receptor were used: 5'-GCCCGAAGGTCT-GTGAGGAAGAA-3' and 5'-GGTACCGGTGCCAGGTTATGA-3'.

### 2.3.2 DNA/RNA sequencing

Sequencing was performed using AB BigDye Terminator v3.1 and capillary electrophoresis on ABI3730XL. Results were compared with the published IGF-1R sequence (Accession No. IGF-1R gene NP034643 and NM010513).

### 2.3.3 SDS/PAGE and Western Blotting

SDS/PAGE and Western blotting were used to separate proteins of interest, determine molecular weight and subsequently stain using

immunofluorescence technique. Stained blots were scanned and signals quantified using Fluor-S MultiImager System (BioRad). Even though it represents powerful tool and could be combined with immunoprecipitation and/or cell fractionation, detection and quantification of the low levels of proteins by WB especially when addressed to their subcellular localization can be limited.

### 2.3.4 Confocal microscopy

Confocal microscopy was used in this study to assess the distribution of the proteins within subcellular compartments under various conditions. For this reason cells grown on cover slips were fixed and subsequently stained according to the protocol. In cells transfected with dsRed-Ha-RasG12V red fluorescence was present in live cells but diminished significantly after fixation and could be neglected after several days.

## 2.4 IGF-1R AND B-ARRESTIN1 TARGETING

### 2.4.1 PPP

Highly purified PPP (99,7%) was used in this study to prevent contamination with related podofyllotoxin (PPT) which normally exist in equilibrium at ration 97,5 to 2,5 (Gensler and Gatsonis 1966).

### 2.4.2 Gene silencing –small interfering and hairpin RNA

Small interfering RNA targeting IGF-1R used in *Paper I* and *Paper II* were purchased from Dharmacon (Lafayette, Colorado). Three constructs (**duplex 6** – 5′-GGCCAGAAAUGGAGAAUAAUU-3′ ; **duplex 8** – 5′-GCAGACACC-UACAACAUCAUU-3′ both against β-subunit and **duplex 17** – 5′-GGACUC-AGUACGCCGUUUAUU-3′ targeting receptor α-subunit) were designed to target human *IGF-1R* but according to our data are also efficient against mouse *IGF-I receptor* gene.
In *Paper III* MISSION vector from Sigma expressing small hairpin RNA (shRNA) (Moffat, Grueneberg et al. 2006) targeting *β-arrestin1* was used. Initially five different constructs were tested but only one most efficient was used in the paper. shRNA targeting human b-arrestin1 (NM_004041) was GCCAGTAGATACCAATCTCAT. The non-target shRNA control vector with the sequence CAACAAGATGAAGAGCACCAA was used as a negative control.

## 2.5 STATISTICAL METHODS

Question that aroused in virtually all our experiments when assessing such parameters as cell survival, colony formation, expression rates of the cDNA or protein, PPP binding and displacement assay etc was: what is the probability that the relationship exists? To answer this question we used determination of statistical significance, namely *Fisher's two-tailed t-test* as calculated by the *Exel.*

Based on probability theory and normal curve this tool gave us the probability that a relationship between two variables was just due to chance occurrence(Ennos 2006).

*Fisher's two-tailed t-test* was used uniformly in our research to determine probability p-value. It was calculated and noted for every observation, repeated for at least three times and p-level was set to be at least 0,05 in all cases were statistical significance was found.

Tests of statistical significance, such as *t-test* constitute powerful yet easily understood tool. It represents a valuable part of our research by letting us to compare our findings with other groups. However it should be noted that this test has no relationship to practical value of our findings neither assures that design of the study was optimal (McKinney WP 1989; Fleiss, Levin et al. 2003).

# 3 AIMS OF THE STUDY

The specific aims of this thesis were:

To investigate potential expression and functions of the IGF-1R in clones of IGF-1R knockout cells.

To explore the effects of the IGF-1R inhibition by PPP on receptor expression and the mechanism controlling these effects.

To investigate the role of β-arrestin mediated signaling of IGF-1R in tumor transformation.

# 4 RESULTS AND DISCUSSION

## 4.1 PAPER I

*IGF-1R tyrosine kinase expression and dependency in clones of IGF-1R knockout cells (R-).*

R- cells are established and seemingly well characterized mouse embryonic fibroblast cell line knockdown for IGF-1R (Sell, Dumenil et al. 1994). Therefore they have been regarded by our group as an ideal control cell line in the experiments where IGF-1R inhibitor cyclolignan PPP was tested. However when running survival assay we could see that in some cases cell survival was affected by PPP treatment. Therefore we investigated the mechanism of PPP sensitivity in two different clones of R- cells; one sensitive to IGF-1R inhibitor PPP (R-s), and the other one resistant (R-r). Since our previous studies have ruled out PPP interactions with the highly homologous insulin receptor and other major tyrosine kinase receptors involved in tumor cell growth, and given the fact that PPP is structurally closely related to PPT, a very potent microtubule inhibitor, we investigated whether this mechanism can explain the sensitivity to PPP. However when tubulin binding capacity of PPP was tested in the R- cells, both direct binding to colchicine and competitive displacement of [$^3$H]PPP could not be detected. Therefore such explanation of the PPP effect was excluded.

Further we focused on possible interference with one or more IGF-I system components. Indeed we could detect expression of the 90 kDa protein reactive to IGF-1R β-subunit antibodies. Similarity with the receptor was further confirmed when siRNA targeting IGF-1R downregulated expression of this protein and several parts of the transcribed mRNA sequences encoding IGF-I receptor protein were discovered in R-s cells. Therefore we concluded that R-s clone of R- cells expresses aberrant 90 kDa IGF-1R or, in particular, its β-subunit. R− cells were created by targeting the ligand-binding domain of the IGF-1R, not the whole receptor. Therefore, R− cells may express a mutant IGF-1R lacking the ligand binding domain. Here the main question raised was whether such a receptor is biologically active.

Further investigation revealed that this protein was weakly but constitutively tyrosine phosphorylated and its downregulation by siRNA significantly decreased cell survival. This later effect was dependent on the nature of siRNA with highest cell death values corresponding to the most efficient siRNA construct 17 (sequence see in *Paper I*).

## 4.2 PAPER II

*Aberrant intracellular IGF-1R β-subunit makes receptor knockout cells (IGF-1R-/-) susceptible to oncogenic transformation*.

It has been demonstrated that R- cannot be transformed by several powerful oncogenes cells undergo spontaneous transformation in culture but at a lower

rate then usual MEFs (Renato Baserga 2003). This led us to the idea that R- cells are being changed when cultured for certain time and this could contribute not only to PPP responsiveness but to virtually all characteristics of this cell line. Therefore in the Paper II we aimed to characterize different clones of R- regarding cell growth, transformation ability, and main downstream signalling effectors of the IGF-1R pathway.

After establishing that subclones of R- can in fact differ in their expression of IGF-1R we further demonstrate that unlike intact IGF-1R usually enriched at the plasma membrane aberrant protein is retained intracellularly. Moreover perinuclear aggregates of this protein observed in basal conditions were redistributed after serum starvation. Therefore the possibility that we detect functionally inactive defective IGF-1R or pro-receptor retained endoplasmatically could be excluded.

Intracellular IGF-1R has been shown in several cases of malignant (Keehn, Saeed et al. 2004; Nakamura, Miyamoto et al. 2004; Harris, You et al. 2007) and normal cells (Mascotti, Caceres et al. 1997; Koda, Sulkowski et al. 2004; Inagaki, Tiulpakov et al. 2007). Hence our observation cannot be considered as an isolated phenomenon only restricted to R-s cells. However mechanism of the expression of the IGF-1R in R-s cells still remains unclear. Based on the sequencing data we confirmed that similar *neo* cassette is replacing part of exon 3 in both R-s and R-r. Here possible explanation of the aberrant expression of the IGF-1R might be an altered transcriptional processing in R-s or instability of the bacterial DNA included in the *neo* cassette.

Another important finding in this study is that expression of the IGF-1R β-subunit allows transformation by H-RasV12 and/or PyMT in the R-s variant of R- cells. In the same time R-r variant, which lacks expression of aberrant β-subunit cannot be transformed by these oncogenes. Consequently downregulation of the protein by siRNA in R-s abrogates colony formation in soft agar induced by oncogenes. How the β-subunit makes the otherwise transformation-resistant IGF-1R knockout cells (Sell, Dumenil et al. 1994; DeAngelis, Chen et al. 2005) susceptible to these oncogenes is still not understood. High steady state activity of Akt (Chang, Lee et al. 2003; Vilgelm, Lian et al. 2006; Yang, Wen et al. 2006; Bader and Vogt 2007) as well as upregulation of EGFR (Maria Sibilia 2007; Schnidar, Eberl et al. 2009) and ErbB3 (Earp, Dawson et al. 1995; Lafky, Wilken et al. 2008) seen in R-s cells might be involved in making these cells permissive for oncogenic transformation. Later finding is consistent with data reported by Spence et al (Spence, Shaffer et al. 2006) showing that late passages of another strain of mouse IGF-1R knockout mouse fibroblast overexpress ErbB3 and can be transformed by SV40 as opposed to early passages. However neither expression of EGFR and ErbB3 nor phosphorylation of Akt and ERKs could be proven to be controlled by the aberrant β-subunit. In this light weak kinase activity by the aberrant receptor and high density of the protein seen in the perinuclear aggregates seem to explain at least some of the mechanisms behind transformation of the R-s cells.

Among other differences between R-s and R-r are the lower overall content of the tyrosine-phosphorylated proteins and decreased expression of Src in R-s. It

is interesting that overall level of Src in R-s was increased after anti-IGF-1R siRNA suggesting aberrant β-subunit might be inducing downregulation of Src as a result of its activation and subsequent ubiquitination.
In conclusion, these results demonstrate that IGF-1R may exert some of its biological effects by acting through non-canonical pathways.

## 4.3 PAPER III

*Picropodophyllin induces downregulation of the insulin-like growth factor 1 receptor: potential mechanistic involvement of Mdm2 and β-arrestin1.*
Cells are interacting with the environment via plasma membrane receptors. Activation of the receptor is inducing intra-cellular signaling events but also triggers mechanisms controlling duration, intensity and biological effects of the signaling. Constitutive and ligand-induced receptor trafficking is therefore an important part of the restriction of the cell sensitivity to the certain stimuli (Shankaran, Wiley et al. 2007). An intriguing property displayed by IGF-1R is that downregulation of the receptor has been associated with high antitumor effect when IGF-1R was used as a target for cancer therapy (Renato Baserga 2003).
PPP has previously been shown to inhibit receptor phosphorylation and interfere with downstream signaling (Girnita, Girnita et al. 2004; Menu, Jernberg-Wiklund et al. 2006; Stromberg, Ekman et al. 2006; Vasilcanu, Vasilcanu et al. 2008). It was also proven to be very efficient in tumor growth inhibition both *in vitro* and *in vivo* (Girnita, All-Ericsson et al. 2006; Vasilcanu, Weng et al. 2006; Economou, Andersson et al. 2008). Therefore we investigated now the PPP effect on receptor downregulation. We also tested the PPP influence on expression of other tyrosine kinase receptors.
Most strategies aiming at IGF-1R inhibition in cancer have been focused on targeting receptor's downstream signaling by inhibiting the receptor tyrosine kinase activity. This approach resulted in the discovery of several tyrosine kinase inhibitors. At the same time monoclonal antibodies against IGF-1R, blocking ligand receptor interaction have been developed. Some of the antibodies used to target IGF-1R retain the agonist properties of the ligand by inducing also IGF-1R downregulation. Such antibodies were very effective; they *in vitro* caused receptor downregulation and tumor growth inhibition *in vivo* (Sachdev, Li et al. 2003). This effect was associated with both inhibition of receptor downstream target molecules phosphorylation and cells being refractory to additional IGF-I stimulation due to receptor downregulation. Therefore it has been suggested that receptor downregulation represents an important mechanism of action (Baserga 2005; Wu, Odman et al. 2005).
Nevertheless, the present paper provides another evidence that IGF-I receptor downregulation is an important factor in tumor growth suppression. Using cyclolignan PPP, developed in our group and known to induce massive apoptosis and tumor regression in xenograft mouse models (Vasilcanu, Girnita et al. 2004; Girnita, All-Ericsson et al. 2006; Menu, Jernberg-Wiklund et al. 2006; Economou, Andersson et al. 2008) we show that PPP causes downregulation of the IGF-1R *in vitro* and *in vivo*. This effect does not involve homologous

insulin receptor or other tyrosine kinase receptors such as VEGFR, EGFR, Kit or PDGFRs. Furthermore, PPP decreases IGF-1R via degradation in a specific manner, without interfering with the general internalisation mechanisms as demonstrated by normal internalisation of the transferrine.
Degradation of the receptor represents an important finding since inhibition of IGF-1R phosphorylation, without accompanying downregulation, leads only to decreased proliferation but not to apoptosis (Baserga 2004; Baserga 2005). The PPP-mediated IGF-1R downregulation was considerable (30–50% after 12 h treatment of cultured cells and 50% in vivo), and clearly contribute to tumor regression.
The molecular mechanisms behind PPP-induced IGF-1R downregulation are is partially understood. It is known that PPP abrogates receptor phosphorylation of tyrosine residue 1136 in the activation loop of the kinase which leads to inhibition of Akt phosphorylation (Vasilcanu, Girnita et al. 2004). At the same time short time PPP treatment induces IGF-1R mediated ERK phosphorylation (Vasilcanu, Vasilcanu et al. 2008). In addition our group found that MDM2 and β-arrestin1 are required as adapters to bind and ubiquitinate IGF-1R (Girnita, Girnita et al. 2003; Girnita, Shenoy et al. 2005; Girnita, Shenoy et al. 2007). Therefore PPP might be considered acting as partial agonist-antagonist to IGF-I receptor leading to its subsequent interaction with the MDM2 E3 ligase, ubiquitination and degradation. Indeed when MDM2 was inhibited by dominant negative constructs or β-arrestin1 was downregulated using shRNA, PPP-induced IGF-1R downregulation was abrogated. This finding provides further support for the involvement of the MDM2 ligase. Additionally we demonstrate also direct physiological impact of this process since blockage of the IGF-1R downregulation decreases PPP-induced cell death *in vitro*.
It has also been shown that c-Cbl (Sehat, Andersson et al. 2008), as well as Nedd4 (Vecchione, Marchese et al. 2003), both act as E3 ligase for the IGF-1R. Whether there is interplay between MDM2, Nedd4 and c-Cbl induced IGF-1R ubiquitination is still unknown. However based on our present study, the PPP-induced downregulation of IGF-1R seems to be dependent on MDM2 since functional inhibition of MDM2 abrogated this response. On the other hand, receptor downregulation following IGF-I stimulation was not fully decreased by dominant negative MDM2.
Taken together, induction of the ligand-independent receptor degradation, through MDM2 and β-arrestin1 supports our previous view on PPP as a potent antitumor drug and adds a new aspect on the mechanism of its action.

## 4.4 PAPER IV

*The role of β-arrestin1 in the Ras induced transformation of mammalian cells*.
Recently we demonstrated that β-arrestin1, is a key factor for ubiquitination and downregulation of the receptor (Girnita, Shenoy et al. 2005; Sehat, Andersson et al. 2007). Moreover it was shown that receptor induced activation of ERK occurred in a β-arrestin1 dependent manner even when tyrosine kinase domain of the IGF-1R was impaired(Girnita, Shenoy et al.

2007). This suggested that β-arrestin1 could play more extensive role in receptor function then it was believed before. We now questioned whether β-arrestin1 could be an important regulatory factor in malignancy. Therefore we investigate the role of β-arrestin1 mediated IGF-1R signaling in oncogene induced transformation.

We used β-arrestin1 knockout cells (KO) stably transfected with three well known oncogenes: H-RasV12, PyMT and v-Src. According to our results v-Src and PyMT can easily transform KO cells whereas in the case of H-Ras β-arrestin1 is required for the fully transformed phenotype. Further, we find that two functionally important Ras related signaling pathways are impaired in KO-Ras cells, MAPK/ERK and PI3K/Akt. The first one, Raf/MEK/ERK pathway is known to be critical contributor to Ras induced transformation in murine cells. It was established (Bonner, Kerby et al. 1985; Stanton, Nichols et al. 1989; Leevers, Paterson et al. 1994; Stokoe, Macdonald et al. 1994) that constitutively activated mutants of Raf or MEK alone are able to cause tumorigenic transformation of NIH3T3 cells. For the second PI3K/Akt pathway, it was shown that dominant-negative mutants can effectively block Ras transformation. Activated variants of PI3-kinase cannot cause transformation of NIH3T3 cells when expressed alone, yet when it cooperates with activated Raf they cause synergistic transformation (Rodriguez-Viciana, Warne et al. 1997). In our system KO-Ras cells failed to activate Akt when grown under anchorage independent or serum free conditions. In the same conditions, ERK phosphorylation was substantially lower then in control cells and required presence of serum as an additional stimulus.

Next we investigated whether IGF-1R impaired signaling could be the reason behind H-Ras failure to transform KO cells. Direct stimulation with the IGF-I showed that all cells respond with proliferation when grown in monolayer. However in suspension ligand stimulation cannot rescue KO-Ras cells from anoikis while MEF-Ras cells demonstrate increased proliferation under the same conditions.

Ras oncoprotein was shown to degrade rapidly when expressed in R-cells growing under anchorage-independent conditions, IGF-1R being an important protective factor (Gatzka, Prisco et al. 2000). However such mechanism cannot explain failure of transformation in the KO-Ras cells since Ras expression is constant in both MEF and KO cells. However in the KO-Ras cells the level of activated Ras is considerably lower when compared to the MEF cells while total expressed protein rates are almost equal. To transduce IGF-I-stimulated signaling, β-arrestin1 has to bind to the C terminus of the IGF-1R and become ubiquitinated by the Mdm2 E3 ligase (Girnita, Shenoy et al. 2005; Sehat, Andersson et al. 2008). This response could be obtained even in receptors lacking binding sites for both the Shc and IRS-1 (Girnita, Shenoy et al. 2007; Sehat, Andersson et al. 2008), two proteins involved in canonical tumor promoting IGF-1R tyrosine kinase signaling (LeRoith, Werner et al. 1995; Baserga 2009).

Mutational analysis of the transforming activity of the IGF-1R demonstrated that C-terminus of the receptor is controlling this property: receptor truncated at residue 1229 (C-terminus) is fully mitogenic, in terms of its response to IGF-I,

but cannot transform cells devoid of endogenous IGF-1R (Hongo, D'Ambrosio et al. 1996). It was also shown that another C-terminus truncated IGF-1R (truncated at residue 1245) alone cannot transform R- cells even when strongly overexpressed (Gatzka, Prisco et al. 2000). However, when it is co-expressed with activated Ras transformation of R- cells is induced. In the present study we show that H-Ras is unable to transform KO cells despite the presence of the IGF-1R and therefore the equivalence between C-terminus signaling of the IGF-1R and β-arrestin1 dependent signaling of the same receptor cannot be made.

In conclusion we demonstrated that oncogenic H-Ras is unable to transform immortalized mouse embryonic fibroblasts in the absence of β-arrestin1. The direct explanation of H-Ras inability to transform cells devoid to β-arrestin1 is the impaired IGF-1R signaling and insufficient activation of the PI3K/Akt and ERK pathways. The present results also suggest a more generalized, alternative mechanism for transformation by Ras and, implicitly, another possible way for targeting Ras in tumor cells.

# 5 CONCLUSIONS AND FUTURE PERSPECTIVES

At present time the IGF-1R inhibitors are in clinical trials for treatment of various types of cancer. Therefore we need to incorporate the knowledge we learned from in vitro and animal models with the clinical lessons learned from the trials involving other RTK inhibition. The first priority here is to identify patients in which anti-IGF-1R therapy will be efficient. The mere presence of IGF-1R may not be sufficient to indicate potential response. Therefore we need to know the exact role as well as mechanisms of receptor action in particular malignancy.

This thesis is trying to add to the current knowledge the role of expression of IGF-1R in malignant cells. Isolated expression of the β-subunit and previously unknown downstream interactions of the adaptor protein β-arrestin1 are proven to be involved in receptor induced transformation. Moreover, receptor inhibitor PPP is shown to act not only by inhibiting signaling but also by downregulation the receptor. However there are still numerous questions that have to be answered in the future. The molecular mechanisms behind expression of the β-subunit of the IGF-1R in R-s, possible intracellular activation of alternative pathways and their importance for malignancy in other cell types are still unknown. Possible strategies disrupting this yet unknown signaling could be important. Next, detailed mechanism of action of PPP could provide new insights not only in biology of IGF-1R but also in designing similar compounds with more potent action. Third, the role of β-arrestin1 in malignant cells should be assessed. Do Ras and β-arrestin1 represent totally independent mechanisms that ensure the maximal number of IGF-1R's downstream molecules activated? Does either of them simply act to localize IGF-1R substrates to the plasma membrane or vice versa and promote receptor access to the substrate? Or do both Ras and β-arrestin1 mutually contribute to the context of activation of IGF-1R? Ideally, disruption of this biochemical signaling pathways within tumor cells could provide useful therapeutic approach. However, involvement of the insulin receptor, growth factor receptors, GPCR and other molecules in these β-arrestin1-dependent transforming stimuli should be assed to assure adequate response to such strategies in terms of tumor growth. Further development of molecular tools for targeting arrestin interactions with individual partners in tumor growth promotion has an enormous therapeutic potential.

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