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The role of JAKs and STATs in chemoresistance

by

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Unless otherwise stated in the text, the present thesis is the result of my own work.

TABLE OF CONTENTS

Abstract	7
Acknowledgements	8
1. Introduction	10
1.1. Cancer	11
1.1.1. Chemotherapy	12
1.1.2. Lung cancer	13
1.1.3. Bone cancer	15
1.1.4. Chemoresistance	17
1.1.5. Apoptosis	17
1.1.5.1. Regulation of apoptosis	18
1.1.5.2. Apoptosis and cancer	21
1.2. The main signalling pathways of cancer	22
1.2.1. The RAS/RAF/MEK/ERK signalling pathway	22
1.2.2. The PI3K/PTEN/PKB/mTOR signalling pathway	27
1.2.2.1. PKCs	31
1.2.3. The JAK/STAT signalling pathway	34
1.2.3.1. Biological role of JAKs and STATs	34
1.2.3.1.1. The JAKs	34
1.2.3.1.2. The STATs	37
1.2.3.2. JAK/STAT activation and signalling	39
1.2.3.3. Regulation of JAK/STAT signalling pathways	43
1.2.3.4. JAKs and STATs in tumour cell proliferation and apoptosis	45
1.2.3.5. Targeting JAK/STAT pathways for cancer treatment	48
1.3. Fibroblast growth factors (FGFs), FGF receptors (FGFRs) and cancer	50
1.4. FGF-2	52
1.4.1. Biological role of FGF-2	53
1.4.2. Activation and signalling in response to FGF-2	54
1.4.3. Negative and Positive Regulators of FGF signalling pathways	58
1.4.4. FGF-2 in cancer	59
1.4.4.1. FGF-2-mediated chemoresistance mechanisms	61
1.4.5. Targeting FGF-2 signalling for cancer treatment	64

1.5. FGF-2 and the JAK/STAT signalling pathway	65
1.5.1. FGF/FGFR in lung and bone cancers	66
1.5.2. JAKs and STATs in lung and bone cancers	67
2. Hypothesis and specific aims	69
3. Material and methods	70
3.1. Materials	71
3.2. Cell lines and culture	72
3.3. Protein extraction	74
3.4. Protein immunoprecipitation	75
3.4.1. Protein co-immunoprecipitation	76
3.5. Sodium dodecyl sulphate polyacrylamide gel electrophoresis and Western blot analysis	77
3.6. Cell death / chemoresistance assays	77
3.6.1. Cell viability assay (WST-1)	78
3.6.2. Cell counting	78
3.6.3. Cell cycle analysis	79
3.6.4. Apoptosis analysis by poly-(ADP-ribose) polymerase and lamin B cleavage	80
3.7. Reverse transcription polymerase chain reaction (RT-PCR)	81
3.8. Crystal violet staining	82
3.9. RNA interference (RNAi)	83
3.10. gp130 detection by flow cytometry	84
3.11. Statistical analysis	85
4. Results	86
4.1. The role of FGF-2 in chemoresistance	87
4.1.1. Introduction	88
4.1.2. FGF-2 response in NSCLC and U2OS cells	89
4.1.3. Expression of canonical FGF-2 receptors in NSCLC and U2OS cells	95
4.1.4. FGF-2 induced proliferation of U2OS but not of NSCLC cells	96
4.1.5. FGF-2 protected U2OS cells from cisplatin-induced cell death but not NSCLC HCC95 and HCC78 cells	101
4.1.5.1 FGF-2 blocked cisplatin-induced apoptosis in U2OS cells but not in NSCLC HCC95 and HCC78 cells	104

4.1.6. FGF-2 protected U2OS cell from cisplatin-induced cell death by a MEK-ERK dependent mechanism	112
4.1.7. FGF-2 induced protein-protein interactions between PKC ϵ , B-RAF and S6K2 In U2OS cells	115
4.1.8. Interactions between PKC ϵ , B-RAF and S6K2 in HCC95 cells	120
4.1.9. FGF-2 induced the up-regulation of anti-apoptotic proteins in U2OS cells	122
4.1.10. Discussion	125
4.2. The role of JAKs and STATs in FGF-2-mediated chemoresistance	133
4.2.1. Introduction	134
4.2.2. JAK1, JAK2 and TYK2 but not STAT1, STAT3 and STAT5 were required for FGF-2-mediated chemoresistance in U2OS cells	135
4.2.2.1. Knockdown of JAK1, JAK2, TYK2, STAT1, STAT3 and STAT5 in U2OS cells	135
4.2.2.2. JAK1, JAK2 and TYK2 were required for FGF-2-mediated chemoresistance	138
4.2.2.2.1. JAK2 activity was required for FGF-2-mediated chemoresistance	145
4.2.2.3. STAT1, STAT3 and STAT5A/B were not required for FGF-2-mediated chemoresistance	149
4.2.3. Activation of STAT1, STAT3 or STAT5A/B by FGF-2 was not observed in U2OS cells	155
4.2.4. FGF-2 induced the phosphorylation of TYK2 and potentially of JAK1 and JAK2 in U2OS cells	158
4.2.5. A requirement for JAKs in FGF-2-mediated chemoresistance mechanism in U2OS cells	165
4.2.5.1. Knockdown of TYK2 affected FGF-2 induced activation of ERK1/2	165
4.2.5.2. JAK1 and TYK2 may be required for the interaction between PKC ϵ and B-RAF induced by FGF-2	167
4.2.5.3 JAK1, JAK2 and TYK2 interacted with PKC ϵ and B-RAF in response to FGF-2	171
4.2.5.4. TYK2 was required for FGF-2-induced up-regulation of the anti-apoptotic protein MCL-1	175
4.2.6. Discussion	177
4.3. Regulation of STAT3 by ERK2	184
4.3.1. Introduction	185
4.3.2. Knockdown of ERK2 by siRNA reduced STAT3 protein levels in U2OS cells	186

4.3.2.1 Knockdown of ERK2 also reduced STAT3 protein levels in other cell lines	191
4.3.3. The inhibition of ERK1/2 activity by MEK inhibitors did not affect STAT3 protein levels in U2OS cells	193
4.3.4. Knockdown of ERK2 affected IL-6 signalling in U2OS cells	195
4.3.4.1. Knockdown of ERK2 reduced gp130 receptor expression	199
4.3.5. Discussion	203
5. General discussion and future directions	209
5.1. General discussion	210
5.2. Future directions	215
6. References	222
Abbreviations	262
Figures and Tables	268
Appendix I – Comparison between the WST-1 assay and cell counting	271
Appendix II – Cisplatin batch-to-batch variation	272
Appendix III – Cell cycle data analysis	273
Appendix IV – FGF-2-mediated rescue in H-510 cells	275

ABSTRACT

Janus kinases (JAKs) and Signal Transducer and Activators of Transcription (STATs) are essential for signalling in response to the Interferons (IFNs) and most cytokines. Aberrant activation of JAKs and/or STATs has been observed in many human cancer cells, and several recent studies have suggested that STAT3 oncogenic pathways are also associated with intrinsic drug resistance.

Chemoresistance is a major cause of failure of cancer treatment. In lung cancer cells chemoresistance can be mediated by Fibroblast Growth Factor 2 (FGF-2). In small cell lung cancer (SCLC) cell lines FGF-2 induces the expression of anti-apoptotic proteins *via* a MEK-ERK-dependent mechanism, which involves the formation of a multi-protein complex constituted by B-RAF, PKC ϵ and S6K2. We investigated the generality of this phenomenon by analysing additional types of cells. Since there have been reports suggesting that activation of JAKs and/or STATs can occur downstream of FGF-2 we hypothesised that JAKs and/or STATs may also play a role in FGF-2 mediated chemoresistance pathway(s).

In U2OS osteosarcoma cells, FGF-2 offers protection against cisplatin-induced cell death. As with SCLC cells this involves B-RAF, PKC ϵ , S6K2, MAPKs and, most notably, JAKs (JAK1, JAK2, or TYK2), but not the STATs. Whereas the non-small lung cancer cells (NSCLC) HCC95 respond to FGF-2, HCC78 do not. In neither case, however, did FGF-2 mediate chemoresistance against cisplatin.

Using RNA interference (RNAi), we have observed that ERK2 knockdown caused a concomitant reduction of STAT3 levels. The data suggest that ERK2, but not ERK1, levels can regulate STAT3 expression, and that ERK1 and ERK2 have non-overlapping functions. It also indicates that ERK2 protein levels can regulate STAT3, an important signal transducer and activator of transcription.

The identification of a novel inter-play between the JAKs and MAPKs in the context of chemoresistance opens new avenues for treatment, which urgently warrant additional studies.

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1. INTRODUCTION

1.1. CANCER

In many countries more than a quarter of deaths are attributable to cancer. In the year 2000, malignant tumours were responsible for 12 per cent of the nearly 56 million deaths worldwide from all causes. According to the World Cancer Report the main cancer risk factors are tobacco consumption, infectious agents, late diagnosis in developing countries, and the Western lifestyle (World Health Organisation, 2003). Cancer control is the aim of many institutions and government policies whose main strategies and goals include prevention, early diagnosis and treatment. Breast cancer is the most prevalent type of cancer in the world (4.4 million survivors up to 5 years following diagnosis) (Parkin *et al.*, 2005). Lung cancer is the most frequently diagnosed type of cancer (1.35 million worldwide *per year*) and the most common cause of death from cancer (1.18 million deaths worldwide *per year*) (Fig. 1).

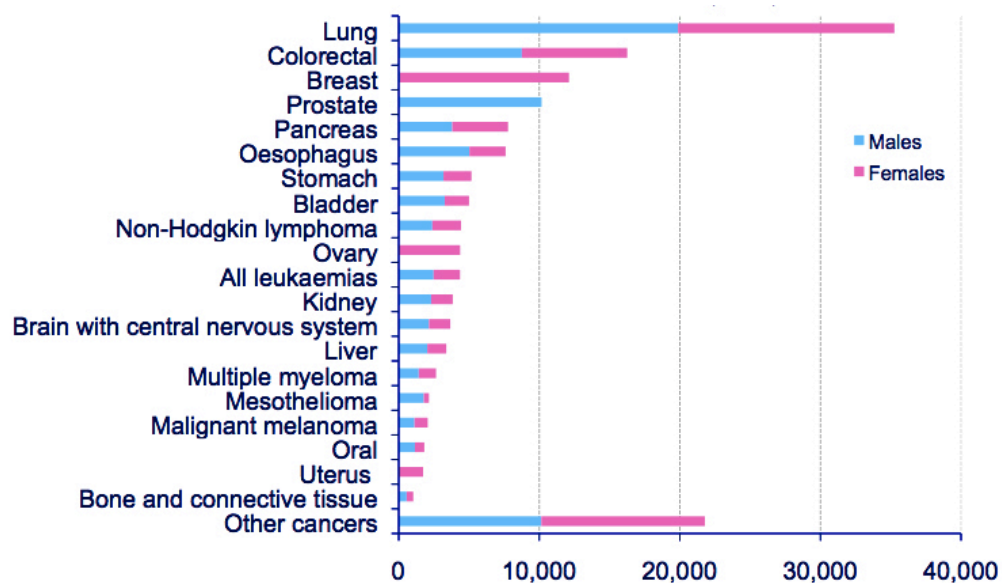


Figure 1. The twenty most common causes of death from cancer in the United Kingdom in 2008. Chart obtained from the Cancer Research UK (CR UK) website (<http://info.Cancerresearchuk.org/cancerstats/mortality/cancerdeaths/>).

In the present study, we have focused on lung cancer and aimed to understand some aspects of the molecular basis of the resistance to chemotherapy. Furthermore, we investigated these mechanisms in another type of cancer, namely bone cancer.

1.1.1. CHEMOTHERAPY

The discovery of chemotherapeutic agents began at the start of the 20th century with the development of models to screen chemicals that could treat cancer (reviewed by DeVita and Chu, 2008). Today, chemotherapy is standard clinical practice in conjunction with surgery and/or radiation to treat cancer patients. Successful treatment has been achieved in subsets of patients with a variety of tumour types such as lymphoma, small cell lung cancer (SCLC), ovarian cancer, and choriocarcinoma. Over the past decade chemotherapy has been widely used for rectal, bladder, breast, gastroesophageal, head and neck cancers, and sarcomas (DeVita and Chu, 2008).

Two commonly used chemotherapeutic drugs used to treat many cancer types are cisplatin and etoposide. Due to their relevance for the present study their mechanism of action is briefly described in this section.

Cisplatin's primary target is DNA and it acts by inducing the formation of DNA adducts (Pinto and Lippard, 1985). Cisplatin's platinum atoms form covalent bonds with the N⁷ positions of purine bases of DNA promoting the formation of DNA-protein and/or DNA-DNA inter- and intra-strand crosslinks (Jamieson and Lippard, 1999). The formation of these lesions activates several signal transduction pathways, including pathways that lead to the activation of apoptosis by mechanisms that are not fully understood (Siddik, 2003). For example, cisplatin was shown to be able to induce apoptosis by promoting the clustering of the death receptor Fas in a Fas ligand-independent fashion on the cell surface (Micheau *et al.*, 1999). Many cells are, however, resistant to Fas-induced apoptosis (e.g. O'Connell *et al.*, 2000; Mizuno *et al.*, 2003; Yang *et al.*, 2007; Bertrand *et al.*, 2009) and thus additional death activating pathways must be triggered by cisplatin. Cisplatin can also induce changes in the fluidity of cellular membranes (Dimanche-Boitrel *et al.*, 2005) and, in addition, interact with the plasma membrane, reducing the activity of certain ion channels and transport proteins (Grunicke and Hofmann, 1992).

The main target of etoposide is DNA-topoisomerase II (Bromberg *et al.*, 2003). Topoisomerase II is a DNA-strand passing enzyme that is able to create a transient

double-strand break in a DNA molecule, allowing the ATP-dependent passage of one DNA strand through another (Wang, 2002). This enzyme participates in several cellular processes such as DNA replication and transcription by reducing DNA superhelicity through the removal of DNA intertwinings that naturally arise from these processes. Topoisomerase II can resolve intertwinings between two catenated DNA molecules, which is a prerequisite for proper chromosome segregation. Indeed, in several organisms, mutations or drugs that block topoisomerase II decatenating activity severely affect chromosome segregation as the concatamers between sister chromatids physically constrain their segregation (Holm *et al.*, 1985; Uemura *et al.*, 1987; Clarke *et al.*, 1993; Gorbsky, 1994; Gimenez-Abian *et al.*, 1995). Etoposide was shown to stabilise the enzyme-cleaved DNA complex that is normally a transient intermediate in the catalytic cycle of topoisomerase II (reviewed by Baldwin and Osheroff, 2005). The accumulation of these cleavage complexes is thought to induce permanent DNA breaks. This, in turn, can trigger programmed death pathways (Kaufmann, 1998; Li and Liu, 2001). The precise mechanisms of action of etoposide, however, remain to be fully elucidated.

The use of cisplatin and etoposide, as well as many other chemotherapeutic drugs, in the clinic aims to activate, by and large, cell death pathways (apoptosis) in cancer cells.

1.1.2. LUNG CANCER

Lung cancer is nowadays the leading cause of cancer mortality (Jemal *et al.*, 2008). The main causal agent is smoking and the disease has no particular symptoms or signs that can allow for its detection at an early stage (Spiro and Silvestri, 2005). Although surgery offers the best chance of cure in lung cancer, only a small proportion of patients are ever suitable for curative resection and the majority must rely on non-surgical and adjuvant therapies. Lung cancer has a median survival period of 6-12 months from the time of diagnosis, with an overall 5-year survival rate of 5-10% (Spiro and Porter, 2002). The cure rate is extremely low largely because the disease readily metastasises and because the tumour eventually becomes resistant to available chemotherapy.

Lung tumours arise from cells of the respiratory epithelium that become malignant. Histopathologically, lung cancer comprises two main tumour types: non-

small cell lung cancer (NSCLC), observed in 75-80% of the cases, and SCLC, which comprises 20-25% of all lung cancer cases (Spiro and Porter, 2002). Although the incidence of NSCLC is higher, SCLC remains the most aggressive form of the lung cancer types, with a median survival time without treatment of 2-4 months (Ilhde, 1995). Surgical resection or radical radiotherapy is able to cure only 10% of all patients with NSCLC. Since SCLC is characterised by the early development of metastases, this type of tumour is usually unsuitable for surgical resection. The cure of many lung cancer patients tends, therefore, to rely on alternative approaches. Chemotherapy has been evaluated as neoadjuvant and adjuvant treatment around surgery, neoadjuvant and adjuvant upon radiotherapy, and as the primary treatment for advanced inoperable disease (Spiro and Porter, 2002). Standard chemotherapy for lung cancer consists of a combination of two or more drugs. Although etoposide and cisplatin are two of the most potent anti-tumour agents, cells often find a means to attenuate DNA damage-mediated apoptotic signals, which can result in the development of chemoresistance. Chemoresistance is, thus, a major limitation in the treatment of lung cancer patients.

Over the last decade newer chemotherapeutic agents have emerged and provided slightly better survival rates in lung cancer. For patients with NSCLC chemotherapy offers only a modest survival improvement. Agents such as navelbine, gemcitabine and taxanes combined with platinum were shown to be better tolerated, resulting in an improved quality of life for patients with advanced NSCLC (Buter and Giaccone, 2005). The management of SCLC, which appeared so promising at the beginning of the 1970s, has advanced very little in outcome over the last 15 years. Indeed, SCLC remains one of the most frustrating cancers to treat (Thatcher *et al.*, 2005). Etoposide, cisplatin, carboplatin, cyclophosphamide, ifosfamide, doxorubicin, vincristine and, more recently, topotecan, irinotecan and paclitaxel are drugs that are being used, or have been trialled, in SCLC patients. Initial treatment of SCLC is often effective but patients usually relapse within a very short period of time (**Fig. 2**).

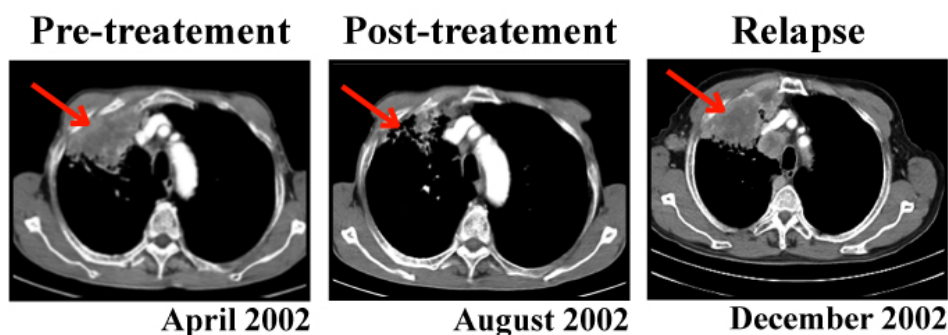


Figure 2. Relapse following successful chemotherapy in a patient with SCLC. Images from a positron emission tomography (PET) scan of a lung cancer patient treated with chemotherapeutic drugs. Pictures correspond to a latitudinal section of the chest showing the lung cavity. After treatment with chemotherapeutic drugs the tumour (indicated with red arrows) was substantially reduced. The patient relapsed just a few months after treatment. Images were courtesy of Professor Michael Seckl.

Although initial chemotherapeutic treatment appears to be effective against lung cancer, treatment with second-line chemotherapy at relapse has a disappointing effect, with few responses and the almost inevitable development of resistance. Continued efforts to understand the biology of the disease and the mechanism by which tumour cells are, or become, resistant to chemotherapy are, however, driving the discovery of new therapies.

Because smoking is the main casual agent of lung cancer in 80-90% of cases, the most important and cost-effective management for this disease is smoking cessation. Nevertheless, for those with the disease, unravelling chemoresistance mechanisms for the development of effective treatment strategies is still a priority.

1.1.3. BONE CANCER

Bone cancer is classified in different types based on its histology: osteosarcoma is the most frequently diagnosed primary sarcoma of bone, followed by chondrosarcoma, Ewing's sarcoma, chordoma and malignant fibrous histiocytoma,

which includes fibrosarcoma (Dorfman and Czerniak, 1995). Osteosarcoma and Ewing's sarcoma may arise at any age but are typically found in children and adolescents. Classical chondrosarcoma typically presents later in adulthood (Bielack and Carrle, 2008). Exposure to radiation is known to be an exogenous risk factor but just occurs in adults and is very rare (Longhi *et al.*, 2003). The majority of osteosarcomas occur without a familial predisposition or a history of exposure to radiation. A number of rare inherited syndromes were linked to this type of cancer and indeed several genetic alterations have been found to be associated with osteosarcoma (Hansen, 1991). Viruses can also induce bone sarcomas in animals and humans (Finkel *et al.*, 1966; Ganem, 2006).

Osteosarcoma can develop anywhere in the skeleton, but the most common places are in the legs or upper arms. It is generally a highly aggressive tumour that metastasises primarily to the lung (Longhi *et al.*, 2006). The application of chemotherapy substantially improved the 5-year survival rate that was only 10% until 30 years ago (Bacci *et al.*, 2000; Longhi *et al.*, 2006; Bielack *et al.*, 2009). Cisplatin, doxorubicin, methotrexate and ifosfamide are considered the most active agents against osteosarcoma (Bielack *et al.*, 2004; Ta *et al.*, 2009). Usually successful treatments involve the combination of two or more drugs. As for many cancers, poor response to chemotherapy is associated with a lower survival rate (Bacci *et al.*, 2000). Second line treatments have been extremely discouraging and local or metastatic recurrences have poor outcomes, with overall survival rates of only 10-20% (Bielack and Carrle, 2008). Nowadays, at relapse, surgery is still the main option when possible. Accordingly, for relapsing patients new strategies must be explored.

There are currently various different ways to treat cancer, which include surgery, radiotherapy, chemotherapy, hormone therapy, immunotherapy, and gene therapy (the latter still being the object of intense research) (Cancer Research UK, 2002). Chemotherapy is one of the most commonly used cancer treatments, but its success is largely affected by some important factors including systemic toxicity (resulting in unpleasant side effects), rapid drug metabolism (resulting in the reduction of drug action duration) and resistance, to mention just a few.

1.1.4. CHEMORESISTANCE

Resistance to chemotherapeutic drugs is probably the major obstacle to the successful treatment of many tumour types. Chemotherapeutic agents were developed based on the observation that tumour cells proliferate faster than normal cells (Johnstone *et al.*, 2002). Therefore, chemotherapeutic treatments used often induce cell cycle arrest and/or cell death through the upregulation of apoptosis, senescence and/or mitotic catastrophe.

Defects in apoptotic pathways or acquired resistance to apoptosis (resistance that occurs as a result of an alteration, such as a mutation in the cell, which favours cell survival) can, however, lead to expansion of a population of tumour cells and/or increase the escape from surveillance by the immune system. This can ultimately lead to resistance to chemotherapeutic agents and treatment failure (Igney and Krammer, 2002). Chemoresistance was initially linked to reduced drug concentrations due to the action of efflux pumps and to detoxifying enzymes. It is now clear that tumour cells also escape cytotoxic treatments by several other mechanisms including enhanced DNA repair activity and reprogramming of survival signalling pathways (e.g. O'Driscoll, 2009). It has been proposed that oncogenes may provide cancer cells with intrinsic resistance capacity ("natural" resistance that is inherent to a cell as a result of the cell's genetic makeup) and that inhibition of apoptosis may confer cells with the ability to survive better in a stressful environment (Johnstone *et al.*, 2002).

Chemoresistance can arise through a number of different molecular mechanisms and, unsurprisingly, evasion from apoptosis is a prominent hallmark of cancer (Hanahan and Weinberg, 2000). Inappropriate control of cell death has also been implicated in the pathogenesis of a number of other human diseases including viral infections, autoimmune diseases, neurodegenerative disorders and acquired immune deficiency syndrome (AIDS) (Thompson, 1995).

1.1.5. APOPTOSIS

Apoptosis is a cell death mechanism that can be induced by exogenous (e.g. radiation and chemotherapeutic agents) and endogenous (e.g. DNA damage, oxidative stress, ATP deprivation) stimuli. Some apoptotic processes are actually "programmed" and part of normal cellular homeostasis. For this reason, apoptosis is sometimes referred to as programmed cell death (PCD). This process is, in fact, crucial for the

development, health and integrity of multicellular organisms contributing to the fine balance between cell proliferation and cell death, thereby allowing the maintenance of a constant total number of cells in the body (Kerr *et al.*, 1972; Wyllie *et al.*, 1980; Kerr, 2002). It is generally accepted that the apoptotic program is the default cellular setting present in a latent form in virtually all the cells in the body, which must be actively suppressed for the cells to survive. Morphologically it is characterised by several cellular modifications such as membrane blebbing, cell shrinkage due to the extrusion of water and chromatin condensation. Intracellular events include fragmentation and loss of DNA content, cytoskeletal alterations and protease activation (reviewed by Danial and Korsmeyer, 2004).

PCD is a multistep process that can be initiated by many different stimuli. The subsequent effector phase converges into a few common pathways that inevitably lead to the loss of cell viability (Kroemer *et al.*, 1995). In mammalian cells, pathways leading to apoptosis converge on the selective proteolytic cleavage of a particular family of proteins termed cysteine-aspartic acid proteases (caspases) (Earnshaw *et al.*, 1999). Caspases are regulated at the post-translational level, which helps to ensure they can be quickly activated following an appropriate stimulus. They are found in the cytosol as inactive pro-enzymes that become activated upon cleavage thereby activating downstream effectors such as other caspases and the nuclear proteins poly(ADP-ribose) polymerase (PARP) and lamin B, which were shown to be useful markers of apoptosis (Lazebnik *et al.*, 1993; Lazebnik *et al.*, 1994). Caspases also play key roles in the regulation of the immune system by virtue of being required for the maturation of cytokines (e.g. caspase 1) and skin development (e.g. caspase 14) (Kuida *et al.*, 1995; Li *et al.*, 1995; Denecker *et al.*, 2008).

1.1.5.1. REGULATION OF APOPTOSIS

Mitochondria play an important role in the regulation of cell death signals, activating caspases and triggering other cell death events (Breckenridge and Xue, 2004). An example of important pro-apoptotic factors released by these organelles is cytochrome *c*. The latter activates caspases through cytosolic apoptotic protease activating factor-1 (APAF-1) (Li *et al.*, 1997) and second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (SMAC/DIABLO), which in turn promote caspase activation by binding to inhibitor of

apoptosis proteins (IAPs) and block their caspase inhibitory activity (Du *et al.*, 2000; Verhagen *et al.*, 2000). IAP family members control apoptosis by regulating the activity of caspases either by inducing their degradation or by directly inhibiting their enzymatic activity (Salvesen and Duckett, 2002; Riedl and Shi, 2004; Vaux and Silke, 2005). The most intensively studied IAP family member, X-linked IAP (XIAP), is a potent inhibitor that directly blocks caspase activity (Eckelman *et al.*, 2006).

There are two main apoptotic pathways: the intrinsic pathway (also called the mitochondrial pathway as these organelles play a pivotal role in the execution of apoptosis) and the extrinsic pathway (also called the death receptor pathway as it is initiated by cross-linking of death receptors) (Green and Evan, 2002). The intrinsic pathway is initiated from within the cell in response to cellular signals that result from severe cell stress, such as DNA damage, viral infection, detachment from the extracellular matrix and hypoxia. It predominantly leads to the activation of caspase 9 (Hakem *et al.*, 1998; Jiang and Wang, 2004). The extrinsic pathway is triggered by death receptors, such as the tumour necrosis factor (TNF) receptor or the TNF-related apoptosis inducing ligand (TRAIL) receptor (reviewed by Papenfuss *et al.*, 2008). These receptors contain an intracellular death domain, which can recruit and activate caspase 8 or 10 through the adaptor protein Fas-associated death domain (FADD), and subsequently downstream effector caspases (Ashkenazi and Dixit, 1998; Debatin and Krammer, 2004). A simplified diagram of the main events involved in intrinsic and extrinsic pathways of apoptosis is shown in **Figure 3**.

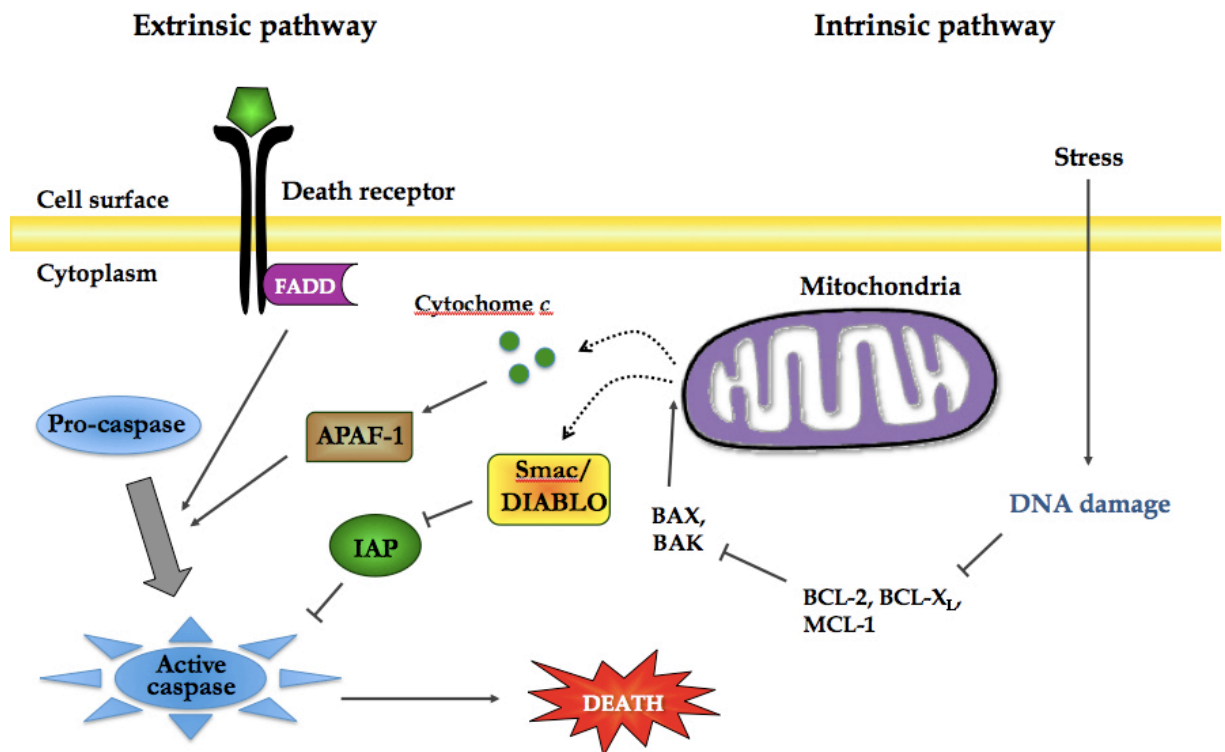


Figure 3. Simplified diagram of the main events in the intrinsic and extrinsic apoptotic pathways. The extrinsic pathway of apoptosis is triggered by death receptors while the intrinsic pathway is initiated from within the cells in response to cellular signals resulting from severe cell stress. Both pathways activate initiating caspases followed by downstream activation of effector caspases to trigger cell death. Pro-apoptotic BCL-2 family members such as BAX and BAK induce permeabilisation of the outer mitochondrial membrane and subsequent release of apoptotic molecules. The anti-apoptotic family members repress apoptosis by inhibiting pro-apoptotic proteins and the mitochondrial release of apoptotic molecules. (Figure adapted from Papenfuss *et al.*, 2008).

The B-cell leukaemia/lymphoma-2 (BCL-2) family of proteins, which reside in the mitochondrial membrane, regulate the permeability of the mitochondrial outer membrane and the release of pro-apoptotic proteins from the intermembranar space (reviewed by Youle and Strasser, 2008). Different family members of the BCL-2 family can have opposing apoptotic activities. Accordingly, BCL-2 proteins can be classified into three classes based on their functions and the presence of BCL-2 homology (BH) domains: **i)** the anti-apoptotic subfamily includes members such as BCL-2, BCL-2-like 1 protein (BCL-X_L) and myeloid cell leukaemia sequence 1 (MCL-1), which are characterised by the presence of four BH domains; **ii)** the pro-apoptotic subfamily encompasses members such as BCL-2-associated X protein (BAX) and BCL-2-

antagonist/killer (BAK), which have three BH domains; and **iii**) the divergent class BH3-only proteins subfamily includes the pro-apoptotic proteins BCL-2-associated agonist of cell death (BAD) and BCL-2-interacting killer (BIK). The interplay between the different classes of BCL-2 family proteins is fundamental in determining the mitochondrial response and thus cellular outcome. Pro-apoptotic family members such as BAX and BAK are crucial for inducing permeabilisation of the outer mitochondrial membrane, and the subsequent release of apoptotic molecules (Wei *et al.*, 2001; Zong *et al.*, 2001). The anti-apoptotic family members such as BCL-2, BCL-X_L and MCL-1 repress apoptosis by inhibiting BAX, BAK and/or BH3-only proteins (e.g. Gross *et al.*, 1999; Cheng *et al.*, 2001). Activation of BCL-2 proteins can occur by different mechanisms, which include **i**) phosphorylation: for example, BAD is activated by loss of phosphorylation in response to growth factor deprivation (Zha *et al.*, 1996); **ii**) proteolysis: for example, BID is activated by caspase 8-mediated cleavage (Li *et al.*, 1998; Luo *et al.*, 1998); or **iii**) regulation of their expression levels: as examples, BCL-X_L can be transcriptionally, or translationally induced by growth factors (Grad *et al.*, 2000; Pardo *et al.*, 2002), and MCL-1 is rapidly degraded by the ubiquitin-proteasome pathway in response to death stimuli (Cuconati *et al.*, 2003; Zhong *et al.*, 2005).

1.1.5.2. APOPTOSIS AND CANCER

Every cell in a multicellular organism has the potential to die by apoptosis, but tumour cells often use alternative pathways that allow them to escape death. Modifications of apoptotic pathways can not only lead to an increase in tumour volume, but also have the potential to confer resistance to therapy (Igney and Krammer, 2002). For example, mutations and over-expression of anti-apoptotic BCL-2 family members has been shown to contribute to tumour progression and to increase resistance to chemotherapeutic drugs. Not surprisingly, such molecular alterations have been identified in numerous types of cancer (Ayhan *et al.*, 1994; Coustan-Smith *et al.*, 1996; Kaiser *et al.*, 1996; Krajewska *et al.*, 1996; Gobe *et al.*, 2002). Because cancer cells often exhibit a dependency on particular pathways for survival through an active process previously described as “oncogene addiction” (Sharma *et al.*, 2006), the identification of oncogenes and a more detailed understanding of the mechanisms involved in cancer cells’ intrinsic resistance capacity is crucial to develop more effective cancer therapies.

1.2. THE MAIN SIGNALLING PATHWAYS OF CANCER

A number of pathways have been reported to be involved in determining life and death at the cellular level by regulating components of the apoptotic machinery. Oncogenic signalling pathways such as the ras viral oncogene homolog (**RAS**)/ raf murine leukaemia viral oncogene homolog (**RAF**)/ mitogen-activated protein kinase kinase (**MEK**)/ extracellular regulated kinase (**ERK**) pathway, the phosphoinositide 3-kinase (**PI3K**)/ phosphatase and tensin homologue deleted on chromosome 10 (**PTEN**)/ protein kinase B (**PKB**)/ mammalian target of rapamycin (**mTOR**) pathway and janus kinase (**JAK**)/ signal transducer and activators of transcription (**STAT**) pathway have been studied in great detail (Bowman *et al.*, 2000; Bromberg and Darnell, 2000; Hanahan and Weinberg, 2000; Ballif and Blenis, 2001; Cantley *et al.*, 2002; Vivanco *et al.*, 2002; Yu and Jove, 2004; Steelman *et al.*, 2008). As these pathways are particularly relevant to this study they will be described in some detail below.

1.2.1. THE RAS/RAF/MEK/ERK SIGNALLING PATHWAY

The RAS/RAF/MEK/ERK signalling pathway is an evolutionarily highly conserved pathway that can control several fundamental cellular processes, such as proliferation, differentiation, survival and apoptosis (reviewed by Kolch, 2000; Ballif and Blenis, 2001). This pathway can be activated by many stimuli and can have distinct biological outcomes depending on a variety of factors that determine the environment in which the signal is generated and received (e.g. concentration of molecules involved; negative feedback mechanisms; particular mutations). Spatial distribution and kinetics of ERK activation are, in fact, critical to the cellular outcome of RAS/RAF/MEK/ERK pathway (Murphy and Blenis, 2006).

RAS proteins are small GTPases that function as molecular switches controlling the signals from cell surface receptors to a panel of intracellular cascades. Upon ligand binding (e.g. growth factors, or cytokines), membrane-bound RAS is activated by the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) by guanine nucleotide-exchange factors (GEFs). This results in a conformational change that reveals an effector interaction site (Omerovic and Prior, 2009). RAS-GTP recruits RAF to the plasma membrane where it becomes phosphorylated on tyrosine residues by membrane-bound or receptor-associated tyrosine kinases (Marais *et al.*, 1995). RAF is a multigene kinase family that comprises A-RAF, B-RAF and C-RAF (Chong *et al.*,

2003). These three proteins play distinct functions (Hagemann and Rapp, 1999; Wellbrock *et al.*, 2004). Knockout mice showed that the three proteins have non-redundant roles in development. Thus, *a-raf*^{-/-} animals are viable but the mice die 7-21 days after birth due to neurological and gastrointestinal defects (Pritchard *et al.*, 1996). In contrast, *b-raf*^{-/-} and *c-raf*^{-/-} embryos die, the first due to growth retardation, and vascular and neuronal defects and the second due to massive liver apoptosis (Wojnowski *et al.*, 1997; Wojnowski *et al.*, 1998; Mikula *et al.*, 2001). RAF proteins are composed of three conserved regions (**Fig. 4**): CR1 that contains a RAS binding domain (RBD) and a cysteine-rich domain (CRD); CR2, which can be phosphorylated and serves as a regulatory domain controlling the localisation and activation of RAF; and CR3, the catalytic kinase domain that is also regulated by phosphorylation (Morrison *et al.*, 1993; Vojtek *et al.*, 1993; Nassar *et al.*, 1995; Morrison and Cutler, 1997; Cutler *et al.*, 1998; Zhang and Guan, 2000; Chong *et al.*, 2001).

Regulation of RAF kinases is a complex process involving membrane recruitment, dimerisation or oligomerisation, interactions with other proteins, conformational changes, and phosphorylation of regulatory and catalytic domains of the protein (Chong *et al.*, 2003). The three RAF isoforms have different repertoires of phosphorylation sites (**Fig. 4**) and the phosphorylation of different sites results in differences in their regulation (reviewed by Wellbrock *et al.*, 2004). Phosphorylation residues are conserved in A-RAF and C-RAF, and these two proteins are activated by similar mechanisms (Marais *et al.*, 1997). B-RAF shares some phosphorylation sites with the other two members but has unique features. In the negative-charge regulatory region (N-region), located in the CR2 domain, B-RAF has a constitutively phosphorylated serine (highlighted with a yellow star in **Figure 4**) and an aspartic acid instead of a tyrosine residue (highlighted with a blue box in **Figure 4**) (Mason *et al.*, 1996; Brummer *et al.*, 2006). This latter residue confers a constant negative charge in the N-region and thus B-RAF has an elevated basal kinase activity compared to other RAF isoforms. Although B-RAF has a conformation typical of active kinases, an interaction between a glycine-rich loop and the activation segment displaces the activation segment (Wan *et al.*, 2004). The phosphorylated threonine and serine residues required for B-RAF activation are located in this segment (Zhang *et al.*, 2000).

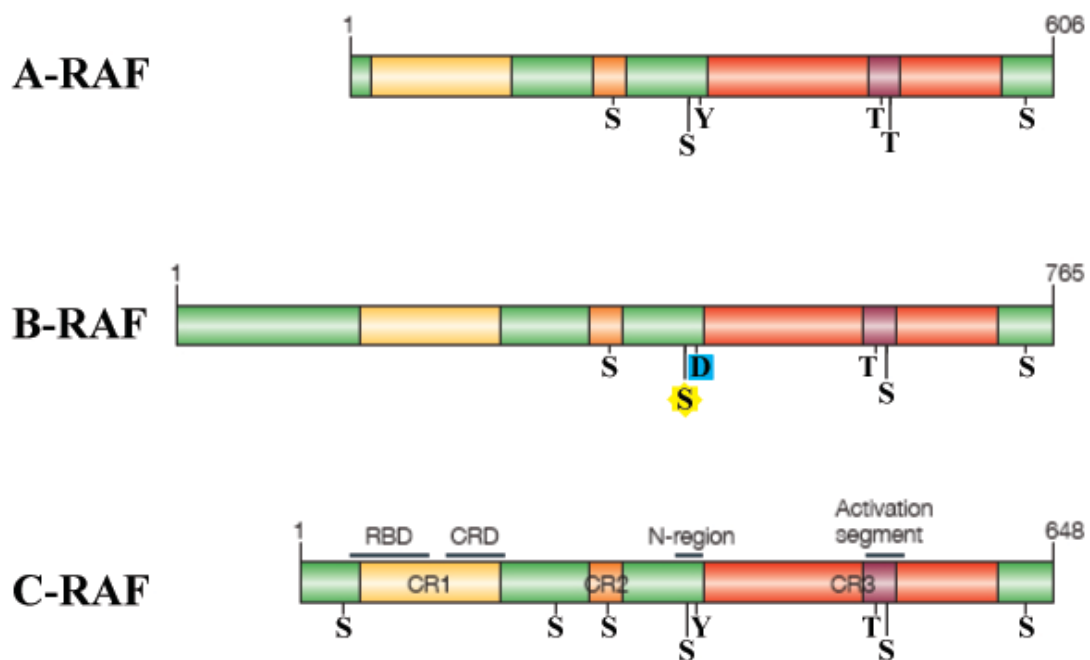


Figure 4. Structural organisation of RAF proteins. Three conserved regions are shared by RAF family members: the CR1 (yellow) that contains a RAS-binding domain (RBD) and a cysteine-rich domain (CRD), the CR2 (orange) that contains a negative-charge regulatory region (N-region) and the CR3 (red) that contains an activation segment (purple). Known phosphorylated sites – tyrosine (Y), serine (S), threonine (T) and aspartic acid (D) – are indicated. The serine in the N-region is conserved among isoforms but is constitutively phosphorylated in B-RAF (highlighted with a yellow star). In the same region, the tyrosine identified in A-RAF and C-RAF is replaced by an aspartic acid in B-RAF (highlighted with a blue box). (Figure adapted from Wellbrock *et al.*, 2004).

The activation of RAF leads to the phosphorylation of MEK on two serine residues (Seger *et al.*, 1992; Alessi *et al.*, 1994; Papin *et al.*, 1995; Schaeffer and Weber, 1999; Kolch, 2000). B-RAF was shown to be the RAF family member that more efficiently phosphorylated MEK (Papin *et al.*, 1996; Marais *et al.*, 1997) and it was therefore proposed to be its main activator (Wellbrock *et al.*, 2004). The expression of an inducible form of B-RAF promoted a more rapid and robust activity of both MEK and ERK than did the equivalent C-RAF and A-RAF forms (Pritchard *et al.*, 1995). In addition, ERK activation can fully occur in mouse embryonic fibroblasts (MEFs) from mice that lack *a-raf* and *c-raf* (Mikula *et al.*, 2001; Huser *et al.*, 2001; Mercer *et al.*, 2002), whereas the same cell type from *b-raf*-null mice have compromised ERK activity (Wojnowski *et al.*, 2000).

Activated MEK phosphorylates ERK1 and ERK2 on specific threonine and tyrosine residues within their activation segments (Boulton *et al.*, 1991). ERK1/2 are serine/threonine kinases that have 83% homology with most differences being mapped to the kinase domains (Chen *et al.*, 2001). Although they are activated by the same upstream kinases, display identical kinetics of action and seem to have the same substrate specificities (Yoon and Seger, 2006), some studies suggest that ERK1 and 2 have critical functional differences that result in non-overlapping functions. For example, mutation of the *erk2* gene in mice causes early embryonic lethality (Saba-El-Leil *et al.*, 2003), while *erk1*^{-/-} mice are viable and reproduce normally, presenting only minor defects such as a deficit in thymocyte maturation (Pages *et al.*, 1999). It has been suggested that *erk2* can compensate for most of the functions of *erk1*, but the contrasting phenotypes seem to indicate that the two kinases also have distinct functions (Aouadi *et al.*, 2006). Interestingly, significant differences between the two erk proteins were found in the control of cell proliferation. While the silencing of *erk1* facilitated cell growth, silencing of *erk2* caused severe cell-proliferation deficits as demonstrated in MEF cells, liver cancer cell lines and murine hepatocytes (Vantaggiato *et al.*, 2006; Bessard *et al.*, 2008; Fremin *et al.*, 2009).

Once activated, ERK1/2, which are also called mitogen-activated protein kinases (MAPKs), can phosphorylate numerous substrates in all cellular compartments including phospholipases, transcription factors, cytoskeletal proteins and protein kinases termed MAPK-activated protein kinases (MKs). MKs include, for example, ribosomal S6 kinases (RSKs) and mitogen- and stress-activated kinases (MSKs) (reviewed by Roux and Blenis, 2004). Upon stimulation a significant population of ERK1/2 accumulates in the nucleus (Khokhlatchev *et al.*, 1998; Lenormand *et al.*, 1998). It has been demonstrated that ERKs continuously shuttle between the cytoplasm and the nucleus depending on their activation status (Ando *et al.*, 2004; Costa *et al.*, 2006; Fujioka *et al.*, 2006). The maintenance of a pool of nuclear active kinases therefore requires continuous activation of the MEK pathway (Costa *et al.*, 2006). ERK1 shuttling through the nuclear membrane was found to be slower than ERK2, which was caused by the presence of a unique region located at the N-terminal (residues 8-39) of ERK1 (Marchi *et al.*, 2008). This difference may explain some of the putative non-redundant roles of ERK1 and ERK2.

In the nucleus, activated ERKs can control transcription at several levels by phosphorylating transcription factors. These transcription factors, once activated, bind to the promoters of many genes required for cell growth and survival and/or initiate

chromatin modifications along inducible genes thereby modulating the transcriptional response (Edmunds and Mahadevan, 2004).

The RAS/RAF/MEK/ERK pathway is tightly regulated through the coordinated action of positive and negative regulators. Activation and deactivation of this signal transduction cascade is regulated by kinases as well as phosphatases. These act at different levels of the signalling cascade, at different times and on different cellular compartments. For example, the regulation of RAF proteins activation is tightly controlled by phosphorylation and dephosphorylation events. The phosphorylation of the residues Ser²⁵⁹ (within the CR2 domain) and Ser⁶²¹ (within the CR3 domain) of C-RAF provides docking sites for 14-3-3 proteins, which regulate its activation through complex mechanisms (Morrison *et al.*, 1993; Muslin *et al.*, 1996). It was proposed that 14-3-3 dimers can maintain C-RAF in a inactive conformation (binding to both Ser⁶²¹ and Ser²⁵⁹), as well as stabilise an active form upon stimulation (binding to Ser⁶²¹) (Tzivion *et al.*, 1998; Hekman *et al.*, 2004). Accordingly, the phosphorylation of Ser²⁵⁹ of C-RAF was shown to inhibit C-RAF activation (Morrison *et al.*, 1993; Dhilon *et al.*, 2002; Kubicek *et al.*, 2002), while the phosphorylation of Ser⁶²¹ seems to be essential for its activation (Tzivion *et al.*, 1998; Hekman *et al.*, 2004). In addition, inactivation of C-RAF can also be mediated by protein phosphatase 5 (PP5) that selectively dephosphorylates the essential activating residue Ser³³⁸ (within the N-region) (von Kriegsheim *et al.*, 2006). Adaptor/scaffold proteins have an important role in modulating the RAS/RAF/MEK/ERK pathway (Kolch, 2000). Scaffold proteins interact with components of the pathway, stabilising and coordinating interactions between them thus increasing the efficiency and fidelity of signal transduction (Morrison and Davis, 2003; Pouyssegur *et al.*, 2003). One example of such a scaffold protein is the kinase suppressor of RAS (KSR) that coordinates the assembly of MEK with C-RAF at the plasma membrane, and then recruits ERK to be subsequently phosphorylated by MEK (Morrison, 2001).

Deregulated RAS/RAF/MEK/ERK signalling has been associated with many human cancers (Hoshino *et al.*, 1999). In particular, oncogenic mutations in RAS and RAF were identified in a significant percentage of cancers. These mutations are believed to contribute to tumourigenesis and cell growth (Bos, 1989; Davies *et al.*, 2002). B-RAF in particular was found mutated in a wide range of human cancers, including 66% of malignant melanomas and in a smaller percentage of lung cancers (3%) and sarcomas (9%) (Davies *et al.*, 2002). Over 45 mutations have been identified in B-RAF and, most importantly, the mutation of the residue 599 (V599E) has been found to account for over 90% of B-RAF mutations and approximately 86% of all mutations in

cancer (reviewed by Garnett and Marais, 2004). In addition, the RAS/RAF/MEK/ERK pathway is absolutely required for the control of cell survival as it modulates the activity of many proteins involved in apoptosis, including BCL-2 family members and caspases (Chang *et al.*, 2003; Troppmair and Rapp, 2003). For example, ERKs can phosphorylate MCL-1 on a threonine residue located at its proline, glutamic acid, serine and threonine (PEST) sequence, a sequence that confers proteins a signal for degradation. This then results in slower turnover of this anti-apoptotic protein, which is normally rapidly degraded (Domina *et al.*, 2004).

1.2.2. THE PI3K/PTEN/PKB/mTOR SIGNALLING PATHWAY

The PI3K/PTEN/PKB/mTOR signalling pathway also plays a central role in the control of cell growth, proliferation and survival and is therefore involved in the pathology of cancer and other disorders including metabolic, inflammatory and cardiovascular diseases (Wymann *et al.*, 2003).

Multiple forms of PI3Ks were identified in higher eukaryotes and these are classified into three classes (I-III) according to their sequence homology and substrates (Wymann and Pirola, 1998). In response to growth factors and hormones, class Ia PI3Ks phosphorylate plasma membrane-located phosphatidylinositol lipids at the 3-D position of the inositol ring converting phosphatidylinositol biphosphate (PIP₂) into phosphatidylinositol triphosphate (PIP₃) (Hawkins *et al.*, 1992). PIP₃ provides an anchor for the assembly of signalling proteins with pleckstrin-homology (PH) domains such as PKB, phosphoinositide-dependent kinase-1 (PDK1) and phospholipase C γ (PLC- γ) amongst others (Wymann and Pirola, 1998; Cantley, 2002). The PH domains specifically bind to D-3 phosphorylated phosphoinositides. PIP₃ levels are tightly regulated in unstimulated cells by phosphatases such as PTEN (Maehama and Dixon, 1998). Loss of the tumour suppressor PTEN protein or function contributes to uncontrolled signalling through PI3K and leads to increased cell survival (Maehama and Dixon, 1999).

Proteins containing a PH domain are located in the cytoplasm of unstimulated cells and when PIP₂ is converted into PIP₃ they are recruited to the plasma membrane. This brings PKB and PDK1 into proximity and promotes the phosphorylation of a threonine residue (Thr³⁰⁸) of PKB by PDK1 (Alessi *et al.*, 1997; Lawlor and Alessi, 2001). The phosphorylation of a serine residue (Ser⁴⁷³) by another kinase has also been shown

to be required for the full activation of PKB (Alessi *et al.*, 1996). PKB, also called AKT, is a member of a multigene family that, following activation by PI3K, can activate numerous downstream targets and exert a wide range of cellular functions (Kandel and Hay, 1999). Most known protein targets of PKB are inhibited by phosphorylation (Cantley, 2002). One such example is the phosphorylation of the pro-apoptotic protein BAD. BAD phosphorylation creates a binding site for the evolutionary conserved inhibitory proteins 14-3-3, which prevent BAD from binding to anti-apoptotic BCL-2 family members (Datta *et al.*, 1997; del Peso *et al.*, 1997).

Activation of PKB has been postulated to phosphorylate over 9000 proteins and plays an important role in cellular metabolic control, proliferation and growth (Lawlor and Alessi, 2001; Hay, 2005; Manning and Cantley, 2007). PKB can transduce anti-apoptotic signals *via* activation of mTOR and ribosomal protein kinases such as p70 S6 kinase (S6K) (e.g. Mahalingam and Templeton, 1996; Jonassen *et al.*, 2004; Edinger and Thompson, 2004). mTOR belongs to the family of phosphatidyl inositol kinase-like proteins, a group of serine/threonine kinases involved in cellular growth and survival, and also in cancer (reviewed by Hay, 2005; Sabatini, 2006). mTOR nucleates two distinct multi-protein complexes. When mTOR associates with the regulatory-associated protein of mTOR (Raptor) it forms a rapamycin- and nutrient-sensitive multiprotein complex [mTOR complex (mTORC) 1] and when it associates with the rapamycin-insensitive companion of mTOR (Rictor) it forms a growth-factor-sensitive but nutrient-insensitive complex (the mTORC2) (reviewed by Guertin and Sabatini, 2007). These two complexes also include other proteins that regulate their function. Activated PKB can induce the activation of mTORC1 contributing to cell growth and proliferation (Laplane and Sabatini, 2009). PKB activates mTORC1 by catalysing the phosphorylation (which leads to the inhibition) of proteins such as tuberous sclerosis complex (TSC) 2 and proline-rich AKT substrate 40 kDa (PRAS40) that are components of the complex and suppressors of its function (Inoki *et al.*, 2002; Potter *et al.*, 2002; Sankat *et al.*, 2007; Vander Harr *et al.*, 2007; Wang *et al.*, 2007a). mTOR when associated to mTORC2 was shown to be the kinase that directly phosphorylated PKB Ser⁴⁷³ residue contributing for its full activation (Sarbasov *et al.*, 2005). Activated mTOR can catalyse the phosphorylation of many other downstream targets including S6Ks (Isotani *et al.*, 1999).

Two S6K proteins were identified, α and β (S6K1/2), which share approximately 70% of homology in their protein sequences (Gout *et al.*, 1998; Shima *et al.*, 1998; Koh *et al.*, 1999; Lee-Fruman *et al.*, 1999). These proteins show a conserved domain structure consisting of four main regions: an acidic N-terminal, a catalytic

domain, a linker domain and an auto-inhibitory C-terminal domain (reviewed by Jastrzebski *et al.*, 2007) (**Fig. 5**). Both *S6K* genes code for two isoforms resulting from alternative splicing at the N-terminal, giving rise to the p70 and p85 isoforms of S6K1 and to the p54 and p60 isoforms of S6K2 (Reinhard *et al.*, 1992). The longer isoforms of S6K1/2 have a N-terminal extension that includes a nuclear localisation sequence (NLS) (Reinhard *et al.*, 1994; Minami *et al.*, 2001). The shorter S6K2 isoform can also localise to the nucleus due to the presence of an additional NLS in its C-terminal regions (Gout *et al.*, 1998; Lee-Fruman *et al.*, 1999). S6K2 is also distinguished by a proline-rich domain, which is not present in S6K1 (Gout *et al.*, 1998).

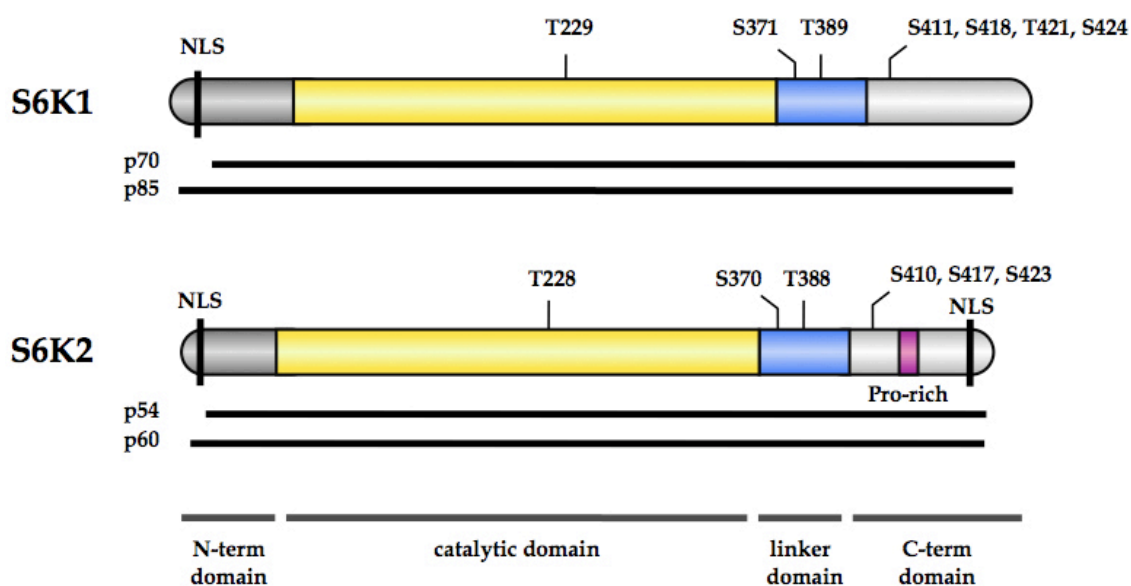


Figure 5. Structural organisation of S6K proteins. The domain structure of S6K1 and S6K2 is depicted in the figure. S6K main domains include a N-terminal (N-term) domain (dark grey), a catalytic domain (yellow), a linker domain (blue) and a C-terminal (C-Term) domain (light grey). Key activating phosphorylation sites – serine (S) and threonine (T) – are indicated. Pro-rich – Proline rich domain, NLS – nuclear localisation sequence. (Figure adapted from Jastrzebski *et al.*, 2007).

Both S6K1/2 can phosphorylate the 40S ribosomal S6 protein thereby promoting an increase of mRNA translation and protein synthesis, which are necessary for growth and proliferation (Dufner and Thomas, 1999). Despite their high homology and apparent similar substrate specificity for S6, several lines of evidence indicate that S6K1 and S6K2 have distinct biological roles. Indeed, knockout mice studies showed that *s6k1*-null mice are small (Shima *et al.*, 1998), whereas *s6k2*-null mice have no obvious phenotype (Pende *et al.*, 2004). Some mechanisms of regulation of S6K1 and S6K2 activity are highly conserved (Martin *et al.*, 2001a; Phin *et al.*, 2003). S6Ks are regulated in a complex manner involving sequential phosphorylation at multiple sites (reviewed by Jastrzebski *et al.*, 2007). Activation of S6K1 requires an initial calcium-dependent priming event that releases the interaction between the autoinhibitory C-terminal and the N-terminal domains exposing four sites (Ser⁴¹¹, Ser⁴¹⁸, Tyr⁴²¹ and Ser⁴²⁴), which are then phosphorylated by mTOR (Isotani *et al.*, 1999; Hannan *et al.*, 2003). This allows the phosphorylation of two residues in the linker domain by mTOR and one residue on the catalytic domain by PDK1 resulting in maximal activation of S6K1 (Alessi *et al.*, 1998; Burnett *et al.*, 1998; Pullen *et al.*, 1998; Isotani *et al.*, 1999). S6K1 and S6K2 were also shown to have some distinct substrates and functions. For example, S6K1 (but not S6K2) phosphorylates S6K1 Aly/REF-like target (SKAR) (Richardson *et al.*, 2004), which contribute for an increase of translation efficiency of spliced mRNA (Ma *et al.*, 2008). In addition, in SCLC cells, S6K2 was shown to play a central role unshared by S6K1 in promoting cell survival and contributing for chemoresistance (Pardo *et al.*, 2006).

S6Ks are mainly regulated by the PI3K pathway but RAS/RAF/MEK/ERK signalling can also contribute to their regulation. Activation of S6K1 was shown to occur downstream of RAS in the absence of PKB activation thought the activation of RSK1 that phosphorylates TSC1, inhibiting its ability to suppress mTOR signalling (Roux *et al.*, 2004). S6K2, but not S6K1, was also shown to be a downstream target of MEK signalling as MEK inhibitors impaired its activity (Martin *et al.*, 2001b; Pardo *et al.*, 2001; Wang *et al.*, 2001).

PI3K-induced cell survival is mainly mediated by its downstream effector PKB. Amplification of the gene encoding for PKB have been reported in many cancers including ovarian, breast and pancreatic (e.g. Bellacosa *et al.*, 1995; Cheng *et al.*, 1996). PKB can block apoptosis by phosphorylating BAD, as previously mentioned, or for example, by inducing the phosphorylation of caspase 9, which inhibits its activity (Cardone *et al.*, 1998). PKB has also been shown to be involved in RAS-mediated

inhibition of apoptosis (Khwaja *et al.*, 1997) and linked to an increased expression of the anti-apoptotic protein BCL-2 (Ahmed *et al.*, 1997).

The PI3K/PTEN/PKB/mTOR pathway is unique in that all of the major elements of the pathway have been found mutated, or amplified, in many cancers (reviewed by Yuan and Cantley, 2008). Indeed, over-activation of oncogenes, such as PI3K and PKB, or the loss of tumour suppressors, such as PTEN are known to contribute to oncogenic transformation (Aoki *et al.*, 1998; Salmena *et al.*, 2008).

PI3K targets also include protein kinases C (PKCs) (Toker and Cantley, 1997). Due to their relevance to the present study, the structure and function of PKCs will be described in detail next.

1.2.2.1. PKCs

PKC represents a family of serine/threonine kinases that transduce signals triggered by various growth factors, cytokines and hormones. They are classified based on the structural motifs that determine their requirements for activation. They are thus divided into three subfamilies that are structurally and functionally different: classic PKCs (PKC α , PKC β I, PKC β II, PKC γ), novel PKCs (PKC δ , PKC ϵ , PKC η , PKC μ , PKC θ), and atypical PKCs (PKC ζ , PKC ι/λ) (reviewed by Parker and Murray-Rust, 2004). PKC α , δ and ϵ are widely expressed, whereas expression of the other PKCs is largely cell-type specific.

PKCs are constituted by four conserved domains (**Fig. 6**): the C1 domain includes one or two cysteine-rich motifs that form a binding site for diacylglycerol (DAG) and phorbol esters (compounds that are analogues of the endogenous ligand); the C2 domain contains a calcium binding site; and the C3 and C4 domains form the catalytic domain with ATP and substrate binding sites (Mackay and Twelves, 2007). Classical PKCs can be activated by calcium, DAG and phorbol esters, whereas novel PKCs can only be activated by DAG and phorbol esters as they lack a calcium binding site. Atypical PKCs are insensitive to both calcium and DAG (Parker and Murray-Rust, 2004).

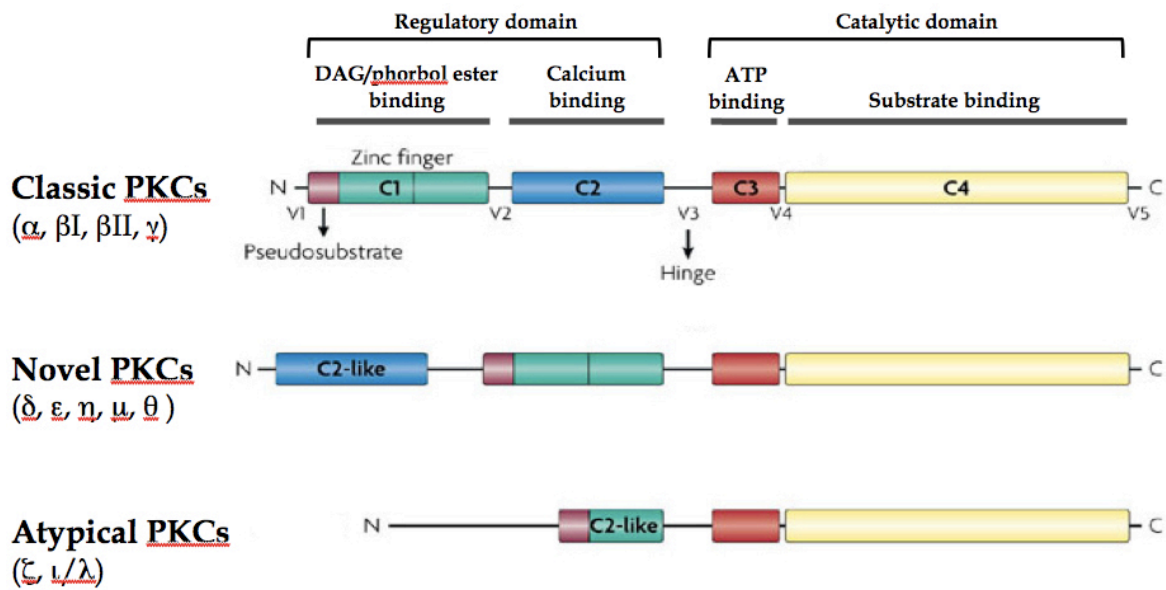


Figure 6. Structural organisation of PKC proteins. Four conserved regions (CR1-4) are shared by PKC family members. The C1 domain (green) includes one or two cysteine-rich motifs that constitute a binding site for diacylglycerol (DAG) and phorbol esters. The C2 domain (blue) contains a calcium binding site. The C3 (red) and C4 (yellow) domains bind ATP and substrate thereby providing catalytic activity to these enzymes. (Figure adapted from Mackay and Twelves, 2007).

Activation of classical and novel PKCs typically involves their recruitment and interaction with DAG. This interaction induces a conformational change that exposes binding sites for substrates and scaffolding proteins (Parekh *et al.*, 2000; Newton, 2003). In the case of classical PKCs, binding of calcium increases the rate of translocation to the plasma membrane where they bind DAG (Schaefer *et al.*, 2001). PKC activation, maturation and stability require the phosphorylation of both serine and threonine motifs. For example, activation of PKC ϵ involves the phosphorylation of the residues Thr⁵⁶⁶, located within the activation loop of the kinase domain and Ser⁷²⁹, located in the C-terminal (Parekh *et al.*, 1999; Cenni *et al.*, 2002). The phosphorylation of the threonine residue within the activation loop appears to be required for optimum activity (Parekh *et al.*, 2000) and is catalysed by PDK-1 through a mechanism similar to that leading to PKB activation previously described (Chou *et al.*, 1998; Le Good *et al.*, 1998). Phosphorylation of residues within the C-terminal loop is critical for stabilising the

kinase core, as it locks this domain in an active conformation (Newton, 2003). In this open conformation, mature PKC binds to substrates and triggers downstream signalling events. Upon stimulation, PKCs can redistribute to the nuclear membrane or to organelles such as the mitochondria or the Golgi apparatus (Wang *et al.*, 1999; Garcia-Bermejo *et al.*, 2002). Their location is determined by scaffold proteins and also by information intrinsic to individual isoforms (such as the presence of NLS) (Parker and Murray-Rust, 2004). Interaction with other proteins not only regulates PKC localisation but also brings it into closer contact with substrate proteins, thereby determining appropriate specificity of action of the different family members (Jaken and Parker, 2000).

PKC isoforms have been shown to regulate a broad range of biological functions including cell growth, differentiation and apoptosis (Dempsey *et al.*, 2000). Different isoforms can play opposing roles. For example, PKC ϵ mediates cell survival in many cell types and it achieves this by different mechanisms, which can involve the activation of PKB (Lu *et al.*, 2006) or be independent of the PI3K pathway (Pardo *et al.*, 2002). In accordance with this, the silencing of PKC ϵ was shown to induce apoptosis of glioma cells and to sensitise melanoma cells to TRAIL-induced apoptosis in melanoma cells (Gillespie *et al.*, 2005; Okhrimenko *et al.*, 2005). In contrast, PKC δ responds to many apoptotic stimuli that induce its translocation to the mitochondria, leading to the release of cytochrome *c* and caspase activation (Li *et al.*, 1999; Majumder *et al.*, 2000; Denning *et al.*, 2002). Atypical PKCs (PKC ι and PKC ζ), for example, were found to be involved in the activation of S6K1, playing an important role in cell cycle progression (Akimoto *et al.*, 1998; Romanelli *et al.*, 1999).

PKCs have also been implicated in drug resistance, as well as in invasion and metastasis of many types of cancer cells (reviewed by Griner and Kazanietz, 2007; Mackay and Twelves, 2007). Increased PKC expression has been, in fact, linked to chemoresistance in various cell types, including ovarian and lung cancer cells (Basu and Weixel, 1995; Ding *et al.*, 2002). In particular, PKC ϵ was shown to mediate prosurvival/chemoresistance in NSCLC cells by preventing the activation of caspase 9 and 3, and mitochondrial cytochrome *c* release (Ding *et al.*, 2002).

1.2.3. THE JAK/STAT SIGNALLING PATHWAY

One of the most studied and best understood signalling pathways are those involving JAKs and STATs (Murray, 2007). JAK/STAT signalling pathways are universal and essential to cytokine receptor signalling. About 40 cytokine receptors signal through combinations of four JAKs and seven STAT family members. These operate in the regulation of expression of many genes that control fundamental biological processes in cells, including cell proliferation, apoptosis, angiogenesis and immune responses and, therefore, play crucial roles in the biology of most organisms (Stark *et al.*, 1998).

1.2.3.1. BIOLOGICAL ROLE OF JAKS AND STATs

Evolutionarily conserved in eukaryotic organisms, JAK/STAT signalling pathways appear to be an early adaptation used to facilitate intercellular communication. These pathways may have co-evolved to give rise to highly adapted, ligand-specific signalling pathways that are able to control gene expression (Aaronson and Horvath, 2002). The evolutionary expansion of JAKs in higher animals is thought to have occurred concurrently with the evolution of innate and adaptive immune cells in fish, which is consistent with the multiple roles that these proteins now have in immune cells (Yamaoka *et al.*, 2004). Chromosomal distribution and homologues in model eukaryotes suggest that the STAT family arose from a single primordial gene as the need for cell-to-cell communication increased (Kisseleva *et al.*, 2002). JAK/STAT signalling pathways are activated and regulated by an infinite number of intrinsic and environmental stimuli, and this can add great plasticity (and presumably speed) to the response of a cell or tissue to any given cue.

1.2.3.1.1. THE JAKs

The four known mammalian family members, JAK1, JAK2, JAK3 and tyrosine kinase 2 (TYK2), are ubiquitously expressed with the exception of JAK3, which is preferentially expressed in leukocytes (reviewed by Schindler and Plumlee, 2008). These kinases feature seven conserved domains, termed JAK homology domains (JH)

1-7 (**Fig. 7**), where only one has catalytic activity (JH1). JH2, a pseudo kinase domain, is devoid of catalytic activity but critically regulates the activity of the kinase domain (c.f. **Section 1.2.3.3**) (Chen *et al.*, 2000; Saharinen *et al.*, 2000; Yeh *et al.*, 2000). JAKs also have a predicted SRC-homology (SH) 2-related domain. In JAK1, this domain does not perform the classical SH2 function of binding specific motifs containing a phosphorylated tyrosine residue, as it was shown to be structurally important for binding to the oncostatin M receptor (OSMR) and efficient OSMR surface expression (Radtko *et al.*, 2005). In the N-terminal region, JAKs have a four-point-one, ezrin, radixin and moesin (FERM) domain that is crucial for the association of JAKs with membrane-proximal receptor motifs (Hilkens *et al.*, 2001; Zhou *et al.*, 2001). As with other kinases, JAK activation is driven by the phosphorylation of critical tyrosine residues, which releases the blockade of the catalytic site (reviewed by Rane and Reddy, 2000; Heinrich *et al.*, 2003; Schindler and Plumlee, 2008). Phosphorylation within the activation loop is predicted to disrupt the interaction between JH1 and JH2, consequently relieving the JH2 domain inhibition of JAK's kinase activity (Saharinen *et al.*, 2003). The phosphorylated tyrosines are adjacent to each other (residues 1022/1023 in human JAK1, 1007/1008 in human JAK2, 980/981 in human JAK3, and 1054/1055 in human TYK2) and are often referred to as the pYY motif (Gauzzi *et al.*, 1996; Feng *et al.*, 1997; Liu *et al.*, 1997; Zhou *et al.*, 1997). The first of these tyrosine residues was shown to be absolutely required for the catalytic function of JAKs (Liu *et al.*, 1997; Feng *et al.*, 1997; Zhou *et al.*, 1997). Unlike what is observed for the other JAKs, the phosphorylation of JAK3 pYY motif can have opposing effects on the regulation of its catalytic activity: the phosphorylation of Tyr⁹⁸⁰ has a positive effect on kinase activity while the phosphorylation of Tyr⁹⁸¹ negatively regulates it (Zhou *et al.*, 1997). Besides these two, many other tyrosine residues that play a role in JAKs regulation were identified (e.g. JAK2 has 49 tyrosine residues but functions have not yet been attributed to all of them). These additional tyrosine residues are both positive and negative regulators of JAK function. For example, the phosphorylation of Tyr²²¹ increases the kinase activity of murine jak2, while the phosphorylation of Tyr⁵⁷⁰, located in the inhibitory JH2 domain, inhibits jak2-mediated cytokine signalling (Argetsinger *et al.*, 2004; Feener *et al.*, 2004). The phosphorylation of JAK2 Tyr⁹⁷² was also shown to play a role in the stabilisation of an active conformation necessary for its maximal function (McDoom *et al.*, 2008). Importantly, the phosphorylation of particular JAK residues was shown to provide binding sites for other signalling molecules necessary for particular biological functions mediated by the JAKs. As an example, JAK2 Tyr²⁰¹ was shown to be required for the interaction of JAK2 and SHP-2, an important tyrosine protein

phosphatase (Godeny *et al.*, 2007). The Tyr⁸¹³ of JAK2 and the correspondent Tyr⁷⁸⁵ of JAK3, located within the pseudokinase domain, were identified as autophosphorylation sites required to create a binding site for the SH2 domain-containing adapter protein SH2B- β that, in the case of JAK2, increases JAK2-mediated phosphorylation of STAT5B (Kurzer *et al.*, 2004).

JAKs play a critical role in many biological responses. *jak1* and *jak2* are essential genes as *jak1* knockout mice die perinatally due to a failure to nurse (Rodig *et al.*, 1998) and *jak2* knockout mice exhibit earlier lethality, presumably because of the critical role that *jak2* plays in erythropoiesis (Neubauer *et al.*, 1998; Parganas *et al.*, 1998). *jak3* and *tyk2* knockout mice survive but have defective cytokine responses. *jak3* knockout mice exhibit severe combined immunodeficiency (SCID)-like defects (Nosaka *et al.*, 1995; Park *et al.*, 1995; Thomis *et al.*, 1995). JAK3 mutations were also identified in patients with autosomal SCID (Russel *et al.*, 1995; Macchi *et al.*, 1995). TYK2-deficient humans exhibit a severe allergic phenotype that has been attributed to an impaired anti-microbial response (Minegishi *et al.*, 2006). In addition, mutations in JAKs are often found in patients with haematological diseases, which are described in **Section 1.2.3.4**.

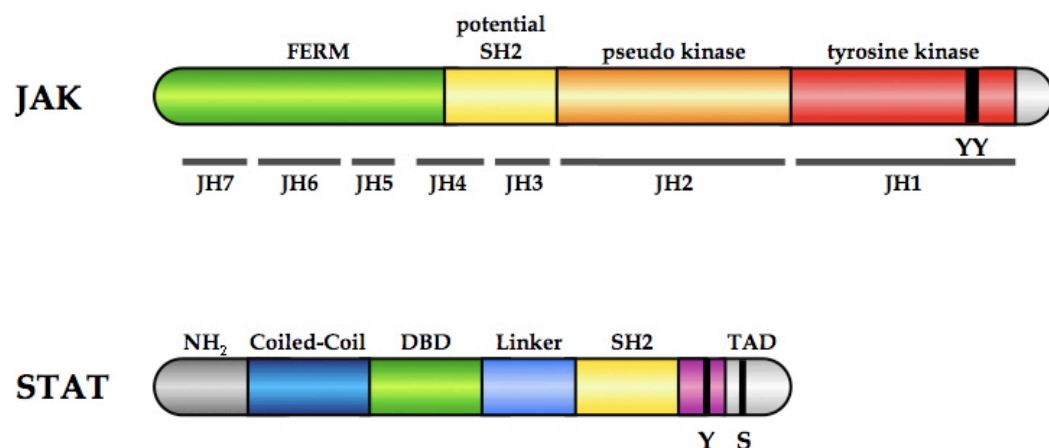


Figure 7. Structural organisation of JAK and STAT proteins. JAKs comprise seven conserved JAK homology domains (JH1-7) that form four main regions: the four-point-one, ezrin, radixin and moesin (FERM) domain (green), the potential SRC homology 2 (SH2) domain (yellow), and the pseudo kinase (orange) and tyrosine kinase domains (red). STATs are composed by seven conserved domains: the N-terminal (NH₂) domain (dark grey), a coiled-coil domain (dark blue), a DNA binding domain (DBD) (green), a linker domain (light blue), a SH2 domain (yellow), a tyrosine activation motif (purple) and a transcriptional activation domain (TAD) (light grey). Relevant phosphorylation sites – tyrosine (Y) and serine (S) – are indicated. (Figure adapted from Heinrich *et al.*, 2003; Schindler and Plumlee, 2008).

1.2.3.1.2. THE STATs

STATs are a family of latent cytoplasmic proteins that are activated by several stimuli, which include interferons (IFNs), most cytokines and a number of growth factors. In mammalian cells, seven members have been identified, namely STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6 (Levy and Darnell, 2002). Different STATs are activated by a distinct set of cytokines and growth factors and are, therefore, implicated in different gene expression programmes (Horvath, 2000).

STATs share seven structurally and functionally conserved domains (**Fig. 7**): the well conserved N-terminal domain promotes the formation of unphosphorylated STAT homotypic dimers; the coiled-coil domain consists of a potentially dynamic four-helix bundle that protrudes laterally from the core and not only associates with regulatory proteins but is also implicated in controlling the process of nuclear import and export; the well conserved DNA binding domain (DBD) mediates binding to gamma IFN-activated site (GAS) elements and is also involved in the regulation of nuclear import and export; the linker domain structurally translates active dimerisation to the DNA binding motif and regulates a process of continual basal nuclear export; the highly conserved SH2 domain mediates specific recruitment to the receptor chains, as well as the formation of active STAT dimers; the tyrosine activation motif that, along with 5–7 specific C-terminal amino acids is exposed on the surface of the inactive homodimer, facilitating its JAK-dependent phosphorylation during receptor recruitment; and the transcriptional activation domain (TAD), which is located at the C-terminal and is highly variable in length and sequence between different STAT family members (reviewed by Schindler and Plumlee, 2008).

STAT activity is predominantly regulated by post-translational modifications (PTMs) (reviewed by Levy and Darnell, 2002). Activation of STATs involves the phosphorylation of a single tyrosine located at the carboxyl end of the molecule (**Fig. 7**). The phosphorylation of this residue induces a conformational change that promotes the formation of STAT dimers *via* reciprocal phosphotyrosine and SH2 domain interactions (Chen *et al.*, 1998; Becker *et al.*, 1998). The dimer conformation gives STATs the ability to recognise specific DNA promoters of target genes. The phosphorylation of a serine residue located within the C-terminal (**Fig. 7**) was also shown to be required for maximal activation of transcription of STATs (Wen *et al.*, 1995; Varinou *et al.*, 2003).

STATs can carry out distinct functions. STAT1 plays a fundamental role in the biological response to type I (IFN α subtypes and IFN β) and type II (IFN γ) IFNs (Durbin *et al.*, 1996; Meraz *et al.*, 1996) and it induces, by and large, the transcription of

target genes associated with the suppression of cellular proliferation (Schindler *et al.*, 2007) and the regulation of different types of immune responses. In marked contrast, STAT3 mediates the transcription of genes that are involved in proliferative and both anti- and pro-inflammatory responses. STAT3 plays an important role in transducing signals elicited by members of the interleukin (IL)-6 and IL-10 families, granulocyte colony-stimulating factor (G-CSF), leptin, IL-21 and IL-27 (reviewed by Schindler and Plumlee, 2008). *stat3* is an essential gene as *stat3* knockout mice exhibit an early embryonic lethal phenotype (Takeda *et al.*, 1997). Constitutive activation of STAT3 can prevent cell cycle arrest and apoptosis, and it has been associated with immune suppression and transformation (e.g. Catlett-Falcone *et al.*, 1999; Kujawski *et al.*, 2008; Bollrath *et al.*, 2009; Grivennikov *et al.*, 2009). Therefore, STAT3 has been reported to be a proto-oncogene (Bromberg and Darnell, 2000) and the encoded protein has been shown to be constitutively phosphorylated in a variety of tumours. STAT1, on the other hand, can play a key role in promoting apoptosis and/or inducing an anti-proliferative effect in a variety of cell types (Stephanou and Latchman, 2005). Paradoxically, it can also play a role in tumour development and drug resistance, depending on cellular context (Kovacic *et al.*, 2006; Patterson *et al.*, 2006). STAT5 has two highly homologous isoforms, STAT5A and STAT5B that derive from closely linked genes and play a critical role in driving both erythropoiesis and lymphopoiesis (Yao *et al.*, 2006). STAT5 mediates the signalling response of members of the IL-3, single-chain and γ_c receptor families (Schindler *et al.*, 2000; Murray, 2007). Like STAT3, STAT5 was also shown to play an important role in the inhibition of apoptosis (Onishi *et al.*, 1998). STAT4 and STAT6 play more restricted and specific roles in lymphocyte function. STAT4 is implicated in IL-12- and IL-23-dependent polarisation of naïve CD4⁺ T-cells and activation of IFN- γ secretion by natural killer (NK) cells (Th1 response). STAT6 plays a critical role in driving IL-4/IL-13 dependent T helper (Th)2-cell polarisation (Th2 response); B-cell function and mast cell activity (Wurster *et al.*, 2000). STAT2 is the most divergent STAT in sequence and function (Park *et al.*, 2000). It responds exclusively to type I and type III (IFN λ) IFNs and it thus operates primarily in immune responses.

The vast range of functions played by different STATs and, in some cases by the same STAT depending on the stimuli, demonstrates the intricate nature of the cellular and molecular regulation of these proteins.

1.2.3.2. JAK/STAT ACTIVATION AND SIGNALLING

JAK/STAT signalling pathway(s) can be activated by diverse stimuli. Ligand binding to cytokine receptors (e.g. IFN and IL receptors) triggers a change in their conformation that promotes sequential phosphorylation events (reviewed by Levy and Darnell, 2002). Unlike many growth factor receptors that have intrinsic tyrosine kinase activity, cytokine receptors do not. Instead, the required tyrosine kinase activity is provided by receptor-associated cytoplasmic proteins, which are often members of the JAK and *v-src* sarcoma viral oncogene homolog (SRC) family kinases. These are usually non-covalently bound to the membrane-proximal region of the cytokine receptors *via* their FERM domains (Chen *et al.*, 1997; Gauzzi *et al.*, 1997; Yamaoka *et al.*, 2004). Receptors with intrinsic tyrosine kinase activity, such as the epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR), can directly phosphorylate STAT proteins thereby bypassing JAK activation (Bowman *et al.*, 2000; Levy and Darnell, 2002). Furthermore, G protein-coupled receptors (GPCR) can also activate the JAK/STAT pathway (Mellado *et al.*, 1998; Wong and Fish, 1998; Vila-Coro *et al.*, 1999).

The JAK/STAT signalling canonical pathway is relatively simple, with only a few main components being required for the response (reviewed by Rawlings *et al.*, 2004). In fact, depending on the stimuli, the receptor-associated JAK can process the signal or, following receptor oligomerisation, other JAKs can be recruited to it. Homo- or heterodimerisation of JAKs is followed by their activation resulting in the propagation of the initial signal (reviewed by Rane and Redi, 2000). Rapid phosphorylation and activation of JAKs has been observed in response to many cytokines and growth factors (Novak *et al.*, 1995; Leaman *et al.*, 1996; Vignais *et al.*, 1996; Gual *et al.*, 1998). For example, IFN γ -induced activation of JAK1 and JAK2 was observed within a few minutes of treatment (Silvennoinen *et al.*, 1993a; Silvennoinen *et al.*, 1993b; Watling *et al.*, 1993; Muller *et al.*, 1993). JAK activation occurs through reciprocal interaction and trans-phosphorylation on tyrosine residues within the kinase activation loop (pYY motif). Several cytokine receptor complexes contain at least two different JAKs and receptor aggregation and transphosphorylation are both important for the activation of the JAKs in response to ligand. For example, IL-2 cannot activate JAK1 in the absence of JAK3 (Oakes *et al.*, 1996) and IFN- γ cannot activate JAK1 without JAK2, which was shown to be the initiating kinase of the cascade (Watling *et al.*, 1993).

Activated JAKs phosphorylate the receptor tail, which provides docking sites for the SH2 domain of STAT proteins. The STAT proteins are recruited to the receptor, whereupon they become phosphorylated on a single tyrosine located at the carboxyl end of the molecule around the residue 700 (cf. **Fig. 7**). This enables them to form homo- or heterodimers, which leads to the formation of a dimer-specific NLS that permits the subsequent translocation into the nucleus (Meyer *et al.*, 2002).

Nuclear import of activated STATs occurs through the nuclear pore after the binding of STAT dimers' NLS to specific importins (for example, STAT1 binds to importin $\alpha 5$ and $\beta 1$, and STAT3 binds to importin $\alpha 3$ or $\alpha 6$ and $\beta 1$) (Reich and Liu, 2006). All STATs can form homodimers and STAT1 can form heterodimers with STAT2 or with STAT3 depending on the nature of the stimuli (Levi and Darnell, 2002). For example, IL-6 stimulation induces the formation of STAT1 and STAT3 homodimers, as well as STAT1:STAT3 heterodimers and the different dimers bind to different DNA sequences thus activating distinct sets of genes (Guschin *et al.*, 1995). The dimer conformation gives STATs the ability to recognise different specific DNA promoters of target genes.

The initial dogma that inactive STATs are present in the cytoplasm as monomers that only dimerise upon tyrosine phosphorylation has been replaced by the understanding that unphosphorylated (and presumably inactive) STATs can also exist as dimers, or in higher order complexes, in the cytoplasm (Ndubuisi *et al.*, 1999; Wenta *et al.*, 2008). Unphosphorylated STAT dimers interact through different domains (N-terminal and core domains) and these interactions are weaker than the interactions that occur between phosphorylated STAT dimers (Mao *et al.*, 2005).

Initial reports suggested that mutations that prevent phosphorylation of the C-terminal key tyrosine residue, or mutations on the SH2 domain that prevent dimerisation, also prevent STAT translocation to the nucleus, showing that these events are critical for the nuclear import of STATs (Shuai *et al.*, 1993, Shuai *et al.*, 1994). Although STAT nuclear accumulation is triggered by dimerisation in response to tyrosine phosphorylation, the phosphorylation of this residue *per se* is not required for nuclear translocation, as “artificially” dimerised unphosphorylated STATs can also accumulate in the nucleus (Milocco *et al.*, 1999). In fact, unphosphorylated STATs have been found in the nucleus (McBride *et al.*, 2000). Unphosphorylated STATs can shuttle between the cytoplasm and nucleus in a constitutive manner (Vinkemeier, 2004) raising questions regarding their nuclear import, as monomeric STATs do not possess a canonical NLS.

Several authors have shown that unphosphorylated forms of STAT1, STAT3 and STAT6 are able to transcriptionally activate target genes, although these are distinctive from those activated upon stimulation with cytokines or growth factors (Chatterjee-Kishore *et al.*, 2000; Yang *et al.*, 2005; Cui *et al.*, 2007). Unphosphorylated STAT1 supports the constitutive transcription of the low molecular mass polypeptide 2 (LMP2), a protein involved in the processing of viral and tumour antigens for presentation in the context of major histocompatibility complex (MHC) class I molecules (Chatterjee-Kishore *et al.*, 2000). Importantly, over-expression of unphosphorylated STAT3 was shown to promote the expression of oncogenes such as muscle RAS oncogene homolog (*MRAS*), found elevated in many cancers and which is believed to contribute to cellular transformation and differentiation (Yang *et al.*, 2005). Unphosphorylated STAT6 can directly bind to the cyclooxygenase-2 (*COX-2*) promoter, a protein that catalyses the production of prostaglandins, which are involved in inflammation and are often found over-expressed in tumours (Cui *et al.*, 2007). In *Drosophila melanogaster*, unphosphorylated STATs can associate with transcriptionally repressed heterochromatin thereby maintaining its stability (Brown and Zeidler, 2008).

As mentioned above, following dimerisation, STATs quickly accumulate in the nucleus where they bind to specific promoter elements of target genes and regulate transcription (e.g. Pranada *et al.*, 2004; Vinkemeier, 2004; Meyer *et al.*, 2007) (**Fig. 8**). STATs can both activate and repress gene expression. One example of negative regulation of transcription is the inhibition of *c-myc* expression in response to IFN- γ , which induces binding of STAT1 homodimers to consensus GAS elements in the *c-myc* promoter. This leads to the inhibition of cell proliferation (Ramana *et al.*, 2000). It is widely accepted that STATs mostly regulate transcription positively (Der *et al.*, 1998). For example, cyclin D1 is upregulated in response to prolactin through the binding of STAT5 to GAS sites in the *cyclin D1* promoter in mammary epithelial cells (Brockman *et al.*, 2002) and also following IL-6 stimulation of most cells as a consequence of STAT3 binding to the *cyclin D1* promoter on v-Sis inducible elements (SIE) sites. Consequently, as cyclin D1 participates in the transition from G1 to S phase of the cell cycle, this results in enhanced proliferation (reviewed by Fu *et al.*, 2004).

Phosphorylation of a serine residue located within the C-terminal of most STATs (**Fig. 6**) was also shown to be required for STAT maximal activation of transcription (Wen *et al.*, 1995). Phosphorylation of STAT1 Ser⁷²⁷ was shown to occur when the protein is assembled on transcriptional complexes bound to chromatin (Sadzak *et al.*, 2008). Importantly, phosphorylation of this serine residue in response to

IFN- γ is dependent on the previous phosphorylation of Tyr⁷⁰¹ (Kovarik *et al.*, 2001). Other PTMs are known to regulate STAT transcriptional activity (Levy and Darnell, 2002). These will be presented in the next section.

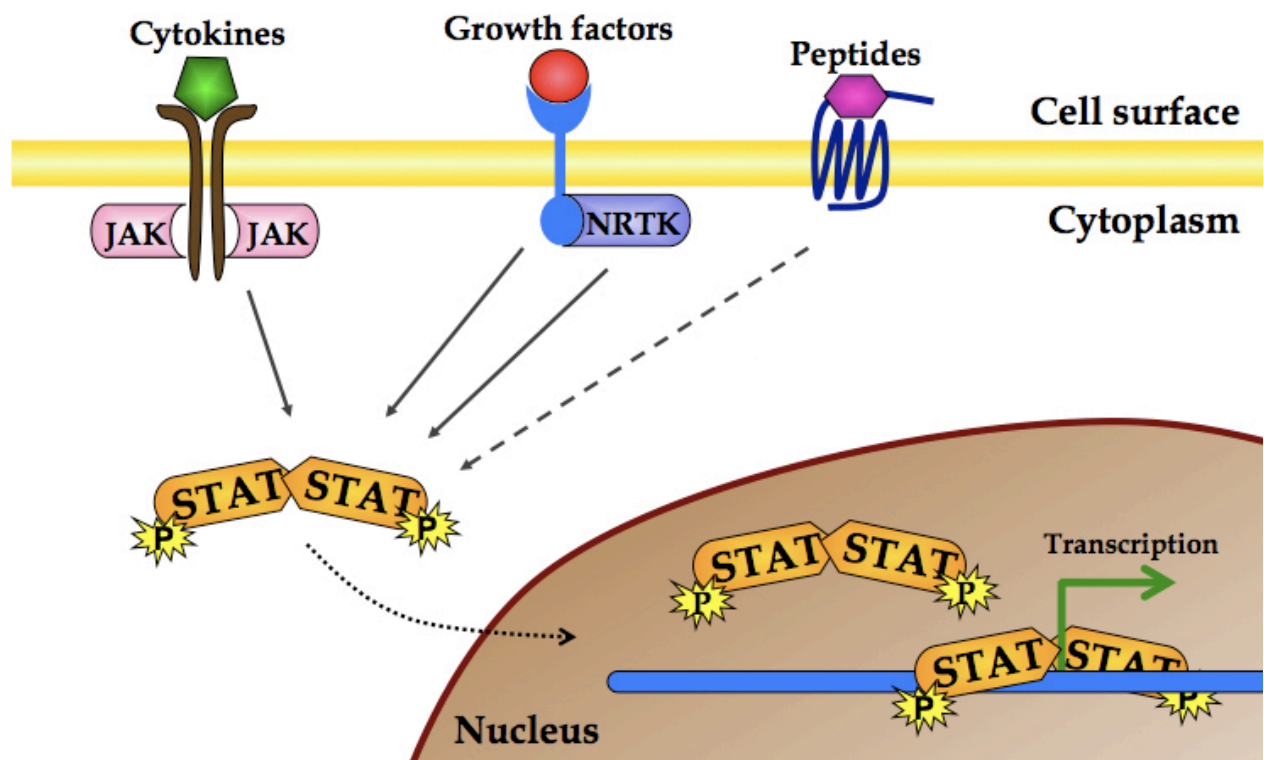


Figure 8. Mechanisms of STAT signalling. Activation of STATs can occur in response to cytokine stimulation of their receptors through JAKs or through receptors with intrinsic tyrosine kinase activity. Latent STAT proteins are recruited to the activated receptors and phosphorylated at a tyrosine residue. Activated STATs translocate into the cell nucleus where they can bind to specific promoter elements of target genes. Grey arrows indicate direct activation. Dashed arrows indicate indirect activation. The dotted curved arrow indicates translocation. (Figure adapted from Levy and Darnell, 2002).

In addition to their function as transcription factors, there is some evidence that STATs also have nontranscriptional roles. STAT3 can affect cell motility through its direct interaction with components of the central machinery for cell motility (reviewed by Gao and Bromberg, 2006). For example, activated STAT3 was found to directly bind components of focal adhesions, such as focal adhesion kinase (FAK) and paxillin and

was thus proposed to have an adaptor or a sensor role in mediating cell motility (Silver *et al.*, 2004). Furthermore, JAK2/STAT3 may play a role in the phosphorylation of these components of focal adhesions (Gao *et al.*, 2009). Another novel, non-canonical, function of STAT3 was recently revealed. STAT3 was found localised in the mitochondria and was demonstrated to regulate metabolic functions (Gough *et al.*, 2009). The accumulation of STAT3 in these organelles supported RAS-dependent malignant transformation, which was associated with sustained altered glycolytic and oxidative phosphorylation activities, two known properties of cancer cells.

Although STATs are the main transcription factors through which the JAKs commonly exert their effects, some responses can be mediated by the JAKs independently of STAT activation. For example, JAK2 can mediate the up-regulation of BCL-X_L in response to erythropoietin (Epo) in cells expressing an Epo receptor defective in activation of the STAT pathway (Packham *et al.*, 1998). In addition, JAK3 was reported to be involved in the regulation of members of the BCL-2 family in stat5-null mice (Wen *et al.*, 2001). Recently, a completely novel role for JAKs was identified in haematopoietic cells (Dawson *et al.*, 2009). JAK2 was found in the nucleus where it was shown to mediate phosphorylation of histone H3 and to prevent its association with heterochromatin protein 1 (HP1), suggesting that JAK2 is able to regulate chromatin architecture around specific promoters. An example of a JAK-regulated gene identified by these authors is LIM domain only 2 (LMO2). Interestingly, LMO2 is an important regulator of transcription in erythropoiesis. It was initially discovered in association with a chromosomal translocation, and consequently aberrantly expressed, and was shown to play a key role in leukaemia's aetiology (Nam and Rabbitts, 2006).

1.2.3.3. REGULATION OF JAK/STAT SIGNALLING PATHWAYS

JAK/STAT signalling pathways, albeit being able to do so, do not usually function autonomously. Rather they cross-talk with many other signalling pathways, such as p38 MAPK (Goh *et al.*, 1999), calcium/calmodulin kinase II (CamKII) (Nair *et al.*, 2002), PI3K (Nguyen *et al.*, 2001) and PKC δ (Deb *et al.*, 2003). These kinases can phosphorylate STATs on particular serine residues within the TAD domain on a conserved PMSP motif (cf. **Fig. 7**). This additional phosphorylation event increases the transcriptional activation potential of STATs and it is necessary for the transcription of particular subsets of genes (Decker and Kovarik, 2000). Similarly, it is possible that

additional unrecognised phosphorylation sites, or other PTMs, may also regulate STAT activity (Levy and Darnell, 2002). Indeed, STAT1 has been shown to be methylated on Arg³¹ which was shown to increase its transcriptional activity (Mowen *et al.*, 2001) and, although this finding is somewhat controversial, it is still plausible that methylation may regulate STAT activity (Meissner *et al.*, 2004; Komyod *et al.*, 2005).

Lysine acetylation has also been reported in STAT1, STAT3 and STAT6. Acetylation of STAT1 residues Lys⁴¹⁰ and Lys⁴¹³ was shown to be required for interaction with the anti-apoptotic nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF- κ B), reducing its DNA binding, nuclear localisation and target gene expression (Kramer *et al.*, 2006). Acetylation of STAT1 is, however, a controversial issue after two other groups, leaders in the JAK/STAT field (D. Levy and U. Vinkemeier Groups), have disputed the validity of these results (A. Costa-Pereira, personal communication). In contrast, acetylation of STAT3 Lys⁶⁸⁵ (within the TAD domain) was shown to be required for STAT3-mediated NF- κ B processing (processing of pro-apoptotic p100 into the anti-apoptotic p52 form) and consequently for cell proliferation and survival (Nadiminty *et al.*, 2006). Acetylation of STAT3 also improves the homodimer stability and increases its DNA binding ability (Wang *et al.*, 2005; Yuan *et al.*, 2005). Acetylation of STAT6 was shown to enhance its transcriptional activity (Shankaranarayanan *et al.*, 2001).

Additionally, STATs are known to interact with proteins that serve as bridges between transcription factors and RNA polymerase II. STATs can interact with members of the evolutionarily conserved eukaryotic minichromosome maintenance (MCM) family (Zhang *et al.*, 1998). The same group showed that STAT1 and MCM proteins are recruited to STAT1 target genes promoters in response to cytokines and, furthermore, that they move along the chromatin with RNA polymerase II during transcription elongation (Snyder *et al.*, 2005). As another example, IFN- α induces the activity of the IFN-stimulated gene factor 3 (ISGF3) transcription complex that is composed of a STAT1:STAT2 heterodimer and a weak DNA-binding protein, IFN-regulatory factor (IRF)-9 (Horvath *et al.*, 1996). IRF-9 binds to STAT1 through a region within the coiled-coil domain (residues 153-239). In response to the same type of IFN, STAT2 can directly bind transcription adaptors such as p300 and CREB-binding protein (CBP) (Bhattacharya *et al.*, 1996).

The duration and degree of gene activation are, unsurprisingly, under strict regulation by a series of negative regulators (reviewed by Greenhalgh and Hilton, 2001; Schindler and Plumlee, 2008). Negative regulation of JAK/STAT pathways can occur by receptor internalisation to endocytic vesicles and subsequent receptor

degradation, and/or by more specific inhibitory signals that lead to the activation of protein tyrosine phosphatases (Aaronson and Horvath, 2002). Protein phosphatases can act at the level of the membrane-associated receptor-kinase complex or in the nucleus, by dephosphorylating activated STAT dimers and promoting the recycling of latent STAT monomers to the cytoplasm. Other regulatory mechanisms involving the suppressors of cytokine signalling (SOCS) and protein inhibitors of activated STATs (PIAS) also exist. SOCS inactivate JAKs and STATs by a number of different mechanisms (Krebs and Hilton, 2001). Expression of SOCS genes can be stimulated by the same cytokines that enhance STAT activation and, therefore, SOCS proteins can act in classic feedback inhibition loops. PIAS bind to phosphorylated STAT dimers, thereby preventing DNA recognition (Shuai, 2000). Importantly, the steady-state and signal-inducible concentrations of all positive and negative regulators determine the intensity and duration of the signal (and thus the cellular response). This is both cell-type and cell context-dependent.

1.2.3.4. JAKs AND STATs IN TUMOUR CELL PROLIFERATION AND APOPTOSIS

JAKs, whose activity is generally required for STAT activation, play critical roles in the pathogenesis of various diseases (reviewed by Verma *et al.*, 2003). Janus kinases have been shown to modulate activation of other pathways implicated in malignant transformation and cell growth, such as RAS/RAF/MEK/ERK pathways (Kumar *et al.*, 1994; Mizuguchi *et al.*, 2000). Malignant transformation by oncogenes, such as *v-abl*, which involves activation of STATs, RAS and PI3K signalling pathways, was also shown to be mediated by JAKs (Danial and Rothman, 2000). JAKs have been implicated in the regulation of p53-dependent cell cycle arrest and apoptosis (Quelle *et al.*, 1998). Moreover, JAKs are able to promote cell survival through enhanced activity of BCL-2 family members thereby modulating apoptosis. JAKs can also mediate the upregulation of anti-apoptotic proteins *via* STAT-independent pathways (Packham *et al.*, 1998; Wen *et al.*, 2001). For example, BCL-X_L can be transcriptionally induced by growth factors, in a JAK/STAT-dependent fashion, to promote cell survival (Grad *et al.*, 2000). For example, STAT5 can bind to the BCL-X_L gene promoter and regulate its expression in response to IL-3 (Dumon *et al.*, 1999). This strongly supports a role for STAT5 in the suppression of apoptosis elicited by cytokines.

A small number of JAK mutations were found in cancer (Constantinescu *et al.*, 2008). A unique somatic mutation in *JAK2* was initially identified in patients with myeloproliferative disorders (MPDs) (Baxter *et al.*, 2005; James *et al.*, 2005; Levine *et al.*, 2005; Kralovics *et al.*, 2005; Zhao *et al.*, 2005). The gain-of-function mutation of a valine to a phenylalanine in the regulatory pseudo-kinase domain (residue 617) (Saharinen *et al.*, 2003), leads to JAK2 constitutive activation and cytokine-independent signalling. The now well known valine mutation has also been found in JAK1 (V658F) and TYK2 (V678F) (Staerk *et al.*, 2005). The equivalent residue was also found mutated in JAK3, but in JAK3 the valine has been substituted with an isoleucine instead of a phenylalanine (V715I) (Jeong *et al.*, 2008). JAK2 mutations, other than the V617F substitution, were identified in the kinase and pseudo-kinase domains in acute lymphoblastic leukemia (ALL) patients (Mullighan *et al.*, 2009). Other *JAK1* mutations were also identified in other types of acute leukemias, lung, colon and breast carcinomas (Lin *et al.*, 2005; Jeong *et al.*, 2008; Kim *et al.*, 2009; Mullighan *et al.*, 2009). *JAK3* mutations have been identified in some leukaemia patients (Walters *et al.*, 2006), and in patients with breast and gastric carcinomas (Jeong *et al.*, 2008). Constitutively activated JAK3 has been reported in several lymphoproliferative disorders (Gee *et al.*, 2001; Yared *et al.*, 2005; Lai *et al.*, 2005; Tomita *et al.*, 2006; Krejsgaard *et al.*, 2006; Marzec *et al.*, 2008). In addition, mutant *JAK3* alleles were identified in acute myeloid leukemia (AML) patients, which resulted in the constitutive activation of JAK3 and a spectrum of downstream pathways that included STAT5, the PI3K/AKT pathway and the RAS/MEK/ERK pathway (Walters *et al.*, 2006).

Chromosomal translocations that fuse the catalytic domain of JAK2 to other molecules have been identified: one involves the oligomerisation domain of ETS translocation variant 6 (*TEL*), a gene encoding a member of the E26 transformation-specific (ETS) transcription factor family, and is thus called TEL-JAK2 (Lacronique *et al.*, 1997; Peeters *et al.*, 1997); another translocation juxtaposes *JAK2* to the coiled-coil dimerisation domain of breakpoint cluster region (*BCR*) gene (Griesinger *et al.*, 2005) and is denoted BCR-JAK2; and another, the PCM1-JAK2, links *JAK2* to the human autoantigen pericentriolar material (*PCM1*) (Reiter *et al.*, 2005). These gene products, which all result in the constitutive activation of JAK2, were initially identified in leukaemia.

As STATs are involved in regulating the expression of many genes that in normal cells control fundamental biological processes, aberrant activation of STAT-mediated signalling can lead to cell transformation and oncogenesis (reviewed by Yu and Jove, 2004). Not surprisingly, constitutive activation of several STATs has been

observed in a wide variety of tumours, including blood malignancies (leukaemia, lymphoma, multiple myeloma) and solid neoplasias (lung, head and neck, brain, breast, pancreas, and prostate cancers) (Buettner *et al.*, 2002; Song *et al.*, 2003).

As previously mentioned (**Section 1.2.3.1.2**), STAT3 mediates cell cycle progression and prevents apoptosis and thus has an important function in the growth and survival of tumour cells (e.g. Catlett-Falcone *et al.*, 1999; Kujawski *et al.*, 2008; Bollrath *et al.*, 2009; Grivennikov *et al.*, 2009). Constitutive STAT3 activity has been attributed to diverse causes such as autocrine or paracrine loops of IL-6 (in myeloma, prostate and in lung cancer cells), transforming growth factor- β (TGF- β), EGFR (in some head and neck cancer cell lines) and hepatocyte growth factor (HGF) signalling in cooperation with SRC kinase (in leiomyosarcoma cells and breast carcinoma cells). SRC family kinases are, in fact, thought to induce constitutive STAT3 activation, which is often observed in many breast cancer, melanoma and lung cancer cell lines (reviewed by Yu and Jove, 2004).

STAT5 was also found to be deregulated in cancer and to promote cell proliferation (Onishi *et al.*, 1998), particularly in leukaemias (Lewis and Ward, 2008). Importantly, the anti-apoptotic BCL-2 family member BCL-X_L is a STAT5 target gene (Dumon *et al.*, 1999; Silva *et al.*, 1999; Socolovsky *et al.*, 1999). Deregulated STAT5 signalling induced by the TEL-JAK mutation has been shown to promote oncogenesis in a number of myelo- and lymphoproliferative diseases (Schwaller *et al.*, 2000). Another oncogenic fusion protein, BCR-ABL, known as the Philadelphia chromosome translocation that is commonly associated with chronic myeloid leukemia (CML), has been shown to activate STAT5 and STAT1, thereby promoting cell survival and transformation of hematopoietic cells (Carlesso *et al.*, 1996; Frank and Varticovski, 1996; Ilaria and Van Etten, 1996; Shuai *et al.*, 1996; Chai *et al.*, 1997). Activation of STAT5 was, in fact, shown to be essential for BCR-ABL-mediated cell transformation (de Groot *et al.*, 1999; Nieborowska-Skorska *et al.*, 1999).

STAT2, STAT4 and STAT6 roles in oncogenesis are not as evident as those of STAT3 or STAT5. In breast cancer cells, STAT6 was shown to mediate IL-4-mediated growth inhibition and induction of apoptosis (Gooch *et al.*, 2002). In primary B cells, however, it can mediate upregulation of BCL-X_L in response to IL-4 and block apoptosis (Wurster *et al.*, 2002). Furthermore, STAT6 signalling was implicated in tumour immunosurveillance (Terabe *et al.*, 2000) and was shown to play a role in the regulation of cell proliferation and apoptosis in colon cancer cells (Zhang *et al.*, 2006). Interestingly, STAT2-mediated signalling was shown to play a critical role in inflammatory responses in carcinogenesis (Clifford *et al.*, 2003; Maher *et al.*, 2008).

Most reports in the past have attributed pro-apoptotic and anti-proliferative properties to STAT1. It seemed, therefore, unlikely that STAT1 could promote tumour cell growth (Bromberg and Darnell, 2000). Indeed, there is a compelling body of evidence suggesting that STAT1 has properties of a *bona fide* tumour suppressor protein (Dunn *et al.*, 2004). More recent findings, however, as previously mentioned, show that, under a particular set of circumstances, STAT1 can also play a role in the development of tumours and in mediating drug resistance (Kovacic *et al.*, 2006; Patterson *et al.*, 2006). Clearly, the cellular context in which any given set of proteins operates strongly determines the outcome of the response.

Interestingly, contrary to the JAKs, no study has identified to date any mutations in STATs and it has thus been postulated that their constitutive activation is due to auto- and paracrine signalling (Constantinescu *et al.*, 2008).

1.2.3.5. TARGETING JAK/STAT PATHWAYS FOR CANCER TREATMENT

An ideal target for cancer therapy should have amongst its properties that of being over-activated in a large percentage of cells in different tumour types and inducing gene expression patterns that determine malignant properties, such as cell survival and proliferation, tumour angiogenesis and immune evasion. Good therapeutic targets should also be susceptible to specific inhibition by drugs and, very importantly, tumour cells should be more dependent on the activity of the target than normal cells. On the basis of these criteria, and despite not being mutated in cancer cells, STAT proteins have been suggested as key molecular targets for cancer therapy (Yu and Jove, 2004). Indeed, the central role that JAK/STAT pathways play in transducing signals downstream of many cytokine receptors and a number of growth factors, and their clear involvement in oncogenesis, makes them attractive targets for drug development (Boudny and Kovarik, 2002; Verma *et al.*, 2003; Yu and Jove, 2004).

Many oncogenic signalling pathways seem to converge on a limited set of nuclear transcription factors (Hahn and Weinberg, 2002) and these transcription factors can be seen as key 'switches' that activate gene expression patterns that may ultimately lead to malignancy. Such properties make transcription factors logical choices as anti-cancer targets (Darnell, 2002). Targeting a single transcription factor, such as STATs (which are both cytosolic and nuclear), can potentially block the effects of a multitude of signalling pathways. In fact, blocking STAT signalling is often sufficient to induce

growth arrest and apoptosis in many different tumour types (reviewed by Yu and Jove, 2004). Importantly, several studies have shown that blocking constitutively activated STAT3 signalling leads to apoptosis of tumour but not normal cells (Bowman *et al.*, 2000; Battle and Frank, 2002; Buettner *et al.*, 2002). This selective inhibition might reflect an irreversible dependence of tumour cells on high levels of activated STAT3 for growth and survival, whereas normal cells might be able to withstand low STAT3 activity or use alternative pathways (reviewed by Yu and Jove, 2004).

At present, while a number of JAK inhibitors have been developed, just a few have been used in the clinic due to issues related to low potency, off-target effects and drug toxicity (reviewed by Wilks, 2008). This may be related to the scarcity in the information available regarding JAKs molecular structures. The crystal structures of the kinase domain (JH1) of JAK2 and JAK3 have already been resolved (Boggon *et al.*, 2005; Lucet *et al.*, 2006), but no structure has been solved for the JAK pseudokinase domain, a domain that has a particularly important role in maintaining the inactive state of the kinase (Saharinen and Silvennoinen, 2002). Treatments with some JAK inhibitors, currently being tested in the clinic, are showing promising results in the treatment of disorders associated with JAK deregulation, such as MPDs (e.g. Pardanani, 2008). Accordingly, more information regarding JAK structure will allow better predictions for the development of more potent and likely selective JAK inhibitors, hopefully with reduced toxicity for the patients (Wilks, 2008).

Constitutive activation of oncogenic STAT3 can be blocked using JAK pharmacological inhibitors, which have been shown to suppress, for example, the growth of human prostate cancer cells (Ni *et al.*, 2000). Therefore, STAT3 seems to assemble a number of properties that make it a particularly well-suited target for cancer therapy. However, the inhibition of STAT3 can also have undesirable consequences. For example, it was shown that in the absence of STAT3, IL-6 can mediate an IFN- γ -like response, including the prolonged activation of STAT1 and the induction of multiple IFN- γ -stimulatable genes (ISG γ) (Costa-Pereira *et al.*, 2002). Since in many cancers, including lung cancer, IL-6 is often found at elevated levels in patients' serum (Yanagawa *et al.*, 1995; Eickelberg *et al.*, 1999; Yeh *et al.*, 2006) the inhibition of STAT3 could lead to an inflammatory response with potentially adverse clinical outcomes. Accordingly, caution is necessary before one can safely use signalling inhibitors of STAT3 in the treatment of patients and more work is required before this can become part of routine clinical practice (Turkson *et al.*, 2001; Karamouzis *et al.*, 2007).

1.3. FIBROBLAST GROWTH FACTORS (FGFs), FGF RECEPTORS (FGFRs) AND CANCER

Growth factors and their receptors play a critical physiological role in cellular growth and differentiation. In cancer, growth factor-induced pathways that mediate normal cellular functions are frequently deregulated leading to malignancy (e.g. Aaronson, 1991). Fibroblast growth factor (FGF) signalling pathways have been the subject of intense investigation not only because of their role in tissue patterning and organogenesis during embryogenesis, but also because of their important role in tumour development (Powers *et al.*, 2000).

Most FGFs are secreted into the extracellular environment and work as paracrine factors, but some FGFs can also function in an autocrine manner. Autocrine FGF signalling seems to be particularly important for the progression of some cancers (reviewed by Beenken and Mohammadi, 2009).

The FGF family consists of a number of small polypeptide growth factors that have certain structural characteristics in common, most of which have a high affinity for heparin and heparan-like glycosaminoglycans (HLGAGs) (Burgess and Maciag, 1989). There are at least 18 identified mammalian fibroblast growth factors (FGF-1-10 and FGF-16-23) that are classified into 6 subfamilies according to phylogeny (Itoh and Ornitz, 2004).

FGFs exert their effects by signalling *via* a set of high-affinity cell surface receptors, the FGF receptors (FGFRs 1-4) encoded by four genes (reviewed by Mohammadi *et al.*, 2005). They are composed of three extracellular immunoglobulin-like domains (D1-D3), a single-pass transmembrane domain and a cytoplasmic tyrosine kinase domain (**Fig. 9**). Between the domains D1 and D2, FGFRs have an acidic, serine-rich sequence (acid box) (Lee *et al.*, 1989). The D1 domain and the acid box were shown to be dispensable for ligand binding and affinity, but play an autoregulatory role in FGFR function (Wang *et al.*, 1995a). It was proposed that the acid box serves as a flexible hinge that allows the D1 domain to interact with the other domains D2 and D3, affecting FGFR's affinity for the ligand and heparin. The immunoglobulin-like domains D2 and D3 constitute the ligand-binding domain (Wang *et al.*, 1995b).

Several FGFR isoforms exist, which are generated by the alternative splicing of *FGFR1*, *FGFR2* and *FGFR3* coding genes, and have different ligand-binding capacities and tissue distributions. Alternative splicing can involve the domain D1 and the acid box determining whether these domains are included in the receptors (Johnson *et al.*,

1990). Another alternative splicing event occurs in the D3 domain where two alternative exons (exons 8 and 9) are spliced in a mutually exclusive manner, giving rise to the FGFR isoforms IIIb and IIIc (highlighted in green in **Fig. 9**) (Johnson *et al.*, 1991; Avivi *et al.*, 1993; Chellaiah *et al.*, 1994). FGFR1-3 isoforms IIIb and IIIc have different ligand specificities (Ornitz *et al.*, 1996). FGFR4 has a unique exon in this region that is more identical to the variant IIIc than to the variant IIIb (Vainikka *et al.*, 1992).

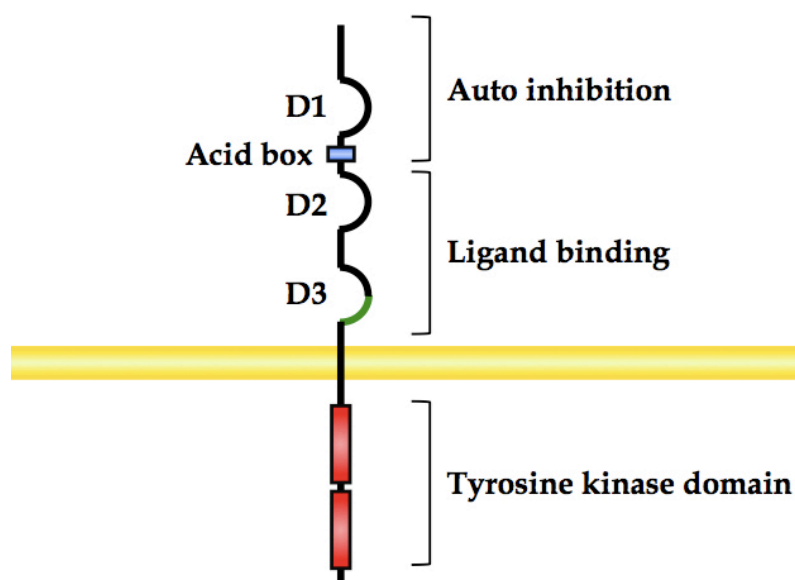


Figure 9. Structural organisation of FGFRs. FGFRs have three extracellular immunoglobulin-like domains (D1–D3), a single transmembrane domain and a cytoplasmic tyrosine kinase domain. Between D1 and D2 domains, FGFRs have a serine-rich sequence (acid box). The green highlighted region within the D3 domain corresponds to the region that can be alternatively encoded by two exons (corresponding to IIIb and IIIc). (Figure adapted from Mohammadi *et al.*, 2005).

FGFs not only provide pro-survival signals, but also confer broad-spectrum resistance to chemotherapeutic drugs (Song *et al.*, 2000) and so have important roles in

cancer. Indeed, FGF/FGFR signalling has been associated with chemoresistance in breast cancer (Manuvakhova *et al.*, 2006; Smaragda Angelidou and Michael Seckl, personal communication), metastatic prostate cancer (Song *et al.*, 2000), and lung cancer (Pardo *et al.*, 2002; Pardo *et al.* 2003; Pardo *et al.*, 2006). Inappropriate expression and secretion of FGFs can contribute to uncontrolled cell proliferation in many cancers including breast, prostate, and pancreatic cancer (reviewed by Dickson *et al.*, 2000; Powers *et al.*, 2000). Deregulation of FGF signalling can also occur as a result of alterations of the expression of their receptors and FGFR mutations have also been associated with tumorigenesis. For example, over-expression of FGFR1 has been reported in breast and prostate cancer (Penault-Llorca *et al.*, 1995; Giri *et al.*, 1999), and over-expression of FGFR2 was found in pancreatic tumours (Nomura *et al.*, 2008). Gain-of-function mutations were found in many tumours. Examples of these include the mutation in the kinase domain of *FGFR1* found in glioblastoma brain tumours (Rand *et al.*, 2005), an activating mutation in the *FGFR2* gene found in some primary uterine tumours (Pollock *et al.*, 2007) and *FGFR3* mutations detected in human bladder, cervical cancers and multiple myeloma (Chesi *et al.*, 1997; Cappellen *et al.*, 1999). A substitution of a glycine residue by an arginine at position 388 in the transmembrane domain of the receptor *FGFR4* was linked to increased motility and invasion in breast and prostate cancer cells (Bange *et al.*, 2002; Wang *et al.*, 2004). Chromosomal translocations giving rise to various fusion genes of *FGFR1* with other genes have been reported in leukaemia, particularly the fusion *bcr-fgfr1*, which can induce CML in mice (Roumiantsev *et al.*, 2004). Isoform-switch was also found to be associated with malignant transformation, as illustrated by the isoform switch of *FGFR2IIIb* to *R2IIIC* that, together with an increased expression of *FGFR1* and several FGF ligands, was found to be associated with the transformation of prostate epithelial cells (Yan *et al.*, 1993).

This study focussed on FGF-2, which is described in more detail in the next section (**Section 1.4**).

1.4. FGF-2

FGF-2 is known to play a role in prostate and breast cancer (Giri *et al.*, 1999; Dickson *et al.*, 2000; Nurcombe *et al.*, 2000; Polnaszek *et al.*, 2003) and has been implicated in driving chemoresistance in a variety of cancer cells, including SCLC cells

(Song *et al.*, 2000; Pardo *et al.*, 2002; Pardo *et al.*, 2003; Pardo *et al.*, 2006). Several studies have established a significant association between expression of FGF-2 and advanced tumour, or poor prognosis, in lung, colon, gastric, pancreatic, liver, bladder, ovarian, and head and neck cancers (Yamanaka *et al.*, 1993; Kitadai *et al.*, 1996; Mise *et al.*, 1996; Takanami *et al.*, 1996; Ueki *et al.*, 1996; Fujimoto *et al.*, 1997; Dellacono *et al.*, 1997; Gazzaniga *et al.*, 1999). Moreover, elevated serum concentration of FGF-2 is an independent prognostic factor for an adverse outcome in SCLC (Ruotsalainen *et al.*, 2002). A significant correlation between serum FGF-2 levels and tumour mass and histological grade was also demonstrated in patients with soft tissue sarcoma (Graeven *et al.*, 1999). In NSCLC, particularly when co-expressed with vascular endothelial growth factor (VEGF) or PDGF, FGF-2 is also associated with reduced survival rates (Donnem *et al.*, 2009). Recently, autocrine signalling of FGF-2 and its receptors has been observed in NSCLC cell lines, in particular in those that are insensitive to the EGFR inhibitor gefitinib (Marek *et al.*, 2009).

1.4.1. BIOLOGICAL ROLE OF FGF-2

FGF-2 (initially named basic FGF, bFGF, for having a basic isoelectric point) is the most extensively studied member of the FGF family (reviewed by Ribatti *et al.*, 2007). Four different FGF-2 polypeptides can be formed from the *FGF-2* gene (Florkiewicz and Sommer, 1989) and it was suggested that the different molecular weight forms of FGF-2 could have distinct functions (Yu *et al.*, 2007). FGF-2 plays key roles in diverse biological processes, including proliferation, differentiation, migration, survival and neovascularisation (Bikfalvi *et al.*, 1997). *fgf-2* knockout mice exhibit loss of vascular tone, indicating that *fgf-2* has an important physiological role in vascular development and homeostasis (Zhou *et al.*, 1998). FGF-2 was also shown to play an important role in astrocyte differentiation (Reuss *et al.*, 2003; Song and Ghosh, 2004). Other possible physiological roles for FGF-2 include inflammation in which stress-induced activation of caspase 1 leads to the release of FGF-2 that then mediates tissue repair and angiogenesis, processes that are required during and after acute or chronic inflammation (Keller *et al.*, 2008). During embryonic development of the lung, FGF-2 regulates the expression of a number of genes (Han *et al.*, 1992). In the adult normal lung FGFRs seem to be expressed at low levels (Ledoux *et al.*, 1991), but deregulated FGF signalling through gain- or loss-of-function mutations of the ligands or receptors

can contribute to many diseases, the most notable being cancer (reviewed by Beenken and Mohammadi, 2009).

1.4.2. ACTIVATION AND SIGNALLING IN RESPONSE TO FGF-2

The mechanism of FGF-2 release is not clearly understood since, unlike other FGFs, FGF-2 does not contain a canonical signal sequence for secretion (Powers *et al.*, 2000). FGFs bind the receptor together with heparan sulphate glycosaminoglycan (HSGAG), which stabilises the ligand-receptor contact and the complex 2:2 FGF-FGFR dimer (Plotnikov *et al.*, 1999; Venkataraman *et al.*, 1999; Schlessinger *et al.*, 2000; Stauber *et al.*, 2000). Like EGF family members, which are known bind to and activate both EGFR homo- and heterodimers (Hackel *et al.*, 1999), the interactions observed at the receptor-receptor interface in response to FGFs are compatible with FGFR homo- and heterodimerisation (Bellot *et al.*, 1991; Ueno *et al.*, 1992; Plotnikov *et al.*, 1999; Schlessinger *et al.*, 2000; Bae *et al.*, 2010). FGFR1 homodimers and FGFR1:FGFR2 heterodimers are the main receptor complexes for FGF-2 (Ornitz *et al.*, 1996; Zhou *et al.*, 2006; Ueno *et al.*, 1992; Plotnikov *et al.*, 1999). Interestingly, FGFRs can also heterodimerise with other receptors. That is the case for FGFR1, which heterodimerises with PDGF receptor (PDGFR) α in response to a dual treatment with FGF-2 and PDGF (Faraone *et al.*, 2006).

Ligand-binding and FGFR dimerisation allows for the trans-phosphorylation of several tyrosine residues on the tyrosine kinase domains of the FGFRs' cytoplasmic tails (Bellot *et al.*, 1991). Phosphorylation sites within the catalytic domain are crucial for the stimulation of receptor kinase activity, while the phosphorylation of other tyrosine residues provides sites for direct binding of secondary messenger molecules with SH2 domains. Binding of SH2-binding proteins to the receptor activates several intracellular signalling pathways, some of which are depicted in **Figure 10** (Dailey *et al.*, 2005). For example, FGFR1 contains seven tyrosine residues on its cytoplasmic domain of which Tyr⁶⁵³ and Tyr⁶⁵⁴ are necessary for the receptor catalytic activity, while the phosphorylation of the other tyrosines likely provides binding sites for scaffolding proteins for further downstream signalling (Mohammadi *et al.*, 1996).

The two main intracellular substrates of FGFR are PLC- γ and FGFR substrate 2 (FRS2). The main pathways activated by FGFs are schematically represented in **Figure 10**. Upon FGF binding, the phosphorylation of FGFR1 provides a binding site for the

SH2 domain of PLC- γ (Mohammadi *et al.*, 1991; Peters *et al.*, 1992). Activated PLC- γ hydrolyses PIP₂ into DAG and inositol triphosphate (IP₃), which leads to the stimulation of PKC and intracellular calcium release, respectively (Mohammadi *et al.*, 1996). FRS2 is constitutively bound to the receptor and is activated after FGF stimulation (Mohammadi *et al.*, 1996). FRS2 forms a complex with growth factor receptor bound-2 (GRB2) and son of sevenless (SOS), leading to the downstream activation of the RAS/RAF/MEK/ERK pathway (Kouhara *et al.*, 1997) (**Fig. 10**). Moreover, FRS2 has also been implicated in the activation of atypical PKCs (PKC ζ and λ) (Lim *et al.*, 1999). Activation of PI3K and mTOR has also been observed in some cells downstream of FGFR and these are known to modulate the activity of S6K1/2 (Kanda *et al.*, 1997; Koh *et al.*, 1999). Of note, mTOR and S6K1/2 have been found highly over-expressed in SCLC tumours downstream of FGF-2 (Arcaro *et al.*, 2002; Pardo *et al.*, 2001).

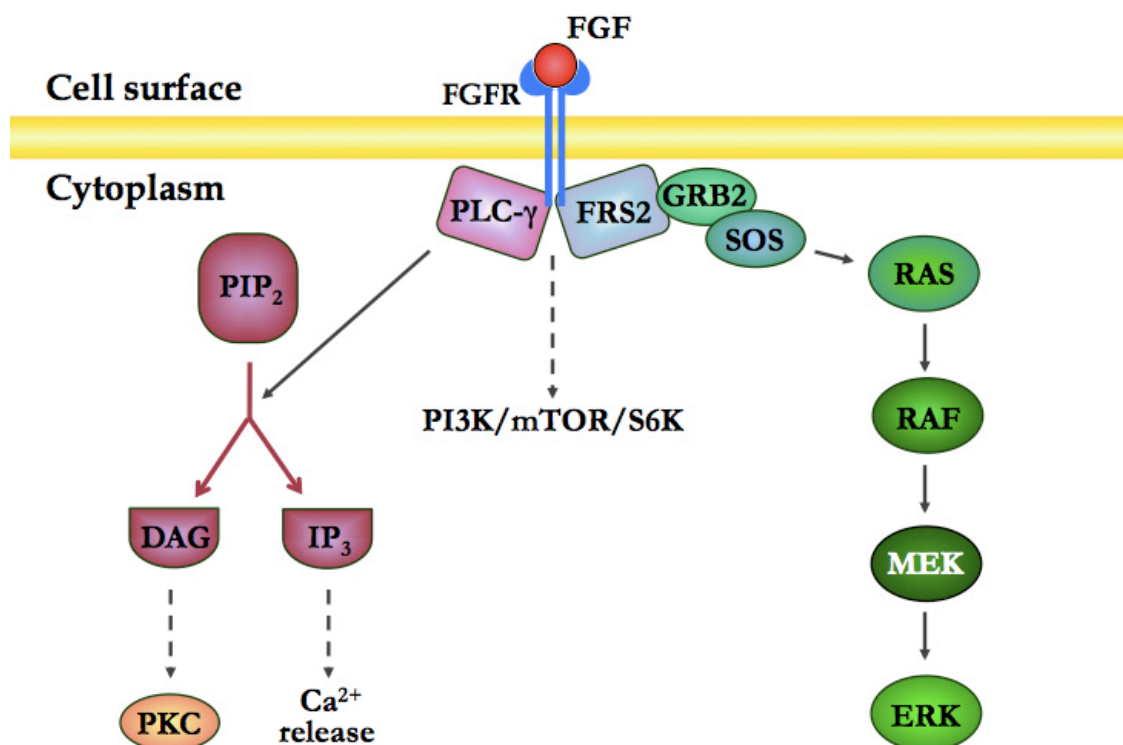


Figure 10. Signalling in response to FGF. Binding of FGF induces FGFR dimerisation and trans-phosphorylation of the cytoplasmic tail, providing docking sites for secondary messenger molecules. The main FGF-activated pathways are represented here. Grey arrows indicate direct activation. Dashed arrows indicate indirect activation. Red arrows indicate hydrolysis.

Under certain circumstances, upon activation of FGFR1, activation of SRC family kinases is also observed and is associated with several effects, including the differentiation of endothelial cells (Klint *et al.*, 1999; Landgren *et al.*, 1995). SRC family members can interact with a variety of receptors, such as receptor tyrosine kinases (RTKs) or GPCRs and regulate survival, proliferation, differentiation and migration (reviewed by Parsons and Parsons, 2004).

Upon binding, FGF-2/FGFR complexes are internalised by endocytosis (Wiedlocha and Sorensen, 2004). Internalised FGF-2 was shown to cross cellular membranes to reach the cytosol and the nuclear compartment (Almaric *et al.*, 1994; Stachowiak *et al.*, 1996; Sperinde and Nugent, 1998). Indeed, FGF nuclear targeting was shown to be involved in cell proliferation and, in particular, FGF-2 entry into the nucleus has been reported as necessary for cell cycle progression and proliferation (Bouche *et al.*, 1987; Bailly *et al.*, 2000). Nuclear FGF-2 has also been suggested to be involved in the survival of carcinoma cells, which are important for the establishment of lung metastases (Thomas-Mudge *et al.*, 2004). After FGF-2/FGFR endocytosis, FGF-2 is translocated through the cytosol through a mechanism that requires PI3K activity (Malecki *et al.*, 2004). The heat shock protein 90 kDa (HSP90) was shown to function as a chaperone required for the FGF-2 translocation from endosomes to the cytoplasm and nucleus (Wesche *et al.*, 2006). Inside the cell, FGF-2/FGFR complexes trigger different signalling pathways from the ones triggered by cell surface receptor/growth factor complexes. FGF-2 in the nucleus can directly stimulate casein kinase 2 (CK2) activity, a ubiquitous serine/threonine kinase involved in the control of cell proliferation (Bonnet *et al.*, 1996). For example, CK2 is required for the induction of nucleolin, a factor involved in control of ribosomal RNA (rRNA) transcription (Bouche *et al.*, 1994). Although FGFR1 also does not contain a detectable NLS, a pool of FGFR1 derived from the cell surface was detected in the nucleus upon FGF-2 binding (Maher, 1996). The uptake of FGFR1 in the nucleus was shown to require importin β , a critical component of multiple nuclear import pathways (Reilly and Maher, 2001). The internalisation and nuclear translocation of FGFR1 does not require activation of receptor tyrosine kinase (Reilly *et al.*, 2004). The same authors suggested that the signalling pathways that are known to be activated downstream of ligand binding are not the ones involved in its internalisation and thus intracellular trafficking may be stimulated by the binding of proteins to the transmembrane domain of FGFR. FGFR1 was demonstrated to serve as a mediator of FGF-2 transport to the nucleus and, together with FGF-2, was shown to regulate the proliferation of astrocytes and glioma cells (Stachowiak *et al.*, 1996; Joy *et al.*, 1997; Stachowiak *et al.*, 1997). Co-localisation of

FGFR1 with nuclear sites of RNA synthesis and processing suggested that the receptor could function as transcriptional regulator (Stachowiak *et al.*, 2003). Examples of genes that can be induced by nuclear FGFR1 are *c-JUN* and *cyclin D*, which encode proteins required for cell division and proliferation (Reilly and Maher, 2001). Interestingly, nuclear FGFR1 transactivates the *FGF-2* gene promoter and induces its expression (Stachowiak *et al.*, 2003). Induction of FGF-2 expression has been previously shown in response to externally added FGF-2 in mouse osteoblastic cells (Hurley *et al.*, 1994). The role of nuclear FGFR2 is less clear. This receptor can also be internalised and translocated into the nucleus but this was shown in response to FGF-9 (Kim *et al.*, 2007). Internalisation of FGFR also results in its degradation or recycling. Receptors that are destined for degradation are ubiquitinated (by virtue of ubiquitin molecules being attached to lysine residues on the cytoplasmic tail) and transported to lysosomes rather than being sorted to recycling endosomes (Haugsten *et al.*, 2005).

Receptor-mediated endocytosis is typically classified into two main pathways: the clathrin-mediated endocytosis (CME) and the clathrin-independent (CI) pathway (reviewed by Doherty and McMahon, 2009). The CME pathway involves the recruitment of clathrin from the cytoplasm to the plasma membrane. Adaptor and accessory proteins, such as epsin family proteins or the adaptor protein-2 (AP2), regulate clathrin recruitment to sites of plasma membrane that are designated to be internalised and promote the polymerisation of clathrin (Hinrichsen *et al.*, 2006; Schmid *et al.*, 2006). Polymerised clathrin-coated membrane pits bud and separate from the plasma membrane with the help of dynamin, which mediates the fission by forming a polymer around the pit constricted neck, thereby giving rise to clathrin-coated vesicles (Praefcke and McMahon, 2004). After internalisation, the vesicles are uncoated and undergo further trafficking within the cell. Accordingly, the receptors can be sorted to different sites of the cell (e.g. to the lysosomes for degradation, or back to the cell surface) (Grant and Donaldson, 2009). CME was for many years believed to be the pathway by which the internalisation of receptors occurred. Although CME is an extremely important endocytic pathway for receptors (including many RTKs), a number of reports have shown that receptor endocytosis can also occur in a clathrin-independent manner (reviewed by Kirkham and Parton, 2005). In addition, the same ligand/receptor complexes can be internalised through both clathrin-dependent and -independent pathways depending on factors, such as ligand concentration, and that can result in distinct signalling outcomes (Di Guglielmo *et al.*, 2003; Sigismund *et al.*, 2005). The best-studied CI endocytic pathway is mediated by caveolae, flask-shaped plasma membrane invaginations characterised by the presence of a caveolin protein

family member and enriched in sphingolipids and cholesterol (reviewed by Mayor and Pagano, 2007). The separation of these pits from the extracellular membrane is dynamin-dependent. Pre-caveolae are formed in the Golgi complex where cholesterol and caveolin oligomerisation takes place. The specific lipid composition of caveolae and the function of other caveolae-associated proteins remain to be firmly established (reviewed by Doherty and McMahon, 2009). Caveolae seem to undergo 'kiss-and-run' fusion and fission with the plasma membrane, transporting the cargo into cellular compartments and then recycling it to the plasma membrane (Pelkmans and Zerial, 2005). Other CI endocytosis can occur independently of caveolae through distinct pathways that require cholesterol and specific lipid compositions. An example of receptor endocytosis that occurs by a clathrin- and caveolae-independent mechanism is the internalisation of the IL-2 receptor (IL-2R) (Lamaze *et al.*, 2001). IL-2Rs are constitutively associated with detergent-resistant membrane (DRM) domains (domains with a specific composition of glycosphingolipids and cholesterol) that were suggested to mediate their endocytosis. The same authors showed that the internalisation of IL-2R is regulated by Rho family GTPases and requires dynamin. In a similar manner, the internalisation of FGF-2/FGFR complexes was shown to occur by a CI mechanism that is also independent of caveolae (Reilly *et al.*, 2004) but it is not fully understood. FGF-2 can also be internalised upon binding heparan sulfate proteoglycans (HSPG) independently of binding to FGFRs (Roghani and Moscatelli, 1992) and may have different intracellular destinations and functions from the internalised FGF-2 in FGF-2/FGFR complexes (Reiland and Rapraeger, 1993). FGF-2/HSPG internalisation was shown to occur by a CI and caveolar-dependent mechanism (Gleizes *et al.*, 1995).

The signalling downstream of FGF-2 and its receptors has been shown to be complex and thus much remains to be fully understood.

1.4.3. NEGATIVE AND POSITIVE REGULATORS OF FGF SIGNALLING PATHWAYS

FGF signalling is tightly controlled through the coordinated action of both positive and negative regulators that function at multiple levels of the signal transduction cascade. The strength and duration of FGF-induced ERK signalling is modulated by factors such as cell-surface receptor density, expression of scaffolding proteins, the surrounding extracellular matrix, and the interplay between kinases and

phosphatases (Murphy and Blenis, 2006). Gene expression is subsequently regulated by the modification of transcription factors by activated ERKs. Positive modulators of FGF signalling include: ETS transcription factors, like ETS-related molecule (ERM) and polyomavirus enhancer activator 3 (PEA3), and the *Xenopus* homolog of the fibronectin-leucine-rich transmembrane protein 3 (XFLRT3). Feedback antagonists of FGF-induced MEK/ERK signalling include family members of Sprouty (SPRY), similar expression to *fgf* (SEF) and MAPK phosphatase (MKP) families (reviewed by Tsang and Dawid, 2004). For example, SEF interacts with FGFRs and prevents the phosphorylation of FRS2 (Kovalenko *et al.*, 2003), while some members of the SPRY family inhibit the recruitment of SOS by binding to either GRB2 or SOS itself (Hanafusa *et al.*, 2002; Ozaki *et al.*, 2005). MKPs, also known as dual specific phosphatases (DUSP), such as MKP3 (DUSP6) can also attenuate MAPK signalling by dephosphorylating critical threonine and tyrosine residues of ERK1 and ERK2 (Zhao and Zhang, 2001). Often key negative regulators of signalling pathways can be induced by the same pathway they regulate. For example, FGF was shown to induce the expression of SPRY family members (e.g. Ozaki *et al.*, 2001) and genes like *erm* and *pea3* are transcriptionally activated by Fgf in zebra fish (Raible and Brand, 2001). Due to the relevance of FGF/FGFR signalling pathways in cancer, it is no surprise that their regulators are also found deregulated in a variety of cancers. These genes have been implicated in a variety of transcriptional regulatory events controlling normal and abnormal cell behaviour, including proliferation, differentiation, and migration (Wasylyk *et al.*, 1998). Indeed, it has been shown that the expression of SPRY family members is often abnormal in breast, prostate and liver cancers (Lo *et al.*, 2004).

The control of FGF pathways is indeed very complex and cross-talk with other pathways further increases the complexity of modulation.

1.4.4. FGF-2 IN CANCER

FGF-2 induces key processes that tumour cells require to successfully survive, proliferate and metastasise and can mediate these wide-range of functions through diverse signalling pathways.

FGF-2 can promote proliferation in many cell types (Bikfalvi *et al.*, 1997) and this can be achieved through different pathways. The activation of MEK/ERK has been shown to be required for FGF-2-induced proliferation in, for example murine lens

epithelial cells (Lovicu and McAvoy, 2001), bladder cancer cell lines (Tomlinson *et al.*, 2009) and some SCLC cell lines (Pardo *et al.*, 2001). In SCLC cells, S6K was also shown to be a major regulator of FGF-2 growth promoting effect, as it can be blocked by rapamycin (a mTOR inhibitor) (Pardo *et al.*, 2001). Proliferation of astrocytes and glioma cells was shown to be regulated by FGF-2 transported to the nucleus together with FGFR1 upon ligand binding and internalisation (Stachowiak *et al.*, 1996; Joy *et al.*, 1997; Stachowiak *et al.*, 1997). Nuclear FGFR1 can induce the expression of genes encoding proteins required for cell division and proliferation such as *c-JUN* and *cyclin D* (Reilly and Maher, 2001).

Another effect of FGF-2 is to confer resistance to therapeutic drugs as demonstrated, for example, in human umbilical vein endothelial cells (HUVECs), prostate, breast and lung cancer cells (Song *et al.*, 2000; Pardo *et al.*, 2002; Alavi *et al.*, 2003; Vandermoere *et al.*, 2005). Due to its relevance for the present study FGF-2 mediated chemoresistance will be described in more detail in the next chapter (**Section 1.4.5**).

Recently, it was shown in mouse brain microvascular endothelial cells (BMVECs) that FGF-2 can mediate migration, invasion and angiogenesis through a mechanism that required STAT5 and involved the activation of JAK2 and ERKs (Yang *et al.*, 2009). In this system, JAK2 but not SRC activity was demonstrated to be required for ERK1/2 phosphorylation. These findings demonstrate a cross-talk between JAK/STAT and MAPK pathways and suggest that this interplay is critical for the FGF-2-mediated angiogenesis.

In addition, FGF-2 can regulate the expression and function of E-cadherin and catenins, which are implicated in the proliferation, invasion and metastasis of a wide variety of solid tumours (El-Hariry *et al.*, 2001). El-Hariry and colleagues (2001) showed that FGF-2 induced E-cadherin phosphorylation, and the expression of E-cadherin and catenins correlated with increased cell adhesion. This led them to suggest that FGF-2 could induce re-localisation of E-cadherin to the plasma membrane. Other authors suggested a conjoint regulation of E-cadherin with FGFR1 and showed that FGF signalling resulted in deregulation of E-cadherin function. Over-expression of E-cadherin, for example, blocked nuclear translocation of FGFR1 (Bryant *et al.*, 2005). In addition, these authors have also shown that these two proteins were internalised in response to FGF-2 (and also FGF-1) stimulation and co-localised in intracellular compartments. Furthermore, FGF-2 can upregulate integrin receptors and potentiate their adhesive and signalling functions (Kinoshita *et al.*, 1993; Miyamoto *et al.*, 1996).

Importantly, FGF-2 was shown to regulate the expression of other factors that modulate cancer cell growth, such as IL-6 (Delrieu *et al.*, 1998). Moreover, this growth factor was shown to promote angiogenesis by inducing the expression of VEGF, a potent mitogen for micro- and macrovascular endothelial cells (Seghezzi *et al.*, 1998). The induction of VEGF expression was shown to be mediated by the RAS/RAF/MEK/ERK signalling pathway, at least in osteoblast cells (Tokuda *et al.*, 2000). Furthermore, externally added FGF-2 can also induce the expression of FGF-2 itself and the production of FGF-2 was suggested to be important as an autocrine and paracrine mediator of cell division, differentiation and function (Hurley *et al.*, 1994).

Interestingly, FGF-2 was also shown to be capable of restoring telomerase activity and consequently maintaining the replicative capacity of cells, a critical property for successful angiogenesis (Kurz *et al.*, 2003). Taken together, these observations demonstrate the pleiotropic nature of FGF-2 and highlight the significance that this growth factor has in cancer.

1.4.4.1. FGF-2-MEDIATED CHEMORESISTANCE MECHANISMS

Signalling downstream of FGF-2 can regulate components of the apoptotic machinery and consequently promote cell survival. FGF-2 has been shown to confer resistance to etoposide-mediated apoptosis in SCLC cells through a PI3K-independent mechanism that requires MEK/ERK activation (Pardo *et al.*, 2002). In contrast, in breast cancer cells, FGF-2-induced survival from ceramide-induced cell death was mediated by PI3K but was independent of MEK/ERK signalling (Vandermoere *et al.*, 2005). FGF-2-protection of breast cancer cells was shown to require the activation of the anti-apoptotic protein NF- κ B by PKB. The PI3K pathway was also demonstrated to be required for FGF-2 protection of osteoblasts against apoptosis induced by low serum concentration (Debiais *et al.*, 2004). Moreover, in HUVECs, FGF-2 also protected cells against apoptosis mediated by the intrinsic pathway independently of MEK/ERK activation (Alavi *et al.*, 2003). In these cells, however, FGF-2 was shown to activate C-RAF and, as a consequence, to promote its mitochondrial translocation where it binds and inhibits the kinase activity of the pro-apoptotic protein apoptosis signal-regulating kinase 1 (ASK1) (Alavi *et al.*, 2007). Together, these reports show that FGF is able to protect cells from apoptosis by different pathways.

Seckl and colleagues have shown that in SCLC cells the mechanism by which FGF-2 mediated chemoresistance required, in addition to MEK activation, B-RAF, PKC ϵ and S6K2 (Pardo *et al.*, 2006). In this cell type, FGF-2 induced the phosphorylation of PKC ϵ at residues Thr⁵⁶⁶ and Ser⁷²⁹, which are known to correlate with activation of this kinase (Parekh *et al.*, 1999; Cenni *et al.*, 2002). In addition, FGF-2 also induced phosphorylation of residue Thr³⁸⁸ of S6K2, which is required for its activation (Phin *et al.*, 2003). FGF-2-induced survival against etoposide was found to involve a translational mechanism that resulted in the upregulation of various anti-apoptotic proteins, such as the apoptosis regulators BCL-2, BCL-X_L, XIAP and the cellular inhibitor of apoptosis 1 (cIAP-1) (Pardo *et al.*, 2002; Pardo *et al.*, 2003). This pro-survival effect was shown to involve the inhibition of SMAC/DIABLO release, but not of cytochrome *c*, from the mitochondria that is usually implicated in the activation of apoptosis.

FGF-2-mediated protection from etoposide-induced cell death in H-510 cells involved the formation of a multiprotein complex composed by B-RAF, PKC ϵ and S6K2 (Pardo *et al.*, 2006) (**Fig. 11**). This is particularly interesting as it further shows that components of different oncogenic signalling pathways can cross-talk and synergise to promote cell survival and resistance to apoptosis. The specific function of each of these proteins in the complex is not yet completely understood. The association of PKC ϵ with B-RAF was suggested to induce MEK/ERK signalling as PKC ϵ was required for ERK1/2 phosphorylation and the expression of PKC ϵ (but not of other PKC family members) was correlated with the ability of FGF-2 to induce MEK/ERK activation and upregulation of anti-apoptotic proteins in SCLC cells (Pardo *et al.*, 2006). Interaction between PKC ϵ and C-RAF had been previously shown in NIH-3T3 cells, where PKC ϵ activation by phorbol esters triggered MEK/ERK signalling (Hamilton *et al.*, 2001). In addition, PKC activity itself was shown to be required for PKB-induced activity of C-RAF through a RAS-independent mechanism, a fact that prevents apoptosis in murine myeloid cells (Majewski *et al.*, 1999). In the SCLC cells, a complex between PKC ϵ and C-RAF was not detected (Pardo *et al.*, 2006).

Furthermore, *in vitro* kinase assays showed that PKC ϵ can directly associate with and phosphorylate S6K2. It is possible that, in SCLC cells, S6K2 is a substrate of PKC ϵ as other authors have shown that S6K2 is phosphorylated by several PKC isoforms (including PKC ϵ), which can in turn regulate S6K2 subcellular localisation (Valovka *et al.*, 2003). The downregulation of B-RAF, PKC ϵ and S6K2 using small interfering RNA (siRNA) demonstrated that while in the absence of B-RAF, PKC ϵ still associated with S6K2, PKC ϵ was required for the interaction of B-RAF with S6K2.

These data suggested that PKC ϵ can directly associate with S6K2, whereas B-RAF required the presence of PKC to join the complex. Nevertheless, other proteins may also bind to these three proteins and have a function in this complex. It is also conceivable that upon FGF-2 several different protein complexes are formed between these, and potentially other, proteins.

The mechanisms by which the B-RAF, PKC ϵ and S6K2 complex interacts with the translational machinery to upregulate the targets previously mentioned and promote FGF-2-mediated chemoresistance remain, however, unknown. It seems plausible that other molecules may bind to the B-RAF, PKC ϵ and S6K2 complex but their molecular identity remains to be unravelled.

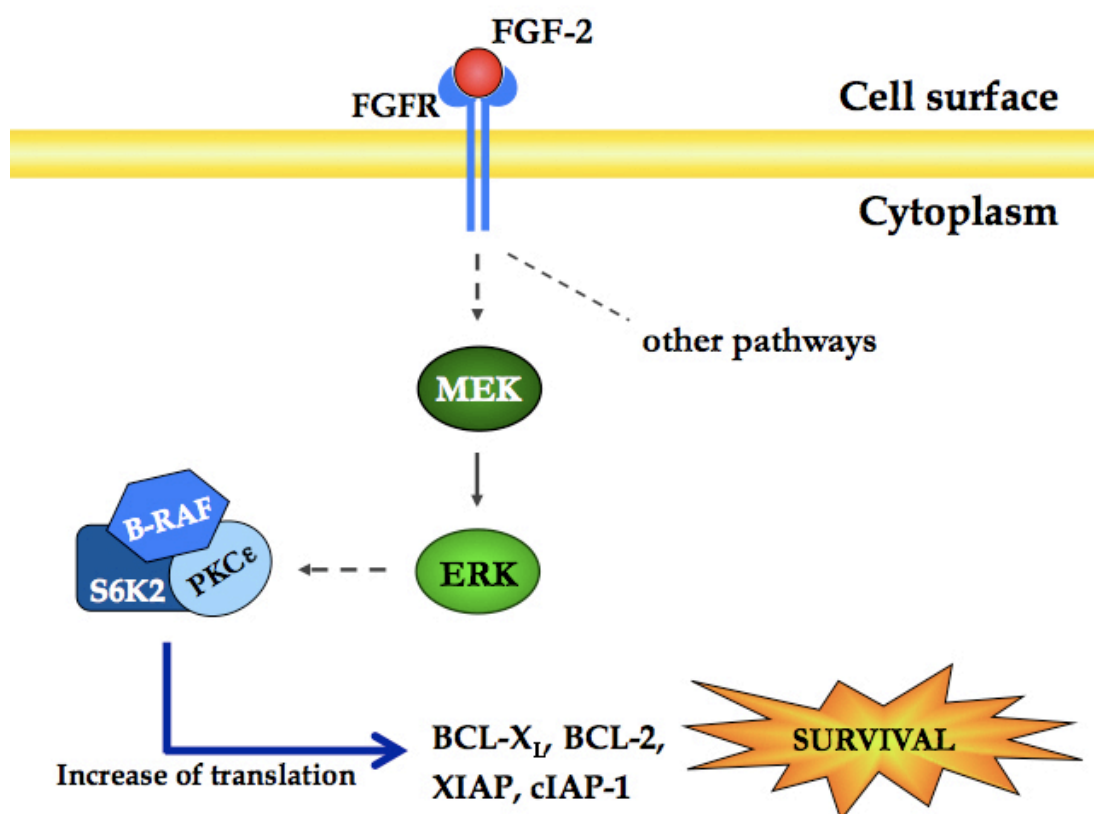


Figure 11. FGF-2-mediated chemoresistance pathways in SCLC cells. FGF-2 signalling *via* MEK-ERK induces the formation of a complex comprising at least PKC ϵ , B-RAF and S6K2. This complex upregulates the expression of anti-apoptotic proteins important for promoting chemoresistance. Grey arrows indicate direct activation. Dashed arrows indicate indirect activation.

1.4.5. TARGETING FGF-2 SIGNALLING FOR CANCER TREATMENT

The inhibition of FGF-2 signalling alone or together with other chemotherapeutic drugs was shown to be an effective therapy for some cancers (reviewed by Beenken and Mohammadi, 2009). One example is thalidomide, an inhibitor of FGF-2-induced angiogenesis that was shown to have therapeutic benefits in prostate and renal cancer patients (Eisen *et al.*, 2000; Figg *et al.*, 2001). Another example is suramin, which interferes with FGF signalling by mimicking heparin, and was shown to be an efficacious treatment in bladder, kidney and prostate cancer patients (Motzer *et al.*, 1992; Myers *et al.*, 1992; Danesi *et al.*, 1993; Eisenberger *et al.*, 1993; Walther *et al.*, 1996). Another compound, sunitinib (SU011248), a tyrosine kinase inhibitor, has been shown to efficiently inhibit several RTKs including FGFR1, PDGFR and VEGFR2, and consequently inhibit tumour cell growth and tumour regression in mouse models of colon cancer, NSCLC, melanoma, and renal and squamous cell carcinomas (reviewed by Chow and Eckhardt, 2007).

Specific FGFR inhibitors have also been developed. One example is PD173074, an ATP pocket inhibitor (Mohammadi *et al.*, 1998). This compound was shown to inhibit FGFR-activation in several cell systems and to promote apoptosis and inhibition of cell growth in cancer cells with activating FGFR mutations (Bansal *et al.*, 2003; Grand *et al.*, 2004; Byron *et al.*, 2008; Dutt *et al.*, 2008). Moreover, recent work by the Seckl and Pardo groups has demonstrated that PD173074 blocks FGF2-induced proliferation and chemoresistance in SCLC cell lines (Pardo *et al.*, 2009). Importantly, treatment with this compound triggered tumour cell apoptosis and cured 50% of mice with H-69 SCLC xenografts and potentiated significantly the efficacy of cisplatin in H-510 SCLC xenografts (Pardo *et al.*, 2009). Accordingly, FGFR inhibitors may provide a good therapeutical strategy for treatments of cancer patients.

Other approach for targeting FGF-2 signalling might be to use MEK/ERK inhibitors, as MEK signalling is a crucial element of FGF-2 signalling pathways (e.g. Tokuda *et al.*, 2000; Lovicu and McAvoy, 2001; Pardo *et al.*, 2001; Pardo *et al.*, 2002; Tomlinson *et al.*, 2009) and, in addition, is a key mediator of proliferation downstream of multiple growth factors (reviewed by Kolch, 2000; Ballif and Blenis, 2001). Indeed, several small-molecule inhibitors of MEK have been developed and these are currently being tested in clinical trials. For example, CI-1040 was the first MEK inhibitor to enter clinical evaluation (Lorusso *et al.*, 2005; Rinehart *et al.*, 2004). Compounds like CI-1040 prevent full activation of MEK1/2 without competing with ATP. The inhibition of activated MEK1/2 prevents the stimulation of ERK1/2 activity and blocks phosphorylation/activation of downstream substrates. CI-1040 first demonstration of

anti-cancer activity was the reduction of human colon cancer xenograft growth (Sebolt-Leopold *et al.*, 1999). This inhibitor showed promising results during a phase I clinical trial. Tumour tissues collected from patients treated with CI-1040 showed a significant reduction in activated/phosphorylated ERK. Importantly, 25% of patients with a variety of tumours had a stable disease for longer than 3 months (Lorusso *et al.*, 2005). However, when CI-1040 advanced to a phase II trial it revealed adverse side effects and did not meet predetermined end points to progress to a phase III trial (Rinehart *et al.*, 2004). Accordingly, clinical development of CI-1040 was stopped. A second-generation agent, PD0325901, was introduced into the clinic but the development of this compound was stopped also because of toxicity-related issues (reviewed by Friday and Adjei, 2008). Another MEK inhibitor compound, AZD6244, was better tolerated and has provided encouraging results in a phase I clinical trial (Adjei *et al.*, 2008). This inhibitor is still undergoing evaluation in phase II trials in combination with other chemotherapeutic agents (Bennouna *et al.*, 2010). Toxicity is one of the major concerns when using small-molecule MEK inhibitors, but additional compounds are now being studied in patients. Nevertheless, early studies and reports of clinical activity are encouraging and put these inhibitors in the forefront of cancer therapeutics (Roberts and Der, 2007; Friday and Adjei, 2008)

1.5. FGF-2 AND THE JAK/STAT SIGNALLING PATHWAY

There have been reports suggesting that activation of the JAK/STAT signalling pathway can be triggered by FGF-2. It has been shown that FGF-2 treatment can induce the activation of STAT1, STAT3 and STAT5 in some cells and that blockage of JAK activity can inhibit FGF-2-induced proliferation (Ebong *et al.*, 2004; Yang *et al.*, 2009). Activation of STATs was also observed downstream of other FGFRs. Constitutively activated FGFR1, FGFR3 and FGFR4 were shown to stimulate the phosphorylation and activity of STAT1 and STAT3 (Hart *et al.*, 2000). Furthermore, activation of STAT1 downstream of FGFR3 was shown to contribute to the inhibition of chondrocyte proliferation and regulation of bone development (Sahni *et al.*, 1999). In addition, STATs can be regulated by proteins that are activated downstream of FGF-2, such as ERK1/2. STAT1, STAT3, STAT4 and STAT5 all have an ERK recognition consensus sequence (PMSP – proline, methionine, serine, proline) located at their C-terminal domain (Wen *et al.*, 1995). ERK1/2 were shown to negatively regulate STAT3

transcriptional activity by phosphorylation of residue Ser⁷²⁷ (located within the PMSP sequence) and ERK2 was demonstrated to physically interact with STAT3 (Jain *et al.*, 1998; Tian and An, 2004). Depending on the cellular context, the phosphorylation of this residue can be induced by several other kinases but when induced by EGF it was shown to be ERK-dependent (Chung *et al.*, 1997).

To the best of our knowledge, no studies have been carried out to determine whether JAKs and/or STATs are involved in FGF-2-induced drug resistance. During my PhD, I have investigated if JAK/STAT signalling pathways impacted on FGF-2-mediated chemoresistance mechanism(s) using lung and bone cancer cell lines as model cell lines.

1.5.1. FGF/FGFR IN LUNG AND BONE CANCERS

As previously mentioned, FGF/FGFR signalling has been shown to be relevant in organogenesis, particularly in the nervous system, the lungs and the limbs (reviewed by Dickson *et al.*, 2000; Powers *et al.*, 2000). For example, FGFR2III-null mice have severe defects of the limbs and lungs due to loss of the progenitor cells by apoptosis (De Moerlooze *et al.*, 2000). FGFs and their receptors play a crucial role in lung development as FGF-10/FGFR2 signalling has been shown to be required for epithelial branching in the mouse lung (Abler *et al.*, 2009). Additionally, FGF-9 has been implicated in the regulation of airway smooth muscle cell differentiation and proximal-distal patterning in the mouse lung (Perl and Gale, 2009; Yi *et al.*, 2009). FGF signalling plays also an important role in the regulation of bone development (Ornitz and Marie, 2002). For example, while FGFR2 can act as a positive regulator of bone growth being essential for osteoblast proliferation and function (Yu *et al.*, 2003), the disruption of the *FGFR3* gene results in bone over-growth (Colvin *et al.*, 1996; Deng *et al.*, 1996). A number of studies have shown that FGF-2 induces cell proliferation and inhibits the differentiation of primary chondrocytes (Kato and Iwamoto, 1990; Hill *et al.*, 1991; Wroblewski and Edwall-Arvidsson, 1995).

FGF/FGFR signalling is not only vital for lung and bone cellular homeostasis, but is also relevant for cancer promotion/progression. Of note, FGFR1 was shown to promote NSCLC progression and invasion (Fischer *et al.*, 2008). A mutation in *FGFR4* was detected in a significant number of NSCLC patients and associated with poor prognosis in node-positive patients (Sasaki *et al.*, 2008). As previously described here

(Section 1.4.4 and 1.4.4.1), FGF-2 has been shown to confer chemoresistance in SCLC (Pardo *et al.*, 2001; Pardo *et al.*, 2002; Pardo *et al.*, 2003; Pardo *et al.*, 2006). Furthermore, FGF signalling was shown to be important for the transformed state and survival of bone cancer cells (Muenke and Schell, 1995; Coffin *et al.*, 1995; Deng *et al.*, 1996; Bodo *et al.*, 2002). For example, FGF-1 expression was correlated with bone tumour growth (Babkina *et al.*, 2009). In addition, FGF-8 was also shown to stimulate the proliferation of osteosarcoma cells (Valta *et al.*, 2006).

Accordingly, given the important role that FGF/FGFR signalling plays in lung and bone cancers, the elucidation of relevant mechanisms of chemoresistance mediated by these growth factors might contribute for the development of more efficient therapies.

1.5.2. JAKs AND STATs IN LUNG AND BONE CANCERS

JAK/STAT signalling pathways were also shown to be critical for lung and bone cancer biology, participating in cell malignant transformation and chemoresistance (Ikuta *et al.*, 2005; Loewen *et al.*, 2005; Geryk-Hall and Hughes, 2009). For example, over-expression of RTKs like EGFR, as well as non-RTKs, such as SRC, are responsible for the aberrant activation of STAT3, which has been implicated in the development of human lung cancer (Haura *et al.*, 2005; Song *et al.*, 2003). In addition, cytokines like IL-6 have been shown to modulate lung cancer cell growth and, in fact, elevated levels of this cytokine seem to be an adverse prognostic factor for patients with this type of carcinoma (Yanagawa *et al.*, 1995; Eickelberg *et al.*, 1999; Yeh *et al.*, 2006). Patients with osteosarcoma, chondrosarcoma, Ewing's sarcoma and giant cell tumour of bone also have significantly elevated levels of serum IL-6 that correlates with a poor overall survival (Rutkowski *et al.*, 2003). IL-6-type cytokines are, in fact, important regulators of bone pathophysiology (reviewed by Blanchard *et al.*, 2009). Although some IL-6-type cytokines, such as OSM, can mediate anti-cancer effects in the bone environment they often induce bone resorption and tumour growth. Importantly, IL-6 has long been known to activate STAT3, which is its main transcription factor (Heinrich *et al.*, 2003). Neuregulin-1 was also found to play an important role in lung epithelial cell proliferation by inducing JAK/STAT activation, in particular JAK3, TYK2, STAT3 and STAT5 (Liu and Kern, 2002).

STAT3 activation enhances the expression of survival proteins involved in preventing cell cycle arrest and senescence. Several recent studies have suggested that the STAT3 oncogenic pathway is not only involved in cell transformation but is also associated with intrinsic drug resistance (Ikuta *et al.*, 2005; Grisko *et al.*, 2006; Barre *et al.*, 2007). In lung cancer cells, over-expression of STAT3 correlated with suppression of apoptotic pathways in cisplatin-resistant NSCLC cells (Ikuta *et al.*, 2005). In addition, it has been shown that the growth and survival of early stage NSCLC tumours may be dependent on the activation of STAT3 by EGFs (Haura *et al.*, 2005). STAT3 was also found to be activated in a subset of the Ewing's sarcoma family of tumours (Lai *et al.*, 2006). Interestingly, activation of oncogenic STAT3 could be induced by Kaposi's sarcoma-associated herpesvirus (KSHV), the infectious cause of Kaposi's sarcoma, and it was thus suggested that it may play a critical role in KSHV pathogenesis (Punjabi *et al.*, 2007).

Somatic EGFR mutations found on NSCLC cells correlated with STAT3 activation, a critical mediator of the oncogenic effects of this growth factor, and importantly STAT3 activity can be downregulated by small-molecule inhibitors targeting EGFR (Alvarez *et al.*, 2006). Regarding the role of STAT3 in promoting survival and proliferation, these authors suggested that EGFR/STAT3 signalling pathway probably limits (or at least contributes to a decrease in) the efficiency of current chemotherapeutic treatments and that the inhibition of STAT3 is likely to be involved in the improved therapeutic effects of, for example, gefitinib.

Since several types of commonly used treatments often fail in tumours where constitutively activated STAT proteins are found, molecular therapies leading to STAT signalling inhibition may increase tumour response to chemotherapy and/or conventional radiotherapy, and these are certainly worthwhile pursuing (reviewed by Calo *et al.*, 2003; Yu and Jove, 2004).

2. HYPOTHESIS AND SPECIFIC AIMS

FGF-2 was shown to be an important player in promoting oncogenesis and driving chemoresistance (Giri *et al.*, 1999; Dickson *et al.*, 2000; Nurcombe *et al.*, 2000; Song *et al.*, 2000; Pardo *et al.*, 2002; Pardo *et al.*, 2003; Polnaszek *et al.*, 2003). It was previously shown in lung cancer cell lines that FGF-2 mediated chemoresistance by inducing the expression of anti-apoptotic proteins *via* a MEK/ERK-dependent mechanism, which involved the formation of a multi-protein complex that contained B-RAF, PKC ϵ and S6K2 (Pardo *et al.*, 2002; Pardo *et al.*, 2006). There have been reports suggesting that activation of JAKs and/or STATs can occur downstream of FGF-2 (Ebong *et al.*, 2004; Yang *et al.*, 2009), molecules that have been implicated in tumourigenesis and chemoresistance mechanisms (e.g. Verma *et al.*, 2003; Yu and Jove, 2004). In addition, constitutive activation of STATs is frequently observed in a variety of tumours (Bromberg, 2002; He *et al.*, 2004). Despite evidence for the deregulation of STAT activation in lung and bone cancer cells, there is remarkably little data regarding the relevance of JAK/STAT signalling during lung and bone carcinogenesis. Since JAKs and STATs represent a point of convergence for many cytokines and, indeed, growth factors, they are also emerging as favoured targets for therapeutic intervention in human cancer therapy. **We hypothesised, therefore, that JAKs and/or STATs play a role in FGF-2-mediated chemoresistance pathway(s).** Accordingly, the main aims of this project were:

- To investigate the generality of FGF-2-mediated chemoresistance by analysing additional cell types, namely NSCLC and osteosarcoma cell lines;
- To establish the role of JAK and STAT proteins in FGF-2-mediated chemoresistance pathways used by different cancer cells;
- To determine the role of mitogenic ERK2 in the regulation of the STAT3 expression, a molecule which is increasingly recognised as a key mediator of tumour cell survival.

3. MATERIALS AND METHODS

3.1. MATERIALS

Foetal calf serum (FCS) was purchased from FirstLink (Birmingham, UK). L-glutamine and penicillin-streptomycin were obtained from Biowhittaker (Verviers, Belgium). Protease inhibitors and JAK inhibitors (JAK inhibitor I and JAK2 inhibitor II) were obtained from Calbiochem (Darmstadt, Germany). MEK inhibitor PD0325901 was provided by Pfizer (Connecticut, USA). Acrylamide was purchased from National Diagnostics (Hull, UK). Phosphate Buffered Saline (PBS) was purchased from OXOID (Hampshire, UK). Glycine, Nonidet-P40 (NP-40) and paraformaldehyde (*p*-FA) were purchased from BDH Chemicals (Poole, UK). Milk was obtained from Fluka BioChemika (Buchs, Switzerland). Bromophenol blue and BioRad protein assay were obtained from BioRad (Munich, Germany). Polyvinylidene difluoride (PVDF) membranes were purchased from Millipore (Bedford, USA). Ethanol, methanol and agarose were obtained from Fisher Scientific (Loughborough, UK). DNA 100 bp ladder was purchased from New England Biolabs (Hertfordshire, UK). Crystal violet was obtained from GURR (High Wycombe Bucks, UK). Enhanced chemiluminescence (ECL) detection reagents were purchased from Amersham (Buckinghamshire, UK). Autoradiography film was purchased from Kodak (Colorado, USA). Xylene cyanol was obtained from Hopkin & Williams (Essex, UK). SYBR green was purchased from Invitrogen (Oregon, USA). Cell viability assays were done using the Calbiochem Rapid Cell Proliferation Kit WST-1 (Darmstadt, Germany). All other reagents were purchased from Sigma-Aldrich (Steinheim, Germany).

Primary antibodies against JAK1 (HR 785), JAK2 (C-20), TYK2 (C-20), STAT3 (N-terminal), STAT5A (L-20), STAT5B (G-2), ERK1 (subdomain XI), B-Raf (H145), BCL-2 (100), BCL-X_L (H-62) and lamin B (C-20) were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). STAT1 (N-terminal) and PY20 antibodies were purchased from BD Transduction Laboratories (Erembodegem, Belgium). Antibodies against JAK1, phosphorylated JAK2 (pJAK2-Tyr^{1007/1008}), phosphorylated STAT1 (pSTAT1-Tyr⁷⁰¹), phosphorylated STAT3 (pSTAT3-Tyr⁷⁰⁵), phosphorylated STAT5 (pSTAT5-Tyr⁶⁹⁴), phosphorylated ERK1/2 (pERK1/2-Thr²⁰²/Tyr²⁰⁴), PARP and ERK5 were obtained from Cell Signalling Technology (Hitchin, UK). Antibodies against MCL-1 and XIAP were from BD Pharmingen (California, USA). 4G10 and PKC ϵ

antibodies were purchased from Millipore (California, USA). The antibody against β -actin was obtained from Sigma-Aldrich (Steinheim, Germany). The PE-conjugated secondary anti-mouse antibody was purchased from DAKO (Glostrup, Denmark). The S6K2 antibody (region between residue 430 and the C-terminus) used for immunoprecipitations (IPs) was obtained from Bethyl (Montgomery, USA) and the Western blot one was a gift from Dr Ivan Gout (University College London, London, UK). The gp130 antibody used for Western blot was purchased from Upstate Biotechnology (New York, USA) and the one used for flow cytometry (BT12) was a gift from Dr J. Wijdenes (Besançon, France).

The human recombinant FGF-2 was obtained from Calbiochem (Darmstadt, Germany). Human IL-6 and soluble IL-6 receptor (sIL-6R) were from R&D Systems (Abingdon, UK). OSM was obtained from PeproTech (London, UK). Etoposide and cisplatin were purchased from Sigma-Aldrich (Steinheim, Germany). IFN- γ was a gift from Dr G. Adolf (Ernst-Boehringer Institut für Arzneimittelforschung, Vienna, Austria).

3.2. CELL LINES AND CULTURE

NSCLC cell lines HCC95, HCC78, A549 and HCC193, the SCLC cell line H-510 and osteosarcoma U2OS cells were a gift from Dr Julian Downward (Cancer Research UK/London Research Institute, London, UK). The JAK1-negative cell line (U4C), the JAK1-negative cells complemented with JAK1 (U4C.JAK1) (Leaman *et al.*, 1998), the JAK2-negative cell line (γ 2A), the JAK2-negative cells complemented with JAK2 (γ 2A.JAK2) (Watling *et al.*, 1993), the TYK2-negative cells (U1A) and the TYK2-negative cells complemented with TYK2 (U1A.TYK2) (McKendry *et al.*, 1991) were a gift from Dr. Ian M. Kerr (Cancer Research UK/London Research Institute, London, UK). Cell line features are summarised in **Table I**.

Table I. Cell lines.

Cell line	Type	Culture	Particular features
HCC95	NSCLC, squamous cell carcinoma	Adherent	-
HCC78	NSCLC, adenocarcinoma	Adherent	-
A549	NSCLC, adenocarcinoma	Adherent	-
HCC193	NSCLC, adenocarcinoma	Adherent	-
H-510	SCLC	Suspension	FGF-2-induced chemoresistance
U2OS	Bone osteosarcoma	Adherent	Express wild type p53
U4C	Derived from the fibrosarcoma cell line HT1080	Adherent	JAK1-deficient
U4C.JAK1	Derived from the fibrosarcoma cell line HT1080	Adherent	JAK1-deficient complemented with JAK1
γ 2A	Derived from the fibrosarcoma cell line HT1080	Adherent	JAK2-deficient
γ 2A.JAK2	Derived from the fibrosarcoma cell line HT1080	Adherent	JAK2-deficient cells complemented with JAK2
U1A	Derived from the fibrosarcoma cell line HT1080	Adherent	TYK2-deficient
U1A.TYK2	Derived from the fibrosarcoma cell line HT1080	Adherent	TYK2-deficient complemented with TYK2

NSCLC, osteosarcoma and JAK-negative and -complemented cell lines are adherent and were grown in Dulbecco's Modified Eagle's medium (DMEM). The SCLC cell line H-510 grows in suspension and was cultured in Roswell Park Memorial Institute (RPMI)-1640. The cells were cultured in media supplemented with 10% (v/v) heat-inactivated FCS, 2 mM L-glutamine, 50 units/ml penicillin and 50 μ g/ml streptomycin in a humidified atmosphere of 10% (NSCLC, osteosarcoma and JAK-negative and -complemented) or 5% CO₂ (SCLC) at 37 °C. Cells were typically passaged twice a week at a 1 in 10 dilution for U2OS, HCC95, A549, HCC193, U4C, U4C.JAK1, γ 2A, γ 2A.JAK2, U1A and U1A.TYK2, 1 in 8 dilution for HCC78 and a 1 in 2-3 dilution for H-510. U4C.JAK1 and γ 2A.JAK2 cells were grown in the presence of 0.5 μ g/ml of puromycin and U1A.TYK2 cells in the presence of 250 μ g/ml of hygromycin B for the selection of plasmid-expressing cells.

For experimental purposes the cells were incubated in starvation media. Osteosarcoma and NSCLC cell lines were grown overnight in serum-free media, or in

DMEM supplemented with 0.5% (v/v) heat-inactivated FCS, 2 mM L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin. The SCLC cell line was grown for 3 days in RPMI-1640 supplemented with 5 µg/ml insulin, 10 µg/ml transferrin, 30 nM sodium selenite, 0.25% (w/v) bovine serum albumin (BSA), 50 units/ml penicillin and 50 µg/ml streptomycin.

3.3. PROTEIN EXTRACTION

Protein extractions were performed using different lysis buffers depending on the proteins under study, and the question being addressed experimentally. Lysis buffers used to extract proteins usually include a buffering agent [such as tris(hydroxymethyl)aminomethane (Tris)-HCl] that controls the pH of the solution, thus preventing denaturation or inactivation of proteins (these agents prevent the change in the pH of the solution by providing the corresponding conjugate acid or base); a salt [such as sodium chloride (NaCl)] that breaks open the cells allowing the release of the proteins by inducing an osmotic shock; and a detergent for lipids solubilisation. Detergents can be non-ionic (such as Tween-20), which solubilises proteins through the establishment of lipid micelles and without altering protein conformation, or ionic [such as sodium dodecyl sulphate (SDS)], which ionise in aqueous solutions and bind to the proteins, normally causing their denaturation (it is usually used for the complete disruption of membranes). Some buffers can also include a thiol compound [such as dithiothreitol (DTT)], which reduces the disulphide bonds of proteins and destroy intra- and inter-molecular bonds between cysteine residues of proteins (and consequently, their main tertiary and quaternary structure), or glycerol, an osmolite which stabilises protein-protein interactions, and which helps protein samples to sink in the loading wells of the gel after loading. Importantly, lysis buffers contain protease and phosphatase inhibitors that prevent protein degradation and the removal of phosphate groups by endogenous enzymes that are released from subcellular compartments during cell lysis (Deutcher, 1990). Commonly used protease and phosphatase inhibitors include aprotinin (a trypsin inhibitor), leupeptin (a lysosomal protein inhibitor), phenylmethanesulphonyl fluoride (PMSF) (a serine protease inhibitor), ethylenediaminetetraacetic acid (EDTA) (a metalloproteases inhibitor that acts by chelating metal ions such Zn^{2+} , Ca^{2+} and Mg^{2+}), sodium fluoride

(NaF) (a serine/threonine phosphatase inhibitor) and sodium orthovanadate (Na_3VO_4) (a tyrosine phosphatase inhibitor).

In this work, Schindler lysis buffer was used in the vast majority of experiments. This buffer was initially developed to detect STATs and extracts mainly cytoplasmic proteins (Schindler *et al.*, 1992). Schindler lysis buffer contains 50 mM Tris pH 8, 150 mM NaCl, 0.5% (v/v) NP-40 (octyl phenoxypolyethoxyethanol), 2 mM DTT, 10% (v/v) glycerol, 0.1 mM EDTA pH 8, 50 mM NaF, 0.1 mM Na_3VO_4 , 100 U/ml aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin and 1 mM PMSF.

For the detection of gp130 receptor, a membrane-bound protein, extractions were performed using a buffer extensively used for that purpose by the Hermanns group (Heike Hermanns, personal communication). This buffer has been shown to efficiently extract gp130 from the plasma membrane. It is composed of 93.2 mM disodium hydrogen phosphate [$\text{Na}_2\text{HPO}_4(*2 \text{ H}_2\text{O})$], 6.8 mM sodium di-hydrogen phosphate [$\text{NaH}_2\text{PO}_4(*\text{H}_2\text{O})$], 0.5% (v/v) Triton X-100, 0.5 mM EDTA, 1 mM Na_3VO_4 , 100 U/ml aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin and 1 mM PMSF.

For co-IPs, proteins were extracted with a lysis buffer that has been shown to successfully preserve protein interactions between proteins under study, and which is extensively used in our laboratory (Pardo *et al.*, 2006). The co-IP lysis buffer contains 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.5, 150 mM NaCl, 0.5% (v/v) Triton X-100, 10% (v/v) glycerol, 2 mM EDTA, 10 mM NaF, 10 mM β -glycerophosphate, 0.1 mM Na_3VO_4 , 100 U/ml aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin and 1 mM PMSF.

Protein lysates were centrifuged at 16.2 xg for 10 minutes and the pellets were discarded. Protein concentrations determined by spectrophotometry using the BioRad protein assay according to the manufacturer's instructions. Extracts were boiled at 99°C for 5 minutes with a modified version of the Laemli buffer [0.5 M Tris, pH 6.8, 2% (w/v) SDS, 100 mM DTT, 10% (v/v) glycerol, 0.001% (w/v) bromophenol blue] before separation by SDS-polyacrylamide gel electrophoresis (PAGE).

3.4. PROTEIN IMMUNOPRECIPITATION

Proteins present in low abundance in cells (such as JAKs) were purified by IP prior to separation by SDS-PAGE. U2OS cells were plated in 15 cm plates ($\sim 4 \times 10^6$ cells/plate), serum-starved overnight in DMEM supplemented with 0.5 % (v/v) of

FCS, penicillin and streptomycin, and then incubated in serum-free media for 2 hours prior to stimulation with FGF-2 (10 ng/ml). Before use beads, which were stored in 20% (v/v) ethanol, were washed 3 times with PBS (by centrifugation at 16.2 xg for 40-60 seconds at 4 °C) and re-suspended in Schindler's buffer. After extracting the proteins with Schindler's lysis buffer (chapter 3.3), 3 mg of whole cell lysates (WCL) were incubated rotating at 4 °C for 1 hour with the appropriate antibody (typically 1.6 µg/ml), followed by 1 hour with protein A-agarose beads (25 µl of a 50% slurry solution). Immunoprecipitates were then washed at least 5 times (by centrifugation at 16.2 xg for 40-60 seconds at 4 °C) with lysis buffer (last wash was done using Schindler's buffer/PBS, 1:1) and boiled for 5 minutes with modified Laemli buffer before separation on a SDS-PAGE gel.

3.4.1. PROTEIN CO-IMMUNOPRECIPITATION

Co-IP was performed to detect protein-protein interactions. Co-IPs were conducted essentially in the same manner as the IPs albeit using a different lysis buffer and time of incubation of the lysate with the antibody and agarose beads. Adherent cells were plated and starved as described in chapter 3.4. H-510 cells were starved (in RPMI-1640 supplemented with insulin, transferrin, sodium selenite, BSA, penicillin and streptomycin) for 3 days and then aliquoted at a density of 5x10⁴ cell/ml (1 ml per aliquot) in serum-free RPMI. All cells were incubated for 1 hour in serum-free media before treatment. After treatment, proteins were extracted using the co-IP lysis buffer (chapter 3.3), which has a composition adequate to perform precipitation of the target protein and co-precipitate binding partners/protein complexes. The antibody (typically 1 µg/ml) and the agarose beads (25 µl of a 50% slurry solution) were previously incubated in 1 ml of 3% (w/v) BSA/PBS (per sample) overnight rotating at 4 °C. Lysates were pre-cleared with agarose beads (25 µl 50% slurry solution per sample) for 30 minutes. After centrifugation at 0.4 xg for 20-40 seconds at 4 °C, the supernatant was transferred to another labelled eppendorf tube. Protein concentration was determined and 0.3-0.5 mg of protein extract was incubated with the antibody and beads for 3 hours, rotating at 4 °C. Immunoprecipitates were then washed 3 times with the same lysis buffer (by centrifugation at 0.4 xg for 20-40 seconds at 4 °C) and boiled for 5 minutes with modified Laemli buffer before separation on a SDS-PAGE gel.

3.5. SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS AND WESTERN BLOT ANALYSIS

Proteins were separated using a 5% acrylamide stacking gel [5% (v/v) acrylamide, 0.125 M Tris, pH 6.8, 0.1% (w/v) SDS, 0.075% (w/v) ammonium persulphate (APS) and 0.083% (v/v) Tetramethylethylenediamine (TEMED)] and a 7.5, 10 or 12.5% acrylamide resolving gel [7.5-12.5% (v/v) acrylamide, 0.375 M Tris, pH 8.8, 0.1% (w/v) SDS, 0.06% (w/v) APS, 0.07% (v/v) TEMED] depending on the size of proteins being analysed. Gels were run using a Tris-glycine running buffer [2.5 mM Tris, 0.2 M glycine and 0.1% (w/v) SDS] at 170 V until the dye reached the bottom of the gel. Proteins were transferred to a methanol-activated PVDF membrane using a Tris-glycine buffer [2.5 mM Tris, 0.2 mM glycine supplemented with 10% (v/v) methanol] at 25 V for 1 hour at 4°C using an electroblotter (Idea Scientific, Minneapolis, USA). Membranes were blocked in Tris-buffered saline/Tween (TBST) [0.01 M Tris, pH 7.4, 75 mM NaCl, 1.25 mM EDTA, pH 8.0, 0.1% (v/v) Tween 20] containing 5% (w/v) BSA, or 5% (w/v) milk prior to incubation with antibodies. Binding of each antibody was detected using ECL. After probing for the phosphorylated protein, the membranes were stripped of antibody using a solution of 2 M of glycine, pH 2.5 and 0.1% (w/v) of SDS and subsequently probed for the total respective protein. β -actin was routinely used as an additional protein loading control.

3.6. CELL DEATH/CHEMORESISTANCE ASSAYS

Cell death and chemoresistance assays were set up similarly for all the cell lines used in this study: cells were plated, incubated in serum free-media the following day (from overnight to 3 days, depending on the cell line), pre-treated with FGF-2 for 4 hours prior to addition of the drug and cell death assessed after 1 to 3 days.

Adherent cells were starved (in serum-free DMEM supplemented with penicillin and streptomycin) overnight and then pre-treated with or without FGF-2 (10 ng/ml) for 4 hours prior to addition of the relevant chemotherapeutic agents. Suspension cells were starved (in RPMI-1640 supplemented with insulin, transferrin, sodium selenite, BSA, penicillin and streptomycin) for 3 days and then pre-treated with or without FGF-2 (0.1 ng/ml) for 4 hours prior to addition of the relevant chemotherapeutic agents (according to Pardo *et al*, 2002).

When carrying out an assay after transfecting cells with small interfering (si) RNA oligonucleotides, in order to avoid variability in cell numbers due to inhibition of different targets, cells were trypsinised, counted and then re-plated at the same cell density.

Determination of viable cells was done using the WST-1 kit (adherent cells only) or by cell counting by trypan blue exclusion. Cell death was also measured by analysing DNA loss by flow cytometry, or by analysing the cleavage of PARP and/or lamin-B by SDS-PAGE/Western blotting.

3.6.1. CELL VIABILITY ASSAY (WST-1)

Cells were plated in 96-well plates (3,000 cells per well) and treated as described in chapter 3.6. After 24 hours, 10 µl of WST-1 mixture was added to each well and cells incubated for 1 hour at 37 °C, essentially as indicated by the manufacturer. In this assay, mitochondrial dehydrogenases cleave the tetrazolium salt WST-1 reducing it into coloured formazan compounds. The intensity of the dye is proportional to the number of viable cells. The optical density (OD) of the samples was read in a microplate reader (Anthos 2001, Bio Whittaker, Maryland, USA) at a wavelength of 460 nm, with a reference filter at 630 nm. Every condition was set up at least in triplicate.

Results obtained using this assay were confirmed by manual cell counting (Appendix I). The results were identical and, due to its ease and higher throughput potential, WST-1 was thus used in most experiments set up to measure cell viability.

Because of its low stability when in solution, the cisplatin used in this study was always freshly prepared, as recommended by the manufacturer. The experiments with U2OS, HCC95 and HCC78 in the first part of this work were performed using the same batch of cisplatin. Each new batch used was titrated before performing experiments because of concerns regarding batch-to-batch variation (Appendix II).

3.6.2. CELL COUNTING

In order to establish the reliability of the WST-1 assay, experiments have been set up where cell death was monitored in parallel by the WST-1 assay and manual cell

counting (using a haemocytometer). The live cell number was determined using the trypan blue exclusion method. This method is based on the principle that in a viable cell most compounds do not pass through the membrane, whereas the membrane in a dead cell is permeable. Dead cells, therefore, show a distinctive blue colour under a microscope and live cells are non-permissive to trypan blue staining. After trypsinisation, 100 μ l of cell suspension was mixed with 100 μ l trypan blue. For each condition, cells were counted from four sets of 16 corner squares. The number of cells in each set of 16 corner squares is equivalent to the number of cells $\times 10^4$ / ml and, thus, the cell concentration of cells was estimated by multiplying the average of the four counts by 10^4 . Final concentration was obtained by multiplying by two (to adjust for the 1:2 dilution in trypan blue).

3.6.3. CELL CYCLE ANALYSIS

Cell death can be measured by analysing DNA content using flow cytometry. This method is based on the principle that dying cells are characterised by morphological changes, and fragmentation and loss of DNA content (reviewed by Danial *et al.*, 2004). After staining with a DNA-binding dye, such as propidium iodide (PI), cells that have lost DNA will take up less stain and this can be evaluated by flow cytometric analysis. Dead cells will appear on the left of the G_1 peak (Sub- G_1 population, **Fig. 12**).

To measure cell death by analysing loss of DNA content, osteosarcoma and NSCLC cells were harvested after 18 hours of drug treatment (trypsinised adherent cells and floating ‘corpses’) and SCLC cells were harvested after 4 days of drug treatment. After cells were harvested, they were washed twice with PBS by centrifugation at 178 $\times g$ for 5 minutes, and fixed and permeabilised in cold 70% (v/v) ethanol (added drop-wise while gently vortexing). After fixing at 4 $^{\circ}C$ for at least 30 minutes, specimens were washed twice with PBS by centrifugation at 178 $\times g$ for 5 minutes. Cells were then resuspended in 400 μ l of PBS, treated with 10 μ g/ml of ribonuclease (RNase) to destroy RNA and incubated briefly with 50 μ g/ml of PI, which intercalates with the DNA. DNA content was measured by flow cytometry in a BD FACS Canto™ (BD Biosciences, Erembodegem, Belgium).

Data mining and analysis was done using FlowJo (Tree Star Inc., Oregon, USA). Cell doublets were excluded using gates based on cell diameter (Appendix III).

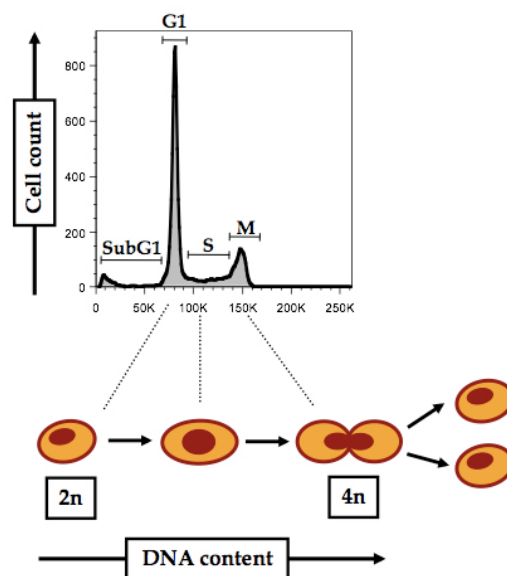


Figure 12. Example of a population cell cycle profile obtained by flow cytometry. Cells were stained with a DNA-binding dye (PI) and DNA content was measured by flow cytometry. G_1 corresponds to the first phase after mitosis and cells have $2n$ DNA content. In S phase, cells synthesise DNA. In G_2/M phase cells are ready to complete cell division and have double DNA content of a cell in G_1 . The Sub- G_1 population corresponds to the percentage of cells with a DNA content of less than $2n$ and thus corresponds to cells that lost DNA and are presumably dead.

3.6.4. APOPTOSIS ANALYSIS BY POLY-(ADP-RIBOSE) POLYMERASE AND LAMIN B CLEAVAGE

One of the key initiating events of the apoptotic pathway is the activation of caspases (cysteine-aspartic acid proteases) followed by cleavage of caspase substrates (Earnshaw *et al.*, 1999). PARP is a 116 kDa protein mainly involved in DNA repair, which is cleaved by members of the caspase family during early apoptosis (Herceg and Wang, 2001). Detection of an 85 kDa, or 31 kDa cleavage fragment of PARP has been shown to be a *bona fide* marker of apoptosis (Lazebnik *et al.*, 1994). Another read-out for apoptosis is the proteolytic cleavage of lamin B (the full length protein is 67 kDa). Nuclear lamin, located at the inner nucleoplasmic surface, is targeted by proteases during the early stages of cell death (Lazebnik *et al.*, 1993). Cleavage of lamin B by the caspases usually generates a fragment of 45 kDa.

To assess apoptosis, cells were harvested after 16 hours of drug treatment. Protein was extracted from all cells (adherent cells and floating 'corpses'). Cleavage of PARP and lamin B was monitored by SDS-PAGE/Western blot using antibodies that recognise both the total and cleaved forms of these proteins.

3.7. REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

RT-PCR was used to determine the expression of two canonical FGF-2 receptors. Primers were designed to amplify a fragment of the FGFR1 and FGFR2IIIc. Primers for FGFR2IIIc amplify only this splice variant since they amplify a segment of the intron 9, unique to the FGFR2IIIc variant.

Total RNA was isolated using RNeasy (Qiagen, West Sussex, UK), as directed by the manufacturer, and RNA concentrations were measured using a NanoDrop ND1000 spectrophotometer (Labtech international, East Sussex, UK). Reverse transcription was performed using a one-step RT-PCR kit (QIAGEN, West Sussex, UK). RT-PCR reactions were carried out in a 50 µl reaction volume containing 30 ng of template RNA, 1x RT-PCR buffer, 1x Q-Solution, 400 µM of each dNTP, 0.6 µM of each primer and 2 µl of RT-PCR enzyme mix, as directed by the manufacturer. The RT-PCR amplification conditions were set up according to the manufacturer's guidelines: 30 seconds at 50 °C, 15 minutes at 95 °C, followed by 35 cycles of 45 seconds at 94 °C, 1 minute at annealing temperature (T_a), 1 minute at 72 °C, with a final extension at 72 °C (10 minutes). The specific primers and correspondent T_a used to amplify the different fragments are shown in **Table II**.

RT-PCR products together with loading dye [5% (v/v) glycerol, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol] were run on a 2% agarose gel containing SYBR green. Products were checked based on their size against a 100 bp DNA ladder. A fragment of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified from all samples as a loading control. An extra reaction without RNA template (blank control) was carried out for each RT-PCR mix in order to detect potential contaminations. No contaminations were ever detected in the blank controls.

Table II. Sequences and annealing temperatures for each set of PCR primers.

mRNA	Primer sequences	T _a	Product size
FGFR1	FGFR1.F ATATTGGACATCCCCAGAAAAGATGG FGFR1.R TCTGGACATAAGGCAGGTTGTCTGG	58	470 bp
FGFR2IIIc	FGFR2IIIc.F GTCGGGGAATATACGTGCTTGGCGG FGFR2IIIc.R CAGGGGCTTGCCCAGTGTCTCAGCTT	58	455 bp
GAPDH	GAPDH.F GGGAAGCTTGTCAATCAATGG GAPDH.R GACTTTGCCTACAGCCTTGG	52	450 bp
JAK1	JAK1.F ATGCCCATCATTACCTCTGC JAK1.R GCAGGATCTGCTTCTTGAGG	52	341 bp
JAK2	JAK2.F GAAACGGTGGAATTCAGTGG JAK2.R ACATTTTCTCGCTCGACAGC	52	463 bp
STAT1	STAT1.F CATTCTCAGAGCTCGTTTGTGG STAT1.R TGGTGGAGTCAGGAAGAAGG	52	538 bp
STAT3	STAT3.F GAGAAGCCAATGGAGATTGC STAT3.R GATTCTCTCCTCCAGCATCG	52	641 bp

3.8. CRYSTAL VIOLET STAINING

Microscopic images from cells were obtained from cells stained with crystal violet. Plates were washed twice with cold PBS and cells were fixed with 4% (w/v) *p*-FA overnight at room temperature (RT). Plates were then washed and the fixed cells stained with 0.075% (w/v) crystal violet solution prepared in 5% (v/v). Plates were carefully rinsed in deionised water (ddH₂O) until colour was no longer coming off in the rinse and dried overnight at RT. Pictures were taken using a Nikon Eclipse TE2000-U microscope (100x magnification) and images were processed using QCapture Pro software.

3.9. RNA INTERFERENCE (RNAi)

For siRNA transfections, cells were plated in 24-well, 6-well or in 10 cm plates. Transfection and knockdown conditions were already optimised in our laboratory for U2OS, A549 and HCC193 cells (**Table III**).

Table III. siRNA transfection conditions.

Cell line	Cell density			Transfection reagent (volume per 100 μ l)
	24-well plates	6-well plates	10 cm plates	
U2OS	4x10 ⁴ cells / well	1x10 ⁵ cells / well	1x10 ⁶ cells / plate	DharmaFECT® II (2 μ l)
A549	4x10 ⁴ cells / well	-	-	DharmaFECT® I (1 μ l)
HCC193	6x10 ⁴ cells / well	-	-	DharmaFECT® I (1 μ l)

Oligonucleotides (siGENOME and siOnTargetPlus) and transfection reagents (DharmaFECT®) were sourced from Dharmacon (Lafayette, CO, USA) and were used in all experiments. Cells were plated, 24 hours later transfected with 75 nM of the appropriate pool of oligonucleotides (**Table IV**) and incubated for 48 hours more. Controls used included untransfected cells, mock-transfected cells (DharmaFECT® only) and cells transfected with a non-specific (NS) siRNA pool (a pool of siRNA oligonucleotides that were individually characterised by genome-wide microarray analysis, which identified minimal off-target signatures). Cells were subsequently treated as required.

Table IV. Sequences of each set of siRNA oligonucleotides (Dharmacon).

Target	Name and accession number	siRNA Product	siRNA oligonucleotide target sequences
ERK1	MAPK3 NM_002746	siGENOME	1. GACCGGAUGUUAACCUUUUAAU 2. AGACUGACCUGUACAAGUUUU 3. GAAACUACCUACAGUCUCUUU 6. GCUACACGCAGUUGCAGUAUU
ERK2	MAK1 NM_002745	siGENOME	1. CCAAAGCUCUGGACUUAUUUU 3. AAACAGAUCUUUACAAGCUUU 4. CAAGAGGAUUGAAGUAGAAUU 9. GUACAGGGCUCCAGAAAUUUU
JAK1	JAK1 NM_002227	siGENOME	5. CCACAUAGCUGAUCUGAAA 6. UGAAAUCACUCACAUUGUA 7. UAAGGAACCUCUAUCAUGA 8. GCAGGUGGCUGUUAUAUCU
JAK2	JAK2 NM_004972	siGENOME	6. GAGCAAAGAUAUCCAAGACUA 7. GCCAGAAACUUGAAACUUA 9. GAUCCUGGCAUUAGUAUUA 10. ACAGAAUGCUGGAACAAUA
STAT1	STAT1 NM_007315	siGENOME	1. AGAAAGAGCUUGACAGUAAUU 3. UAAAGGAACUGGAUUAUUCUU 4. GAGCUUCACUCCCUUAGUUUU 5. GAACCUGACUUCUCCAUUCGCUU
STAT3	STAT3 NM_003150	siGENOME	1. CCAACGACCUGCAGCAAUAUU 2. GGAGAAGCAUCGUGAGUGAUU 3. CCACUUUGGUGUUUCAUAAUU 4. UCAGGUUGCUGGUCAAUUUUU
STAT5A	STAT5A NM_003152	siGENOME	1. GCACGUGGAGGAACUCUUAUU 2. ACAGAACCCUGACCAUGUAUU 5. CUACUGUGCUGUGGGACAAUU 6. GUACUACACUCCUGUGCUGUU
STAT5B	STAT5B NM_012448	siGENOME	1. GGACACAGAGAAUGAGUUUUU 2. GAAGUUUGAUUCUCAGGAAUU 3. CCAAGUCUCUGCUCAAGAAUU 5. GAGAACACCCGCAAUGAUUUU
TYK2	TYK2 NM_003331	siGENOME	6. GCAUCCACAUUGCACAUAA 7. UCAAAUACCUAGCCACACU 8. CAAUCUUGCUGACGUCUUG 21. CACAAGGACCAACGUGUAU

3.10. GP130 DETECTION BY FLOW CYTOMETRY

Cell surface levels of gp130 receptor were measured by flow cytometry. Forty-eight hours after siRNA transfection, cells were removed from the plates with trypsin-EDTA and washed with ice-cold PBS. Cells were incubated initially with an anti-gp130 antibody (BT12) for 1 hour and, after two washes with cold PBS, cells were incubated with a PE-conjugated secondary antibody for 45 minutes. Cells were subsequently

washed three times with ice-cold PBS, fixed with 1% *p*-FA (w/v) and analysed on a FACSCanto machine (BD, Erembodegem, Belgium).

Data mining and analysis was done using FlowJo. Differences between the geometric mean values of cells stained with BT12 antibody and cells stained with secondary antibody only were compared between samples. The geometric mean calculates the n^{th} root of the product of n measurements and gives the average of the logarithmic values of a data set, converted back to a base 10 number. It estimates the quantity of molecules expressed per cell and is a more applicable metric for a logarithmically normal distribution than the percentage of cell population.

3.11. STATISTICAL ANALYSIS

Mean and standard error of the mean (SEM) of independent experiments were calculated. Student's *t*-test was used to determine the statistical significance of the differences observed between conditions. A two-tailed *p*-value below 0.05 was considered significant.

4. RESULTS

4.1.

THE ROLE OF FGF-2 IN CHEMORESISTANCE

4.1.1. INTRODUCTION

Nowadays many chemotherapeutic agents are available and survival rates of cancer patients have slightly improved. However, development of drug resistance by the tumour cells limits the use of these agents and eventually leads to failure to cure the patients (Hanahan and Weinberg, 2000). This problem is exemplified by lung cancer, which is almost never cured with initial therapy and is mostly chemoresistant upon relapse. These two factors contribute to a very low overall survival. Consequently, understanding the mechanisms underlying chemoresistance is critical for the development of new therapeutic approaches that might extend patient survival.

FGF-2 is a growth factor that plays an important role in cancer biology (Giri *et al.*, 1999; Dickson *et al.*, 2000; Nurcombe *et al.*, 2000; Polnaszek *et al.*, 2003) and, indeed, it has been implicated in driving chemoresistance in cancer cells, including lung and breast cancer cells (Pardo *et al.*, 2002; Pardo *et al.*, 2003; Song *et al.*, 2000; Vandermoere *et al.*, 2005; Smaragda Angelidou and Michael Seckl, personal communication). FGF-2 has been shown to confer resistance to etoposide-mediated apoptosis in SCLC cells *via* a MEK-dependent pathway that results in the up-regulation of various anti-apoptotic proteins such as BCL-X_L, BCL-2, XIAP and cIAP. Indeed, Seckl and colleagues (Pardo *et al.*, 2006) have shown that FGF-2-mediated chemoresistance requires the formation of a multi-protein complex consisting of at least B-RAF, PKC ϵ and S6K2.

In the first part of this work, **our main aim was to investigate the generality of the FGF-2-mediated chemoresistance mechanism by analysing additional cell types, namely osteosarcoma and NSCLC cells.** This was done using cisplatin, a clinically relevant chemotherapeutic drug commonly used to treat many cancer types. To understand the role of FGF-2 in cisplatin-mediated cell death three cell lines were used: two NSCLC cell lines (HCC95 and HCC78) and an osteosarcoma cell line (U2OS). The HCC95 and HCC78 cell lines were derived from patients with squamous cell and adenocarcinoma, respectively. The U2OS cell line is morphologically classified as epithelial and was used to extend the original findings to other types of cancers commonly treated with cisplatin (Bielack *et al.*, 2004; Ta *et al.*, 2009) and in which FGF-2 may also have an important role in disease progression (Graeven *et al.*, 1999). In addition, the U2OS cell line has been extensively utilised by the Costa-Pereira Group,

and, as such, many experimental protocols were already developed/ optimised for this cell line.

The first step was to determine how the three cell lines responded to FGF-2. The kinetics of FGF-2-induced ERK1/2 activation were initially characterised in the NSCLC HCC95 and HCC78 cells, and the osteosarcoma U2OS cell line by performing dose- and time-responses with FGF-2. The expression levels of FGF-2 canonical receptors were also determined in order to ensure that a defect in the response to FGF-2 was due to an intracellular defect rather than due to lack, or mutation, of the appropriate receptor(s). Subsequently, we tested whether FGF-2 could rescue these cells from cisplatin-induced cell death. Furthermore, major players involved in FGF-2-mediated chemoresistance previously described for the SCLC cell line H-510 (Pardo *et al.*, 2006) were also investigated.

4.1.2. FGF-2 RESPONSE IN NSCLC AND U2OS CELLS

FGF-2 is known to activate ERK1/2 in many cell types including lung cancer and osteosarcoma cells (Wang *et al.*, 2000; Pardo *et al.*, 2001; Kuhn *et al.*, 2006). The activity of these kinases can be monitored by Western blot using an antibody that recognises both phosphorylated ERK1 and ERK2. To investigate if FGF-2 activated ERK1/2 in the osteosarcoma U2OS cell line and the NSCLC cell lines HCC95 and HCC78, cells were incubated with increasing doses of FGF-2 for different periods of time. Treatments were performed after serum starvation (0.5% FCS overnight and serum-free media for 1 hour prior to FGF-2 treatment) in order to reduce the basal ERK1/2 phosphorylation potentially induced by FCS components.

Using this approach, we observed that FGF-2 induced the phosphorylation of ERK1/2 in U2OS cells (**Fig. 13**). A statistically significant increase of ERK1/2 phosphorylation ($p < 0.05$ - 0.005 for ERK1 and $p < 0.05$ - $p < 0.01$ for ERK2) was observed after treatment with all doses tested (1-50 ng/ml) (**Fig. 13 A**). Treatment with 10 ng/ml of FGF-2 induced maximal ERK1/2 phosphorylation, which was on average for ERK1 13.2-fold and for ERK2 4.3-fold above basal levels (**Fig. 13 A**, 3rd bar in the graphs).

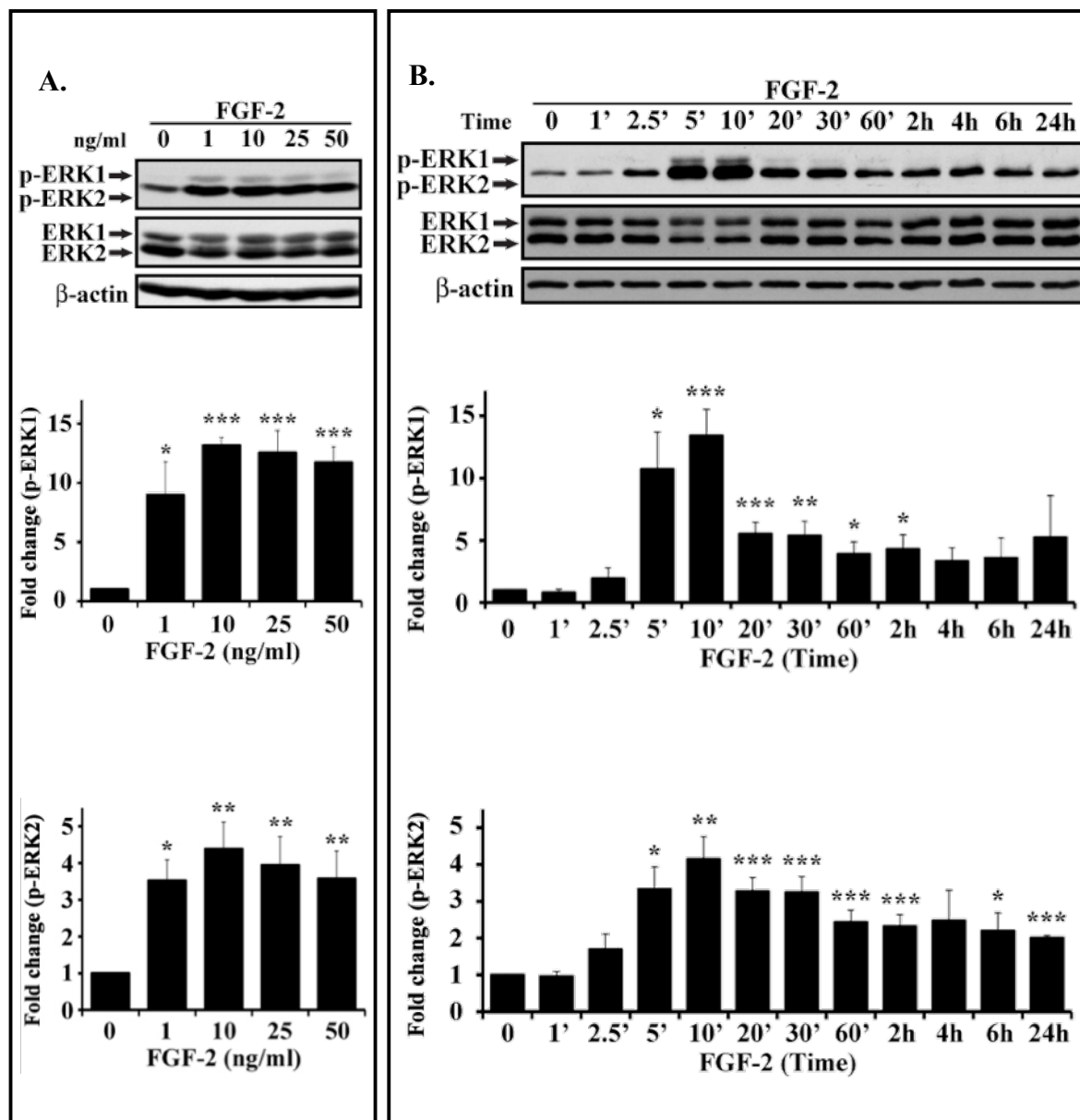


Figure 13. ERK1/2 phosphorylation in response to FGF-2 in U2OS cells. U2OS cells were serum-starved (0.5% FCS) overnight and then incubated in serum-free media for 1 hour. Cells were stimulated with FGF-2 for 10 minutes with the indicated concentrations (**A.**) and with 25 ng/ml of FGF-2 for the indicated times (**B.**). Proteins were extracted and separated on a 10% SDS-PAGE gel. Western blotting analysis was performed on total cell lysates using antibodies against pERK1/2-Thr²⁰²/Tyr²⁰⁴ and total ERK1/2. β -actin was used as a loading control. Representative Western blots and mean $\pm$ SEM of densitometric values of at least three independent experiments are shown in graphs. Values are expressed as fold change relative to untreated controls. Statistical analysis was performed using Student's t-test (* - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.005$ versus untreated control). ' - minutes, h - hours.

Phosphorylation of ERK1/2 increased at 5 minutes and peaked at 10 minutes ($p < 0.005$ and $p < 0.01$ for ERK1 and ERK2, respectively) (**Fig. 13 B**, 4th and 5th bars in the graphs). After 10 minutes, the phosphorylation levels decreased but were sustained above basal for up to 24 hours (particularly that of ERK2).

Membranes were subsequently probed for total ERK1/2 and β -actin. Some differences in total ERK1/2 levels could be observed between FGF-2 untreated and FGF-treated samples (**Fig. 13**). These differences may be related to the ERK1/2 band shift due to phosphorylation, which may be responsible for a less defined band in FGF-2 treated samples. Alternatively, the differences may be due to incomplete stripping of the anti-phospho-ERK1/2 antibody (this antibody is particularly difficult to remove from the membrane by stripping). This could interfere with the binding of the total ERK1/2 antibody. Accordingly, densitometric values of all conditions were normalised using β -actin.

FGF-2 also activated ERK1/2 in the HCC95 cell line in a dose dependent manner (**Fig. 14 A**). In these cells, peak activation of ERK1/2 was also observed at 10 minutes, with an average 7.9-fold induction for ERK1 ($p < 0.005$) and 5.1-fold induction for ERK2 ($p < 0.01$) (**Fig. 14 B**, 5th bar in the graphs). Contrary to U2OS cells (**Fig. 13**), phosphorylation of ERK1/2 in response to FGF-2 was transient: induction of ERK1 phosphorylation was not statistically significant beyond 2 hours of FGF-2 treatment (**Fig. 14 B**, 10th-12th bars in the top graph) and the increase in ERK2 phosphorylation was not statistically significant past 30 minutes of stimulation (**Fig. 14 B**, 8th-12th bars in the bottom graph). In particular, ERK2 phosphorylation was below the basal level after incubation with FGF-2 for 24 hours ($p < 0.005$) (**Fig. 14 B**, 12th bar in the bottom graph).

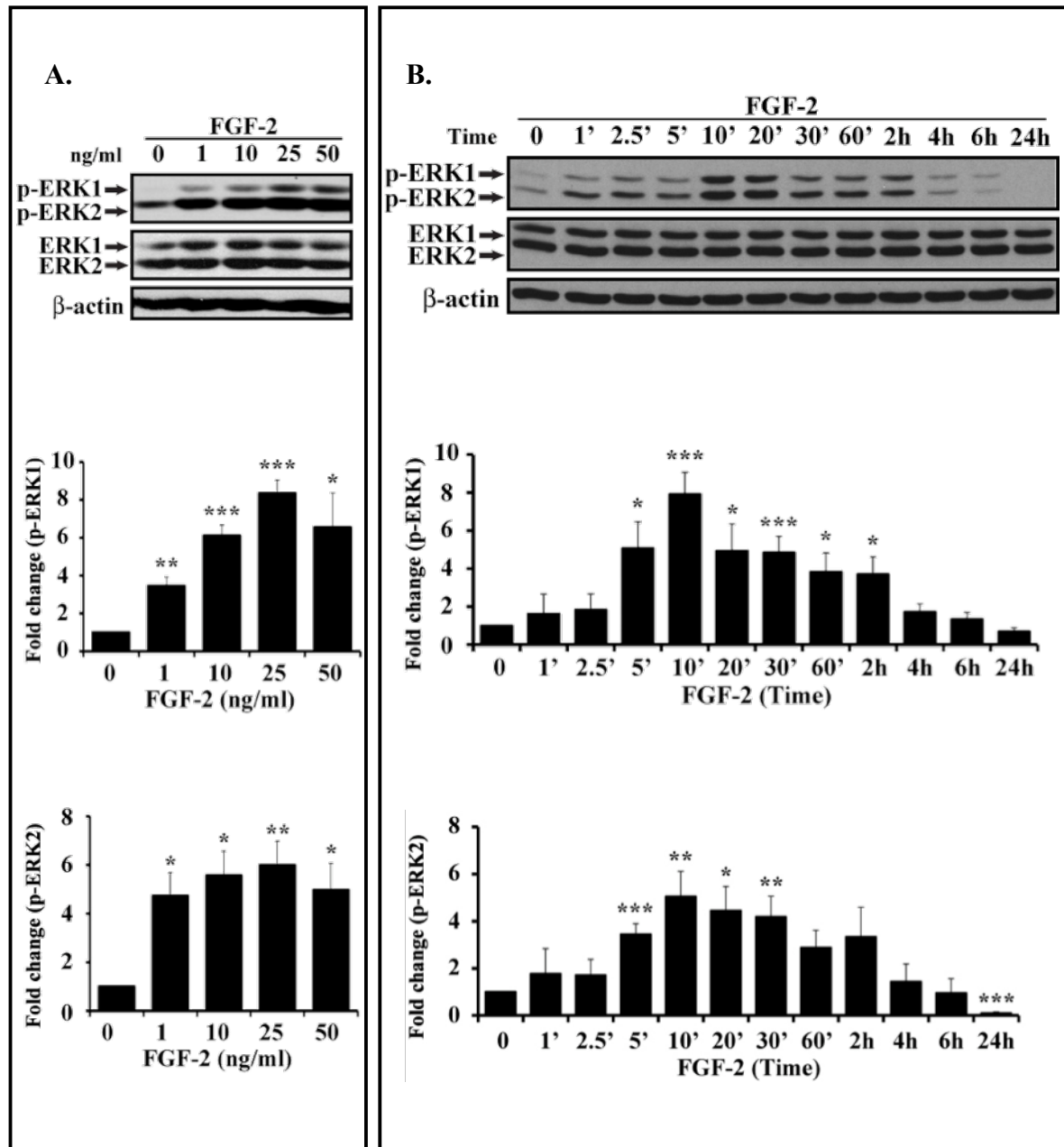


Figure 14. ERK1/2 phosphorylation in response to FGF-2 in HCC95 cells. HCC95 cells were serum-starved (0.5% FCS) overnight and then incubated in serum-free media for 1 hour. Cells were stimulated with FGF-2 for 10 minutes with the indicated concentrations (**A.**) and with 25 ng/ml of FGF-2 for the indicated times (**B.**). Proteins were extracted and separated on a 10% SDS-PAGE gel. Western blotting analysis was performed on total cell lysates using antibodies against pERK1/2-Thr²⁰²/Tyr²⁰⁴ and total ERK1/2. β-actin was used as a loading control. Representative Western blots and mean ± SEM of densitometric values of at least three independent experiments are shown in graphs. Values are expressed as fold change relative to untreated controls. Statistical analysis was performed using Student's t-test (* - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.005$ versus untreated control). ' - minutes, h - hours.

In marked contrast to U2OS and HCC95 cells, FGF-2-induced phosphorylation of ERK1/2 in HCC78 cells was very modest (**Fig. 15**). The increase of ERK1/2 phosphorylation observed in response to FGF-2 was not statistically significant in the dose-response experiments performed (**Fig. 15 A**). Results, however, still suggest that the ERK1/2 phosphorylation was slightly increased upon FGF-2 treatment, measuring an average of 1.4-fold for ERK1 and 1.3-fold for ERK2 (**Fig. 15 B**). Although the induction of ERK1/2 phosphorylation by FGF-2 was much smaller than that observed in the other cell lines studied, the effect reached statistical significance at some time points: at 10 and 20 minutes for ERK1 ($p < 0.05$ and $p < 0.01$, respectively) (**Fig. 15 B**, 5th and 6th bars in the top graph) and at 5 minutes for ERK2 ($p < 0.05$) (**Fig. 15 B**, 4th bar in the bottom graph). The phosphorylation of ERK1/2 was reduced compared to basal levels after 20 minutes treatment with FGF-2 (**Fig. 15 B**, 7th-12th lane in the Western blot), presumably due to a negative control mechanism (e.g. phosphatase activity). This decrease, however, was not statistically significant.

Although the increase in ERK1 phosphorylation reached statistical significance in the treatment with 25 ng/ml of FGF-2 for 10 minutes ($p < 0.05$) (**Fig. 15 B**, 5th bar in the top graph), the equivalent conditions in the dose-response experiments (same concentration and time of FGF-2 stimulation) did not reach statistical significance (**Fig. 15 A**, 4th bar in the top graph). This reflects variation between experiments. Such a small increase in phosphorylation is less robust than a larger increase and it could have been affected by many factors. Although every effort was made to standardise the experimental method in order to avoid variation associated with cell density, or sample handling, other factors such as cell cycle are more difficult to control.

These observations suggest that in HCC78 cells, FGF-2 only led to a weak activation of the MEK/ERK pathway.

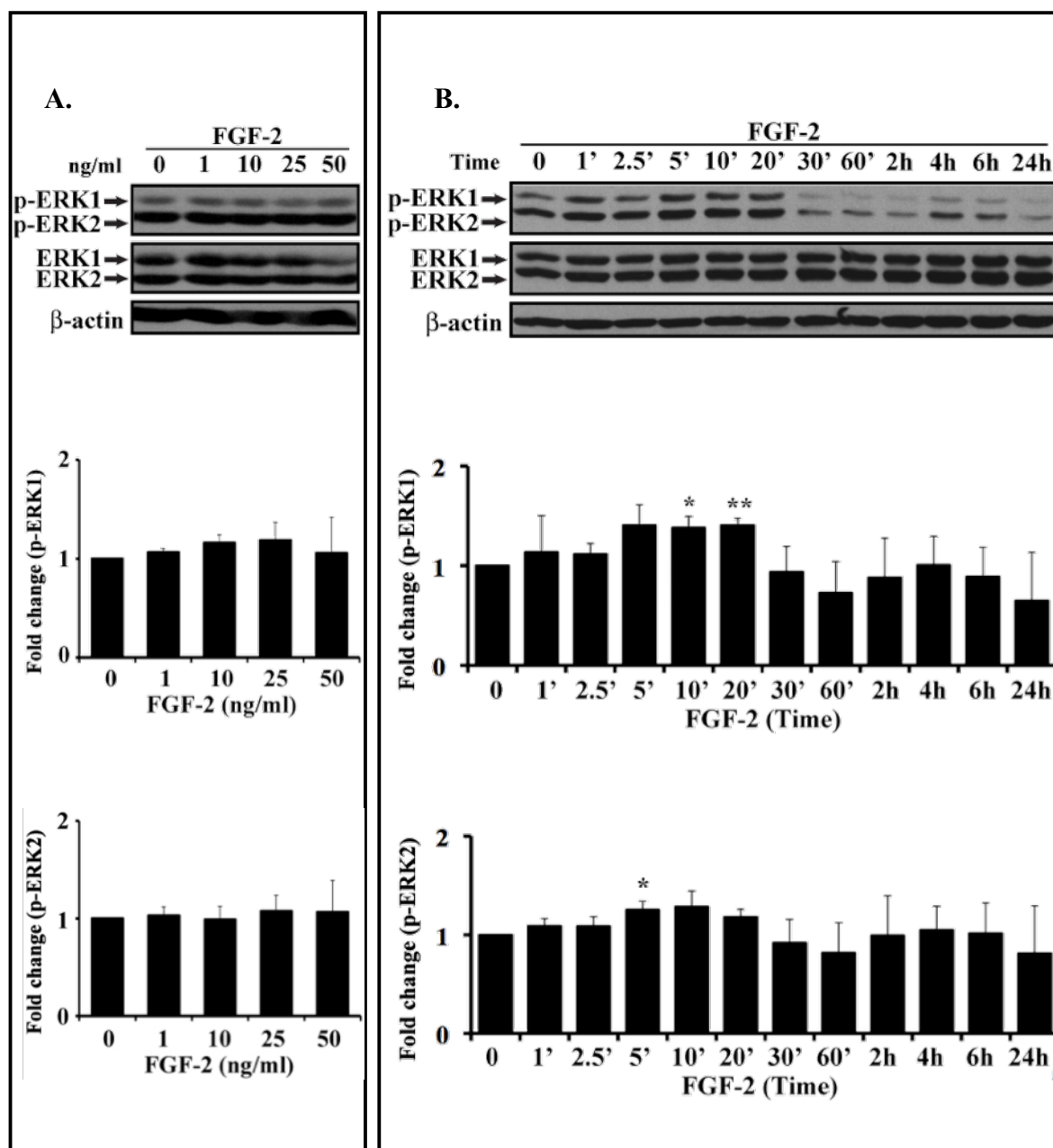


Figure 15. ERK1/2 phosphorylation in response to FGF-2 in HCC78 cells. HCC78 cells were serum-starved (0.5% FCS) overnight and then incubated in serum-free media for 1 hour. Cells were stimulated with FGF-2 for 10 minutes with the indicated concentrations (**A.**) and with 25 ng/ml of FGF-2 for the indicated times (**B.**). Proteins were extracted and separated on a 10% SDS-PAGE gel. Western blotting analysis was performed on total cell lysates using antibodies against pERK1/2-Thr²⁰²/Tyr²⁰⁴ and total ERK1/2. β-actin was used as a loading control. Representative Western blots and mean ± SEM of densitometric values of at least three independent experiments are shown in graphs. Values are expressed as fold change relative to untreated controls. Statistical analysis was performed using Student's t-test (* - $p < 0.05$, ** - $p < 0.01$ versus untreated control). ' - minutes, h - hours.

4.1.3. EXPRESSION OF CANONICAL FGF-2 RECEPTORS IN NSCLC AND U2OS CELLS

FGFRs 1 and 2 are the main receptors for FGF-2 (Ornitz *et al.*, 1996). In order to determine the expression levels of FGF-2 receptors FGFR1 and FGFR2IIIc (a FGFR2 isoform used by FGF-2) in U2OS, HCC95 and HCC78 cell lines, total RNA was extracted from cells, reverse-transcribed into cDNA and fragments from the FGFRs amplified by RT-PCR. Prostate cancer LNCaP cells, which are known to express both receptors (Susan Willis and Michael Seckl, personal communication), were used as a positive control. Similar RNA levels of FGFR1 were detected in all of the three cell lines studied (**Fig. 16**). FGFR2IIIc was amplified successfully in U2OS and HCC95 cells, but it was not detected in the HCC78 cell line even after 35 cycles of amplification. These results suggest that the receptor FGFR2IIIc is not expressed in HCC78 cells. This, together with our previous data (**Fig. 15 B**), suggests that FGF-2, in these cells, may bind to a receptor complex different from the one bound by FGF-2 in U2OS and HCC95 cells. The precise identity of which FGFRs might mediate FGF-2 action was beyond the scope of this work and was therefore not pursued.

The detection of FGFR by RT-PCR does not reflect protein levels or cell surface localisation of the different receptors. This method establishes which receptors are transcribed and thus potentially expressed in the cells. Detection of FGFRs by Western blot would have made possible to determine if the receptors were effectively expressed in these cell lines and flow cytometric analysis of unpermeabilised cells would have established if they were present on the cell surface and, if so, in what abundance. Unfortunately, FGFR antibodies currently commercially available do not discriminate between the multiple FGFR isoforms and are unsuitable for flow cytometry. When better antibodies become available, these will be crucial experiments to determine the expression of canonical FGF-2 receptors. Alternatively, we could have designed siRNA molecules targeting a single FGFR isoform and used them to silence each of them independently. The cells (U2OS, HCC78 and HCC95) could then be treated with FGF-2 and phosphorylation of ERK1/2 monitored. An inhibition/significant reduction of ERK1/2 phosphorylation, when compared to untransfected cells and cells transfected with a non-targeting siRNA molecule, would have been suggestive of the isoforms required for signalling in response to FGF-2. The main caveat here is that due to the lack of appropriate isoform-specific antibodies the specific knockdown could only be assessed at the RNA level.

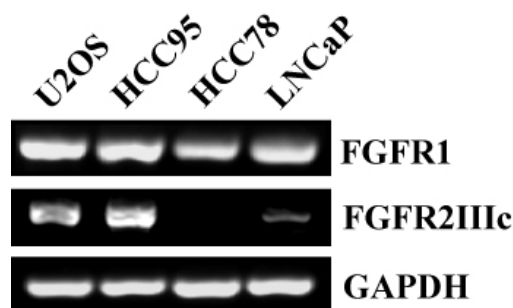


Figure 16. Expression of FGF-2 receptors FGFR1 and FGFR2IIIc in NSCLC HCC95 and HCC78 and U2OS cell lines determined by RT-PCR. Fragments of FGFR1 and FGFR2IIIc were amplified by RT-PCR in U2OS, HCC95 and HCC78 cells. RT-PCR products were run on an agarose gel. LNCaP cells were used as a positive control. GAPDH was amplified and used as a loading control. These results are representative of two independent experiments.

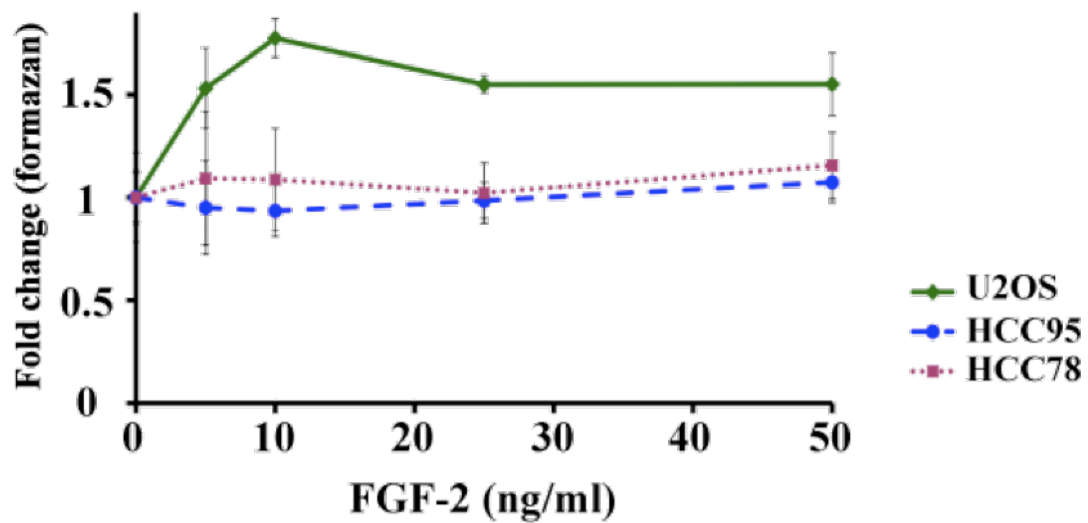
4.1.4. FGF-2 INDUCED PROLIFERATION OF U2OS CELLS BUT NOT OF NSCLC CELLS

FGF-2 was shown to promote proliferation in some SCLC cell lines (Pardo *et al.*, 2001). This was also shown in other cells lines, such as lung, prostate and pancreatic cancer cells, and human embryonic stem cells (hESCs) (Yamanaka *et al.*, 1993; Fukui *et al.*, 2003; Polnaszec *et al.*, 2003; Xu *et al.*, 2005; Berger *et al.*, 1999). To assess the effect of FGF-2 on the proliferation of the osteosarcoma cell line U2OS and the NSCLC cell lines HCC95 and HCC78 we used a cell viability assay (WST-1) (cf. Material and methods). This assay measures the cleavage of the tetrazolium salt WST-1 (light red) to formazan compounds (dark red) by mitochondrial dehydrogenases in living cells. The intensity of the dye is proportional to the number of viable cells. This cell viability assay has a number of clear advantages when compared to other methods [e.g. $[^3\text{H}]$ -thymidine or 5-bromo-2'-deoxyuridine (BrdU) incorporation] such as the fact that the cells do not need to be washed or harvested, no radioactivity or volatile solvents are required, it is more sensitive than the 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay and the absorbance correlates with the amount of cells alive. We tested the correlation between the results obtained using the WST-1 assay and cell

counting using trypan blue and a light microscope by performing the experiments in parallel. The results obtained were identical (Appendix I) and WST-1 was thus used in most experiments to measure cell viability.

HCC95, HCC78 and U2OS cells plated in 96-well plates were treated with increasing concentrations of FGF-2 for 24 hours after an overnight starvation in serum-free media. Cell viability was measured using WST-1. All treated samples were compared to the respective untreated controls. Proliferation of U2OS cells significantly increased ($p < 0.005$) with the addition of FGF-2, in a dose-dependent manner, reaching a peak with 10 ng/ml (**Fig. 17 A and B**). In contrast, under the experimental conditions used, increased proliferation of the two NSCLC cell lines studied (HCC78 and HCC95) in response to FGF-2 was not observed (**Fig. 17 A and B**). These results suggest that FGF-2 has a functional effect on the growth of U2OS cells but not on the growth of HCC95 or HCC78 cells.

A.



B.

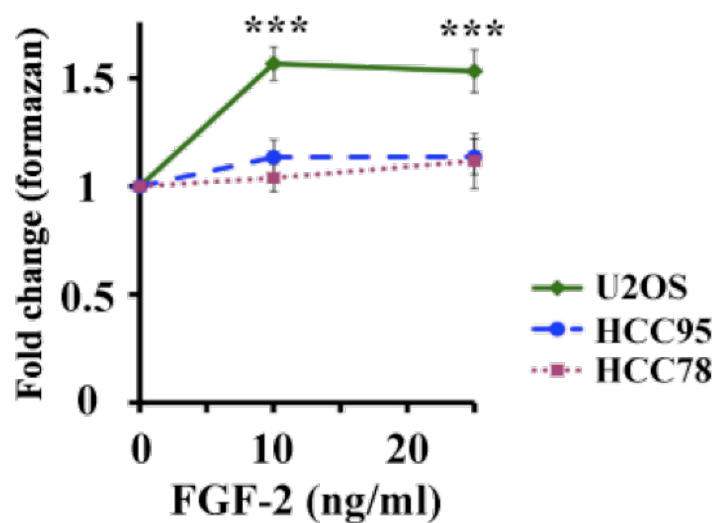


Figure 17. FGF-2 induced proliferation of U2OS but not of NSCLC cells. Cells were plated in 96-well plates (3,000 cells per well), starved overnight (in serum-free media) and then treated with FGF-2 at the indicated concentrations for 24 hours. Cell viability was measured using the WST-1 cell viability assay. **A.** Results from one experiment are presented as fold induction relative to untreated controls. Values correspond to the OD of coloured formazan compounds derived from cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases. Each condition was set up in triplicate, and the results are shown as mean $\pm$ standard deviation. **B.** Mean $\pm$ SEM of three independent experiments performed in triplicate are shown in a graph. Values are expressed as fold change relative to untreated controls. Statistical analysis was performed with Student's t-test (***) – $p < 0.005$ versus untreated control).

FGF-2 treatment for longer periods of time (48 and 96 hours) had no significant effect on NSCLC whereas in U2OS cells, increased proliferation was still observed at 48 hours and, to a lesser extent, at 96 hours (data not shown). At these later time points the cells were at a much lower density and appeared to exhibit a different morphology compared to the cells incubated in media supplemented with 10% FCS (**Fig. 18 A**). Cells incubated for 96 hours in FCS-supplemented media exhibited a spindle-shaped appearance while the cells incubated in serum-free media for the equivalent period of time exhibited a more elongated shape and were sparser. After 96 hours of starvation cell viability was also significantly reduced by 0.8-fold ($p < 0.005$) when compared to the number of cells incubated in fully complemented media (**Fig. 18 B**). A reduction in cell viability could already be observed after 24 (0.5-fold decrease, $p < 0.005$) and 48 hours (0.7-fold decrease, $p < 0.005$). The differences in cell morphology and density observed were likely due to the long-term starvation of the cells. Therefore, all subsequent experiments were performed within a 24 hours window.

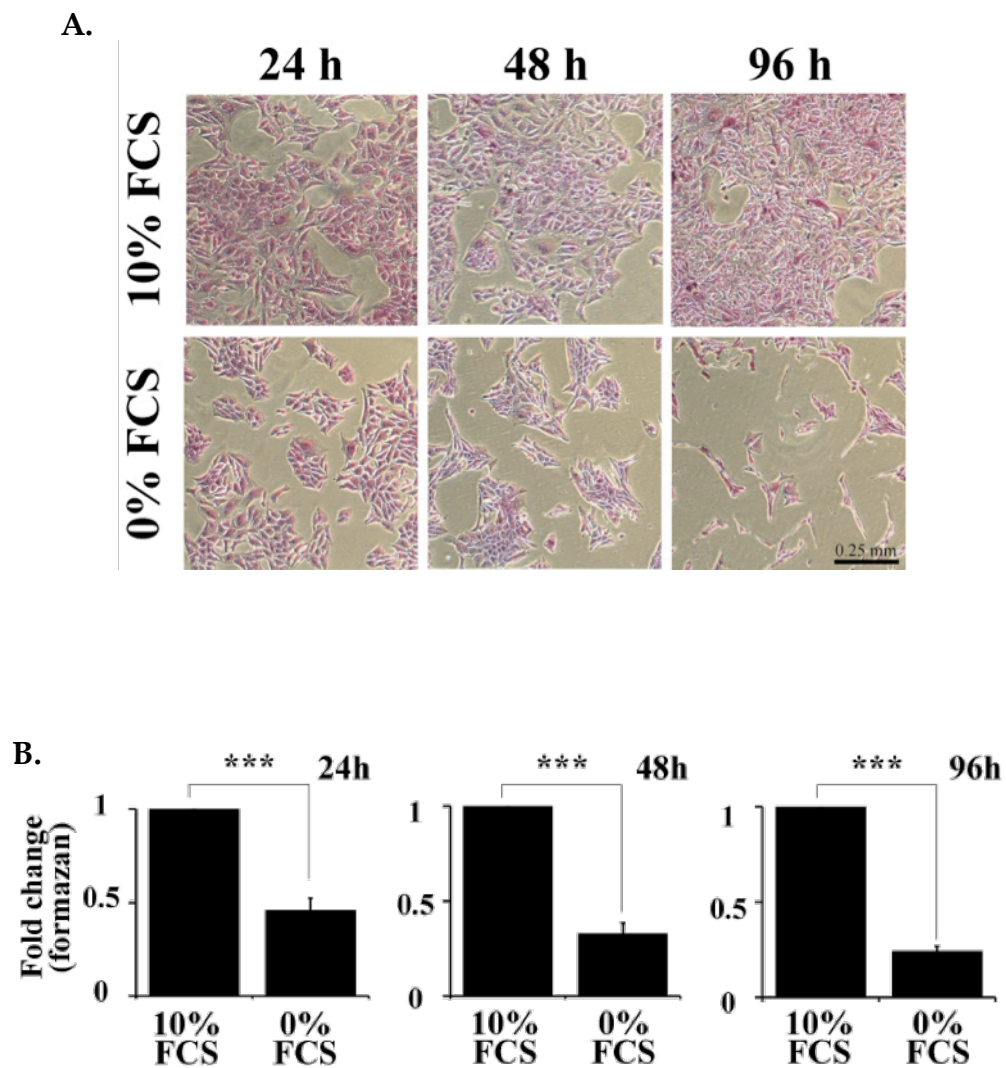


Figure 18. Effect of serum starvation on cell morphology and density of U2OS. Cells were plated in 96-well plates (3,000 cells per well), incubated in serum supplemented with 10% FCS or serum-free media (0%FCS) for 24, 48 and 96 hours. **A.** Cells were fixed in *p*-FA and stained with crystal violet. Samples were visualised using a Nikon Eclipse TE2000-U microscope (100x magnification) and images were processed using QCapture Pro software. The scale bar refers to all the pictures. **B.** Cell viability was measured using the WST-1 cell viability assay. Values correspond to the OD of coloured formazan compounds derived from cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases and are expressed as fold change over the 10% FCS condition for each time point. Mean $\pm$ SEM of three independent experiments performed in triplicate are shown in graphs. Statistical analysis was performed with Student's *t*-test (***) – $p < 0.005$ versus 10% FCS condition). h – hours.

4.1.5. FGF-2 PROTECTED U2OS CELLS FROM CISPLATIN-INDUCED CELL DEATH BUT NOT NSCLC HCC95 AND HCC78 CELLS

In order to investigate if FGF-2 induced chemoresistance in the osteosarcoma cell line U2OS and the NSCLC cell lines HCC95 and HCC78 cells were treated with cisplatin, a clinically relevant chemotherapeutic drug commonly used to treat NSCLC and osteosarcoma (Bielack *et al.*, 2004; Buter *et al.*, 2005; Ta *et al.*, 2009). To assess which doses of cisplatin induced around 50% of cell death in these cell lines, cells were treated with increasing concentrations of the drug for 24 hours (**Fig. 19**). Cell viability was determined using the WST-1 cell viability assay. The LD₅₀ (lethal dose that caused 50% killing of the cells) was approximately 120 μ M for U2OS, 100 μ M for HCC95 and 400 μ M for HCC78. These observations suggest that HCC95 and U2OS cells were more sensitive to cisplatin than HCC78 cells. Indeed, 100 μ M of cisplatin reduced the viability of U2OS cells by approximately 20% ($p < 0.005$) and of HCC95 cells by approximately 50% ($p < 0.005$) without significantly affecting HCC78 cells.

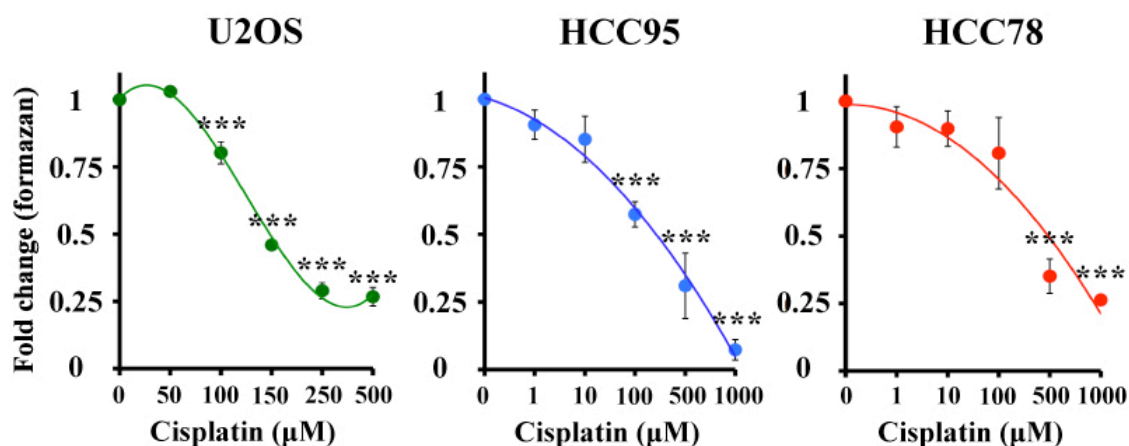


Figure 19. Cisplatin-induced cell death in U2OS, HCC95 and HCC78 cells. Cells were plated in 96-well plates (3,000 cells/well) and incubated in serum-free media overnight. Cells were treated with increasing doses of cisplatin for 24 hours. Cell viability was measured using the WST-1 cell viability assay. Values correspond to the OD of coloured formazan compounds derived from cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases. Mean $\pm$ SEM of three independent experiments, each performed in triplicate, are presented as fold induction over untreated controls. Statistical analysis was performed with Student's t-test (***) – $p < 0.005$ versus untreated control).

To investigate the effect of FGF-2 on cisplatin-induced cell death, cells were pre-treated with or without FGF-2 for 4 hours prior to addition of cisplatin (cf. Material and methods). Previous experiments showed that four hours were required for the induction of early cell survival signalling pathways necessary for inducing chemoresistance (Pardo *et al.*, 2002 and our own observations, **Fig. 28, Section 4.1.9**). Henceforth, these experiments will be referred to as “rescue experiments”. Rescue experiments were performed with 10 ng/ml of FGF-2. This dose was chosen based on its activity as measured by ERK1/2 phosphorylation (**Fig. 13, 14 and 15**). Individual LD₅₀ doses of cisplatin were chosen in order to assess whether FGF-2 could prevent drug-induced cell death in HCC95, HCC78 and U2OS cell lines.

The cisplatin used in this study was always freshly prepared, as recommended by the manufacturer, and each batch titrated before performing experiments. This was because cisplatin has low stability when in solution and because of concerns regarding batch-to-batch variation (Appendix II). Such precautions were taken in order to reduce variability between experiments.

As previously observed (**Fig. 17**), FGF-2 significantly induced proliferation of U2OS cells ($p < 0.005$) (**Fig. 20 A**, 1st *versus* 2nd bar). Treatment of U2OS cells with cisplatin alone for 24 hours induced 50% of killing ($p < 0.005$) (**Fig. 20 A**, 1st *versus* 3rd bar). Incubation of U2OS cells with 10 ng/ml of FGF-2 for 4 hours prior to adding cisplatin, substantially blocked cell killing induced by 24 hours treatment with cisplatin, suggesting that FGF-2 confers protection to U2OS cells against cisplatin-induced cell killing (**Fig. 20 A**, 3rd *versus* 4th bar). The difference between cells treated with cisplatin alone and cells treated with FGF-2 before cisplatin was statistically significant ($p < 0.01$).

As expected, cisplatin treatment also induced 50% of killing in the NSCLC cells HCC95 ($p < 0.01$) and HCC78 ($p < 0.005$) (**Fig. 20 B and C**, 1st *versus* 3rd bar). However, in these cell lines, pre-treatment with FGF-2 did not block cisplatin-mediated killing as shown by the lack of statistical significance between cells treated with cisplatin alone and cells treated with FGF-2 prior to cisplatin (**Fig. 20 B and C**, 3rd *versus* 4th bar). These results thus suggest that, unlike U2OS cells, FGF-2 did not rescue HCC95 and HCC78 cells from cisplatin-mediated cell death.

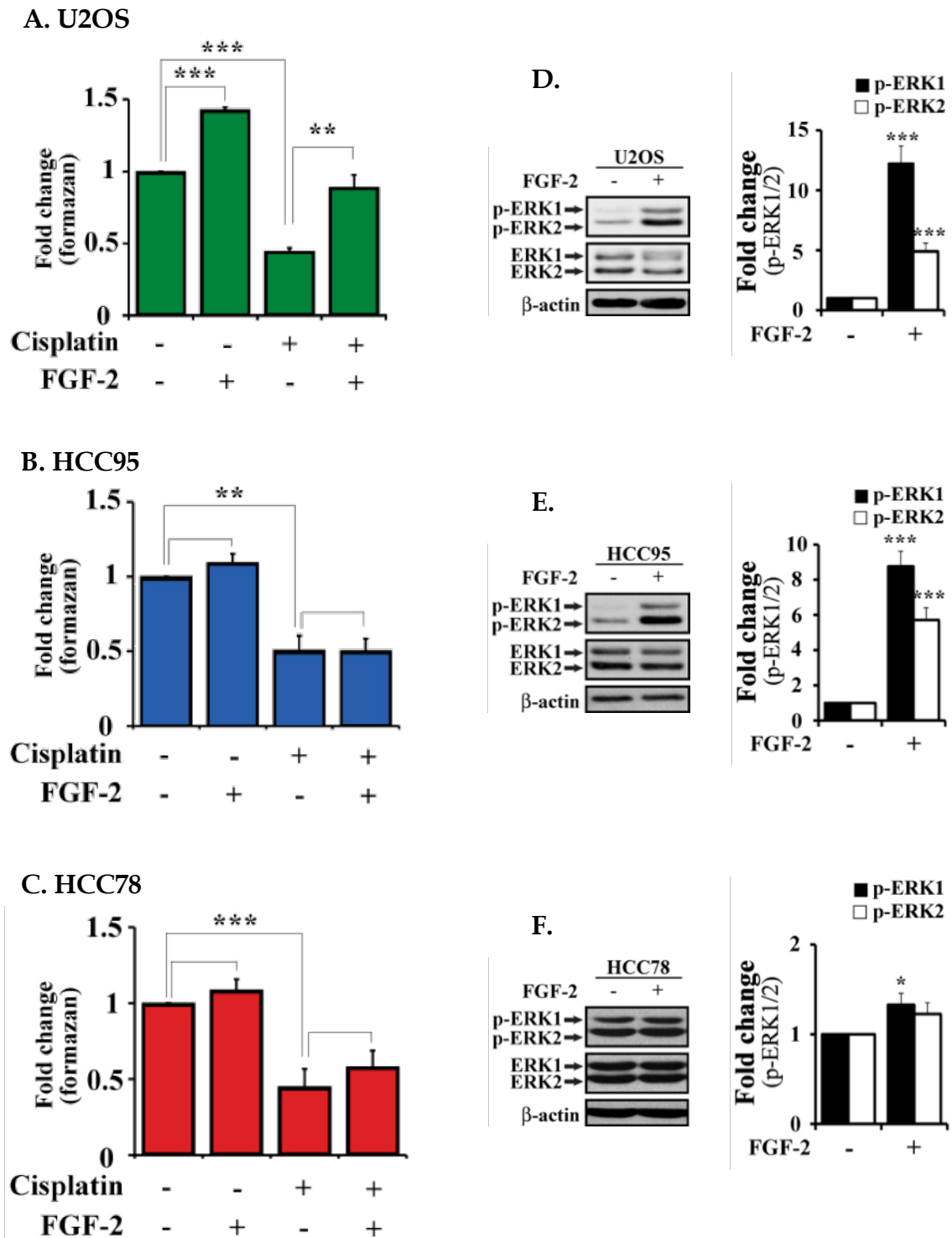


Figure 20. FGF-2 protected U2OS but not NSCLC HCC95 and HCC78 cells from cisplatin-mediated cell killing. A., B. and C. Cells were plated in 96-well plates (3,000 cells/well) and incubated in serum-free media overnight. After 4 hours pre-treatment with FGF-2 (10 ng/ml), each cell line was treated with the LD₅₀ of cisplatin for 24 hours. Cell viability was measured using the WST-1 cell viability assay. Mean $\pm$ SEM of three independent experiments performed in triplicate are presented as fold induction relative to untreated controls. Values correspond to the OD of coloured formazan compounds derived from cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases. Values are expressed as fold change over untreated controls. Statistical analysis was performed using Student's t-test (** - $p < 0.01$, *** - $p < 0.005$ versus untreated control). D., E. and F. Cells were stimulated with 10 ng/ml of FGF-2 for 10 minutes, proteins extracted and separated on a 10% SDS-PAGE gel. Western blotting analysis was performed on total cell lysates using antibodies against pERK1/2-Thr²⁰²/Tyr²⁰⁴ and total ERK1/2. β -actin was used as a loading control. Mean $\pm$ SEM of densitometric values from three independent experiments are shown in graphs. Values are expressed as fold change over untreated controls. Statistical analysis was performed using Student's t-test (* - $p < 0.05$, *** - $p < 0.005$ versus untreated control).

Functionality of FGF-2 was monitored concomitantly in all rescue experiments by assessing the phosphorylation of ERK1/2. Whole cell extracts from cells treated with FGF-2 for 10 minutes were run on a 7.5% SDS-PAGE gel and the membranes were probed with an antibody against phosphorylated ERK1/2 (**Fig. 20 D, E and F**). The antibody was stripped of the membrane, which was subsequently probed with an ERK1/2 antibody that detects both the phosphorylated and unphosphorylated forms of ERK1 and ERK2. The Western blot membrane was also probed with an antibody against β -actin, which served as a loading control. In all cases, it was established that the FGF-2 was active.

Using the WST-1 assay we estimated the number of viable cells by measuring mitochondrial activity. With this method, however, it is not possible to discriminate if a reduction in cell number is due to apoptosis, necrosis, or due to a decrease in cell division. Accordingly, we used other experimental methods to establish if the observed effect(s) of FGF-2 in cisplatin-mediated cell death were due to inhibition of apoptosis. For that purpose, the rescue experiment was performed and cell death was assessed by flow cytometry, which allows measuring the loss of cellular DNA content and cell cycle alterations. In addition, cleavage of caspase' substrates, such as PARP and lamin B, which can be used as 'read-outs' for apoptosis (Lazebnik *et al.*, 1993; Lazebnik *et al.*, 1994), was analysed by Western blot.

4.1.5.1 FGF-2 BLOCKED CISPLATIN-INDUCED APOPTOSIS IN U2OS CELLS BUT NOT IN NSCLC HCC95 AND HCC78 CELLS

Having shown that FGF-2 promotes chemoresistance in U2OS cells but not in the NSCLC cell lines HCC95 and HCC78 using the WST-1 method (**Fig. 20**), we confirmed these observations by analysing DNA content and cleavage of caspases substrates in cells upon cisplatin treatment.

Cell cycle profiles were analysed by flow cytometry. Using this method we assessed cell death by quantifying the sub-G₁ peak of the cell cycle profile, which corresponds to cells that have lost DNA (cf. Materials and methods). After an overnight starvation in serum-free media, cells were pre-incubated for 4 hours with FGF-2 and, subsequently, with cisplatin for 18 hours. Cells were harvested (adherent, alive cells and floating "corpses"), the cell membrane permeabilised and the cells incubated with PI, which intercalates within the DNA.

In U2OS cells we observed an average 2-fold increase of cells in the sub-G₁ peak after treatment with cisplatin ($p < 0.005$) (**Fig. 21 A**, top-left *versus* top-right histogram, and **B**, 1st *versus* 3rd bar), indicating that cisplatin induced apoptosis. Pre-incubation with FGF-2, however, substantially reduced the cell death induced by cisplatin, as shown by the reduction of the sub-G₁ population ($p < 0.005$) (**Fig. 21 A**, top-right *versus* bottom-right histogram and **B**, 3rd *versus* 4th bar). The percentage of cells within the sub-G₁ population of cells pre-treated with FGF-2 prior cisplatin (7.1%) was very similar to the sub-G₁ population of untreated cells (5.5%) (**Fig. 21 A**, top-left and bottom-right histograms, and **Fig. 21 B**, 1st and 4th bars). Importantly, “rescued cells”, when re-plated, survived for at least a couple of weeks but did not proliferate further (data not shown). It remains to be determined, however, if given fresh FGF-2, these cells would re-enter the cell cycle.

Cells treated with FGF-2 alone exhibited a decrease in basal cell death when compared to the untreated control ($p < 0.005$) (**Fig. 21 A**, top-left *versus* bottom-left histogram and **B**, 1st *versus* 2nd bar). Basal levels of apoptosis observed in the untreated cells were likely caused by serum starvation. Another way of analysing these data would be to normalise the cisplatin treatments to the respective controls (with or without FGF-2). However, with the current experimental settings, that would not be conclusive, as cells treated with FGF-2 for 24 hours will present lower levels of apoptosis due to protection from serum starvation-induced cell death and proliferation.

These data suggest that FGF-2 was able to protect cells from apoptosis induced by different cues, namely cytotoxicity and serum starvation. In addition, they clearly demonstrated that treatment with FGF-2 induced two different biological responses: on one hand, it induced cell proliferation and, on the other hand, it protected the cells against apoptosis.

To further establish that FGF-2-mediated rescue reflected protection from apoptosis rather than increased proliferation we have determined whether caspases, the main executors of apoptosis, were activated. For that purpose, cleavage of caspases substrates such as PARP and lamin B was analysed, as these are routinely used as caspase ‘read-outs’ (Lazebnik *et al.*, 1993; Lazebnik *et al.*, 1994). PARP is a protein that protects the cell from apoptosis when the cell is able to repair damaged DNA. Its cleavage is an indication that the cell was unable to repair the DNA (Oliver *et al.*, 1998) and it is thus often used as a marker of cells undergoing apoptosis (Lazebnik *et al.*, 1994). Lamin B is located in the inner nucleoplasmic surface and it is targeted by

proteases during early stages of cell death (Lazebnik *et al.*, 1993). The cleavage of these proteins can be monitored by Western blot using antibodies that recognise both uncleaved and/or cleaved forms. Cells were serum-starved overnight and pre-treated with or without FGF-2. After drug treatment overnight, proteins were extracted from all cells (adherent, alive cells and floating “corpses”), separated by SDS-PAGE gel and analysed by Western blot.

In U2OS cells, cisplatin induced the cleavage of PARP, which can be seen by the increase of cleaved PARP (85 kDa) compared to untreated cells (**Fig. 21 C**, 1st versus 3rd lane). This was statistically significant ($p < 0.005$) and corresponded to an average 5.2-fold increase over basal levels (**Fig. 21 D**, 1st versus 3rd bar). Pre-incubation of U2OS cells with FGF-2 substantially reduced cisplatin-induced PARP cleavage ($p < 0.005$) (**Fig. 21 C**, 3rd versus 4th lane, and **B**, 3rd versus 4th bar). Pre-treatment with FGF-2 also reduced the basal cell death ($p < 0.005$) (**Fig. 21 C**, 1st versus 2nd lane, and **D**, 1st versus 2nd bar). Similar results were obtained assessing lamin B cleavage (data not shown). These results establish an anti-apoptotic role for FGF-2 in U2OS cells that have been challenged with the cytotoxic effects of cisplatin or subjected to serum-starvation.

As exemplified by the Western blot shown in **Figure 21 C**, total (116 kDa) and cleaved (85 kDa) forms of PARP were not the only bands detected by the PARP antibody. These bands could be either unspecific or could correspond to other forms of PARP. The different intensities observed between samples suggest that they might be proteins responding to the treatments performed. The identity of these bands is currently unknown but it could be investigated by isolating the proteins from the gel and examining them by using mass spectrometry.

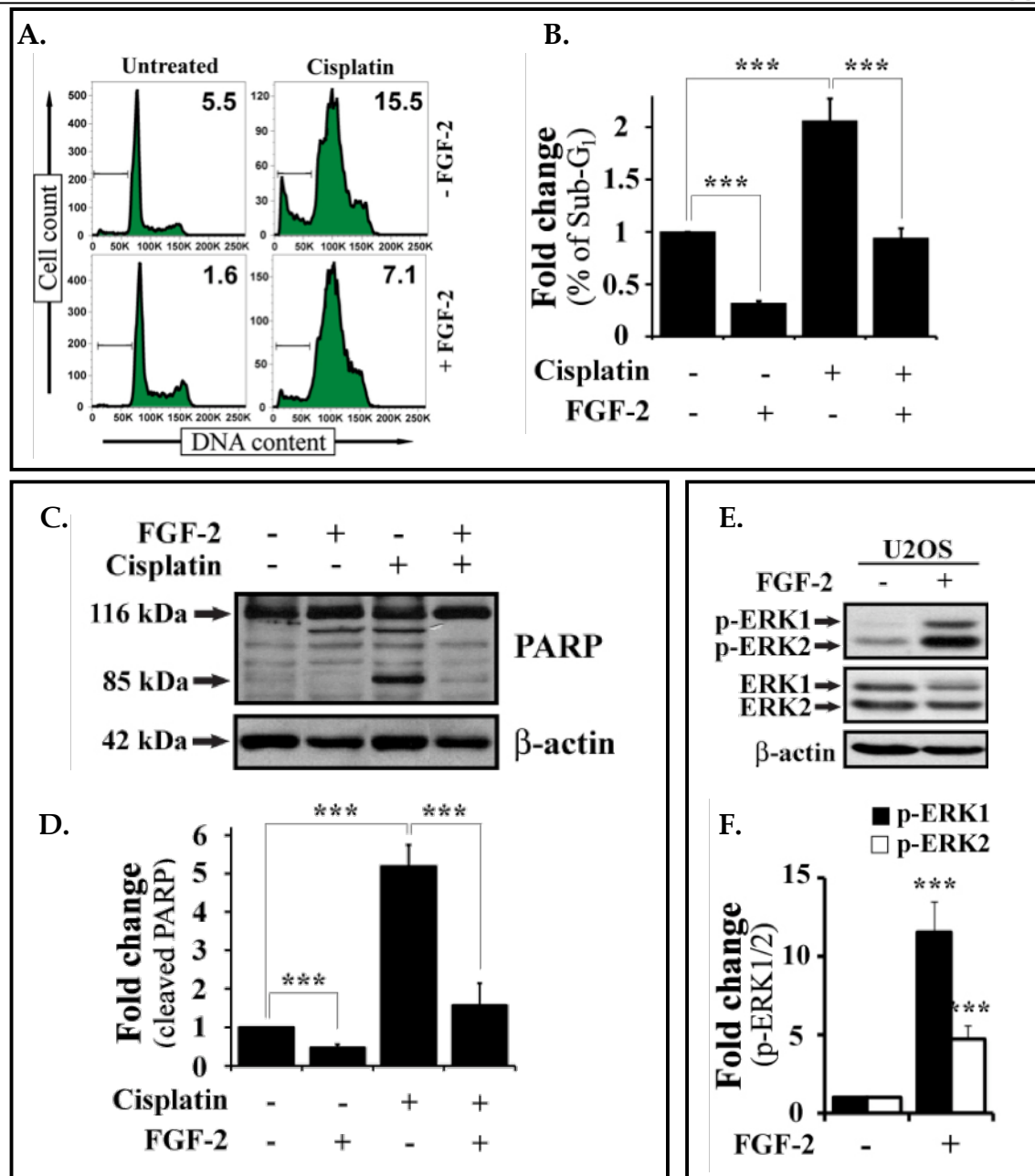


Figure 21. FGF-2 rescued U2OS cells from cisplatin-induced cell death. U2OS cells were incubated in serum-free media overnight. **A.** After 4 hours pre-treatment with 10 ng/ml of FGF-2, cells were treated with the LD₅₀ of cisplatin for 18 hours. Subsequently, cells were harvested, permeabilised and stained with PI (50 µg/ml). Cell death was assessed by flow cytometric analysis using the FACSCanto and the results were analysed using FlowJo. Cell cycle profiles from one experiment are shown. Numbers refer to the percentage of dead cells represented within the sub-G₁ population. **B.** Mean ± SEM of five independent experiments are shown in a graph. Values are expressed as fold change of sub-G₁ populations over the untreated control. Statistical analysis was performed with Student's t-test (***) - $p < 0.005$). **C.** After 4 hours pre-treatment with 10 ng/ml of FGF-2, cells were treated overnight with the LD₅₀ of cisplatin. Subsequently, cells were harvested, proteins were extracted and separated on 7.5% SDS-PAGE gel. Western blotting analysis was performed using total cell lysates (adherent alive cells and floating "corpses") and antibodies against PARP. β-actin was used as a loading control. **D.** Mean ± SEM of densitometric values (cleaved PARP) from five independent experiments are shown in a graph. Values are expressed as fold change over untreated controls. Statistical analysis was performed with Student's t-test (***) - $p < 0.005$). **E.** Cells were stimulated with 10 ng/ml of FGF-2 for 10 minutes and proteins were extracted and separated on 10% SDS-PAGE gel. Western blotting analysis was performed on total cell lysates using antibodies against pERK1/2-Thr²⁰²/Tyr²⁰⁴ and total ERK1/2. β-actin was used as a loading control. **F.** Mean ± SEM of densitometric values from five independent experiments are shown in a graph. Values are expressed as fold change over untreated controls. Statistical analysis was performed with Student's t-test (***) - $p < 0.005$ versus untreated control).

In parallel, phosphorylation of ERK1/2 in response to FGF-2 was monitored in order to confirm the functionality of FGF-2 (**Fig. 21 E and F**). Proteins from cells treated with 10 ng/ml of FGF-2 for 10 minutes were run on a 7.5% SDS-PAGE gel and analysed by Western blot. Membranes were probed with an antibody against phosphorylated ERK1/2 and upon stripping re-probed with an ERK1/2 antibody. β -actin was also probed and used as a loading control.

In HCC95 and HCC78 cells, the treatment with cisplatin induced an increase of the sub-G₁ peak ($p < 0.05$) (**Fig. 22 A**, top-left *versus* top-right histogram, and **B**, 1st *versus* 3rd bar, and **Fig. 23 A**, top-left *versus* top-right histogram, and **B**, 1st *versus* 3rd bar). FGF-2, however, did not protect these cells from cisplatin-induced apoptosis (**Fig. 22 A**, top-right *versus* bottom-right histogram, and **B**, 3rd *versus* 4th bar, and **Fig. 23 A**, top-right *versus* bottom-right histogram, and **B**, 3rd *versus* 4th bar). Indeed, in the case of HCC95 cells FGF-2 appeared to have potentiated the apoptotic effect of cisplatin (**Fig. 22 A**, top-right *versus* bottom-right histogram). The latter was, however, without statistical significance (**Fig. 22 B**, 3rd *versus* 4th bar).

A significant increase in PARP cleavage was observed in response to cisplatin in the HCC95 cell line ($p < 0.05$) as shown by the increase of the 85 kDa band (**Fig. 22 C**, 1st *versus* 3rd lane and **D**, 1st *versus* 3rd bar). In HCC78 cells, the cleavage of lamin B was analysed instead (**Fig. 23 C**) because although cisplatin treatment caused a decrease of the upper band of PARP, an increase in the lower band (cleaved form) was not detected under the conditions used (data not shown). As shown in **Figure 23 C** (1st *versus* 3rd lane) and **D** (1st *versus* 3rd bar), HCC78 cells treated with cisplatin exhibited an increase of the lamin fragment (45kDa) ($p < 0.005$). Confirming what was previously observed using the WST-1 cell viability assay (**Fig. 20 B and C**) and cell cycle analysis (**Fig. 22 A and B**, and **23 A and B**) FGF-2 was unable to prevent the cisplatin-induced cleavage of PARP or lamin B in these NSCLC cells (**Fig. 22 C**, 3rd *versus* 4th lane and **D**, 3rd *versus* 4th bar, and **Fig. 23 C**, 3rd *versus* 4th lane and **D**, 3rd *versus* 4th bar).

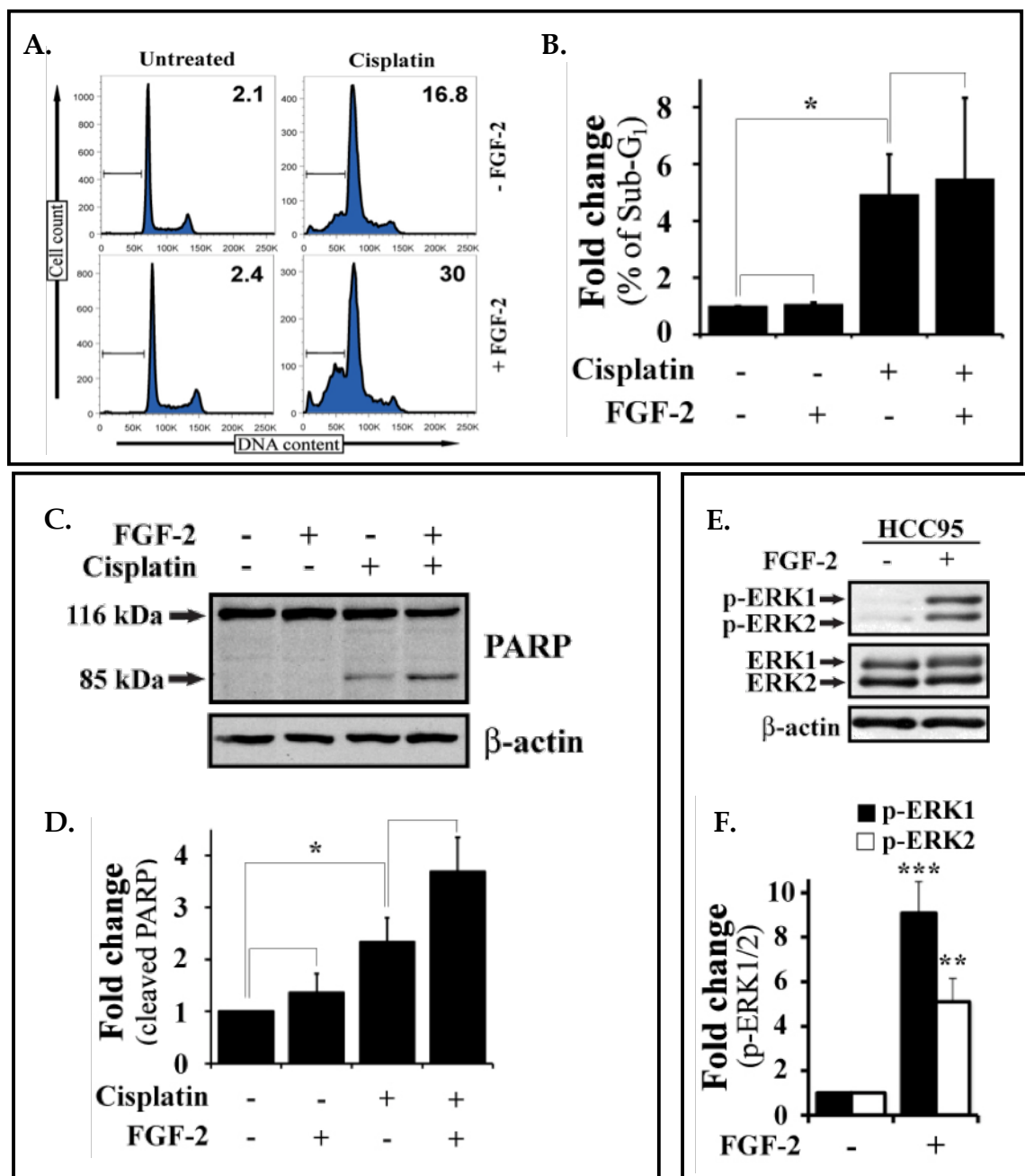


Figure 22. FGF-2 failed to reduce cisplatin-induced cell death in HCC95 cells. HCC95 cells were incubated in serum-free media overnight. **A.** After 4 hours pre-treatment with 10 ng/ml of FGF-2, cells were treated with the LD₅₀ of cisplatin for 18 hours. Subsequently, cells were harvested, permeabilised and stained with PI (50 µg/ml). Cell death was assessed by flow cytometric analysis using the FACSCanto and the results were analysed using FlowJo. Cell cycle profiles from one experiment are shown. Numbers refer to the percentage of dead cells within the sub-G₁ population. **B.** Mean ± SEM of four independent experiments are shown in a graph. Values are expressed as fold change of sub-G₁ populations over the untreated control. Statistical analysis was performed with Student's t-test (* - p<0.05). **C.** After 4 hours pre-treatment with 10 ng/ml of FGF-2, cells were treated overnight with the LD₅₀ of cisplatin. Subsequently, cells were harvested, proteins were extracted and separated on 7.5% SDS-PAGE gel. Western blotting analysis was performed using total cell lysates (adherent alive cells and floating "corpses") and antibodies against PARP. β-actin was used as a loading control. **D.** Mean ± SEM of densitometric values (cleaved PARP) from three independent experiments are shown in a graph. Values are expressed as fold change relative to untreated controls. Statistical analysis was performed with Student's t-test (* - p<0.05). **E.** Cells were stimulated with 10 ng/ml of FGF-2 for 10 minutes and proteins were extracted and separated on 10% SDS-PAGE gel. Western blotting analysis was performed on total cell lysates using antibodies against pERK1/2-Thr²⁰²/Tyr²⁰⁴ and total ERK1/2. β-actin was used as a loading control. **F.** Mean ± SEM of densitometric values from three independent experiments are shown in a graph. Values are expressed as fold change over untreated controls. Statistical analysis was performed with Student's t-test (***) - p<0.005).

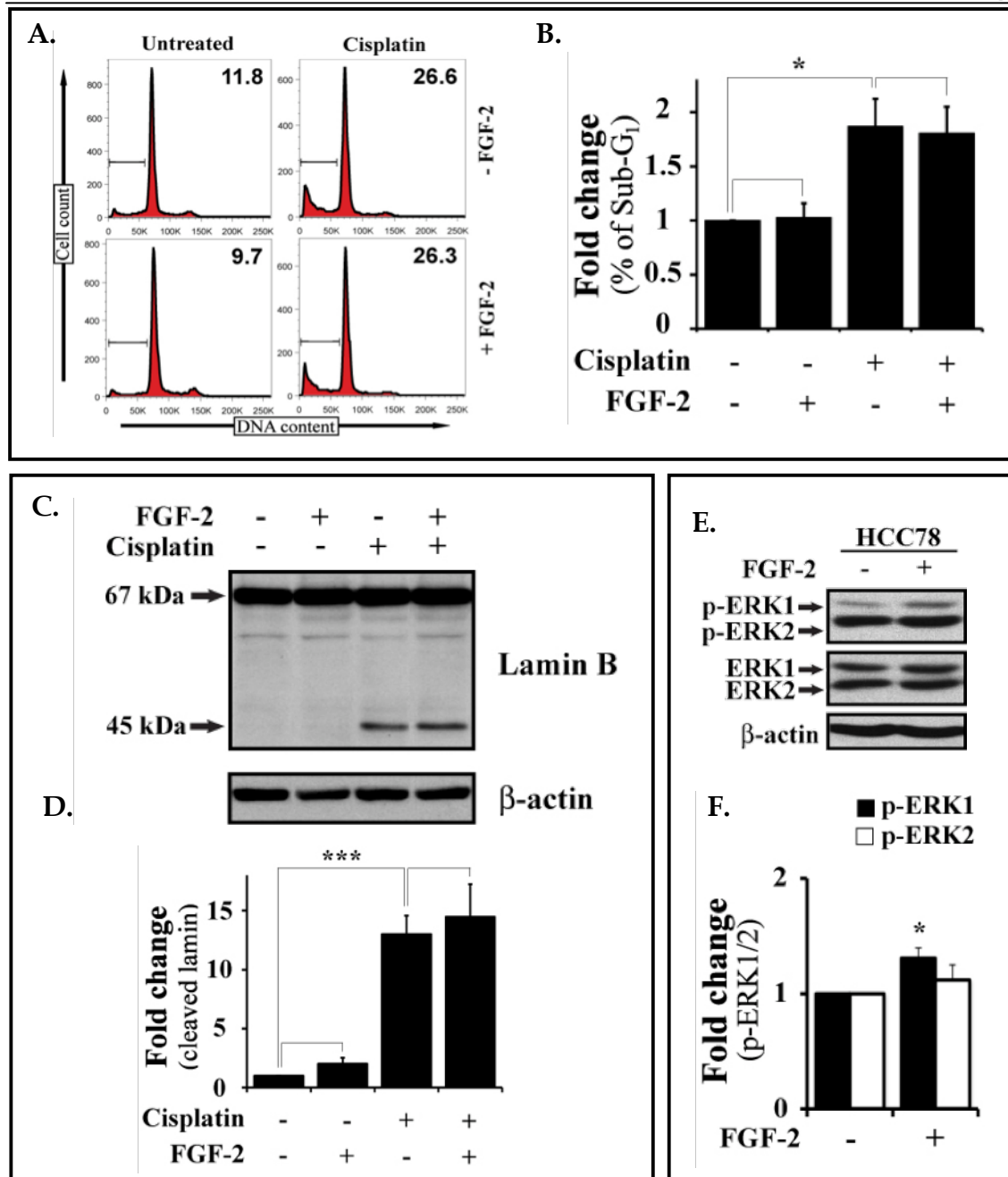


Figure 23. FGF-2 failed to reduce cisplatin-induced cell death in HCC78 cells. HCC78 cells were incubated in serum-free media overnight. **A.** After 4 hours pre-treatment with 10 ng/ml of FGF-2, cells were treated with the LD₅₀ of cisplatin for 18 hours. Subsequently, cells were harvested, permeabilised and stained with PI (50 µg/ml). Cell death was assessed by flow cytometric analysis using the FACSCanto and the results were analysed using FlowJo. Cell cycle profiles from one experiment are shown. Numbers refer to the percentage of dead cells within the sub-G₁ population. **B.** Mean ± SEM of three independent experiments are shown in a graph. Values are expressed as fold change of sub-G₁ populations over the untreated control. Statistical analysis was performed with Student's t-test (* - p<0.05). **C.** After 4 hours pre-treatment with 10 ng/ml of FGF-2, cells were treated overnight with the LD₅₀ of cisplatin. Subsequently, cells were harvested, proteins were extracted and separated on 7.5% SDS-PAGE gel. Western blotting analysis was performed using total cell lysates (adherent alive cells and floating "corpses") and antibodies against lamin B. β-actin was used as a loading control. **D.** Mean ± SEM of densitometric values (cleaved PARP) from three independent experiments are shown in a graph. Values are expressed as fold change relative to untreated controls. Statistical analysis was performed with Student's t-test (***) - p<0.005). **E.** Cells were stimulated with 10 ng/ml of FGF-2 for 10 minutes and proteins were extracted and separated on 10% SDS-PAGE gel. Western blotting analysis was performed on total cell lysates using antibodies against pERK1/2-Thr²⁰²/Tyr²⁰⁴ and total ERK1/2. β-actin was used as a loading control. **F.** Mean ± SEM of densitometric values from three independent experiments are shown in a graph. Values are expressed as fold change over untreated controls. Statistical analysis was performed with Student's t-test (* - p<0.05).

As previously mentioned, phosphorylation of ERK1/2 in response to FGF-2 was also monitored by Western blotting (**Fig. 22 E and F**, and **Fig. 23 E and F**). As observed before (**Fig. 14**), FGF-2 induced a significant phosphorylation of ERK1 ($p<0.005$) and ERK2 ($p<0.01$) in HCC95 cells. In the HCC78 cell line, FGF-2 induced a small (an average of 1.3-fold) but statistically significant ($p<0.05$) increase in ERK1, but not ERK2, upon 10 minutes of stimulation.

The FGF-2 rescue experiment was also performed in SCLC H-510 cells, where the FGF-2 chemoprotection was initially described (Pardo *et al.*, 2002). These cells were pre-treated with 0.1 ng/ml of FGF-2 and treated with etoposide for 96 hours as described in Pardo *et al.*, 2002. Subsequently, the DNA content was measured by cell cycle analysis and Lamin B cleavage assessed by Western blotting (Appendix III). This experiment was carried out initially to reproduce the original observation and ascertain that the results obtained with additional cell lines were *bona fide*.

FGF-2 rescue experiments have been extensively repeated in the NSCLC HCC95 and HCC78 cells using multiple permutations but under the conditions used in our study no chemoprotection was ever observed. The study of additional NSCLC cell lines will be crucial to establish if FGF-2 is able to mediate chemoprotection in this type of lung cancer cells. It is possible that had other NSCLC cell lines been chosen we might have observed chemoprotection. These two cell lines were originally chosen because one had high levels (HCC78) and the other very low levels (HCC95) of constitutively activated STAT3. This was relevant to this project because we wanted to investigate whether JAKs and STATs were involved in mediating chemoresistance and, in cancer cells, STAT3 is the most frequently de-regulated JAK/STAT signalling molecule. Had FGF-2 conferred protection against cisplatin in these cells, they would have been very useful to address many of the questions asked initially in this work.

In contrast to HCC95 and HCC78 cells, treatment of U2OS cells with FGF-2 protected them from cisplatin-induced apoptosis. This finding is particularly interesting given that FGF signalling was shown to have an important role in this type of cancer (Muenke and Schell, 1995; Coffin *et al.*, 1995; Deng *et al.*, 1996; Bodo *et al.*, 2002). In addition, because the osteosarcoma U2OS cell line had been extensively utilised by the Costa-Pereira Group, some experiments (such as RNAi) were already optimised for this cell line. This cell line was thus subsequently used in this work to investigate the major players in FGF-2-mediated chemoresistance pathway(s) and further our understanding of this complex signalling pathway.

4.1.6. FGF-2 PROTECTED U2OS CELLS FROM CISPLATIN-INDUCED CELL DEATH BY A MEK-ERK DEPENDENT MECHANISM

FGF-2 was shown to confer resistance to etoposide-mediated apoptosis in SCLC cells *via* a MEK-dependent pathway (Pardo *et al.*, 2002). Here we investigated if in U2OS cells the activation of MEK-ERK pathway was also required for the FGF-2 chemoprotection from cisplatin. We used PD0325901, a MEK1/2 specific inhibitor, which was added to cells prior to performing the rescue experiment. This inhibitor is a significantly improved analogue of CI-1040, the first MEK inhibitor to enter clinical evaluation (Lorusso *et al.*, 2005; Rinehart *et al.*, 2004). Like its analogue, PD0325901 is non-competitive with ATP, and its specificity and high potency against activated MEK1 and MEK2 has been demonstrated *in vitro* and *in vivo* (Sebolt-Leopold *et al.*, 2004).

To assess the functionality of PD0325901, U2OS cells were pre-treated with this inhibitor and then stimulated with FGF-2 for 10 minutes. Proteins were extracted, run on a 7.5% SDS-PAGE gel and analysed by Western blot using antibodies against phosphorylated ERK1/2, ERK1/2 and β -actin. Pre-treatment of U2OS with this inhibitor for 30 minutes was sufficient to block the phosphorylation of ERK1/2 by FGF-2 in a dose dependent manner (data not shown). Addition of 0.5 μ M of PD0325901 was sufficient to maximally inhibit FGF-2-induced activation of ERK1/2 in U2OS cells ($p < 0.005$) (**Fig. 24 A**, 2nd *versus* 4th lane, and **B**, bars compared with red asterisks).

The requirement for the MEK/ERK pathway for the FGF-2-mediated rescue was then investigated by performing the rescue experiment in cells previously incubated with 0.5 μ M of the inhibitor PD0325901. As a control, cells were incubated with the drug vehicle [0.005% (v/v) of DMSO]. Similarly to what was observed before (**Fig. 21 C and D**), in cells incubated with the drug vehicle (untreated), cisplatin induced the cleavage of PARP ($p < 0.005$) (**Fig. 24 C**, 1st *versus* 3rd lane, and **D**, 1st *versus* 3rd bar in the 1st graph) and this cleavage was blocked by the pre-treatment with FGF-2 ($p < 0.005$) (**Fig. 24 C**, 3rd *versus* 4th lane, and **D**, 3rd *versus* 4th bar in the 1st graph). In cells previously incubated with PD0325901, however, FGF-2 was not able to prevent cisplatin-induced PARP cleavage (**Fig. 24 C**, 7th *versus* 8th lane, and **D**, 3rd *versus* 4th bar in the 2nd graph).

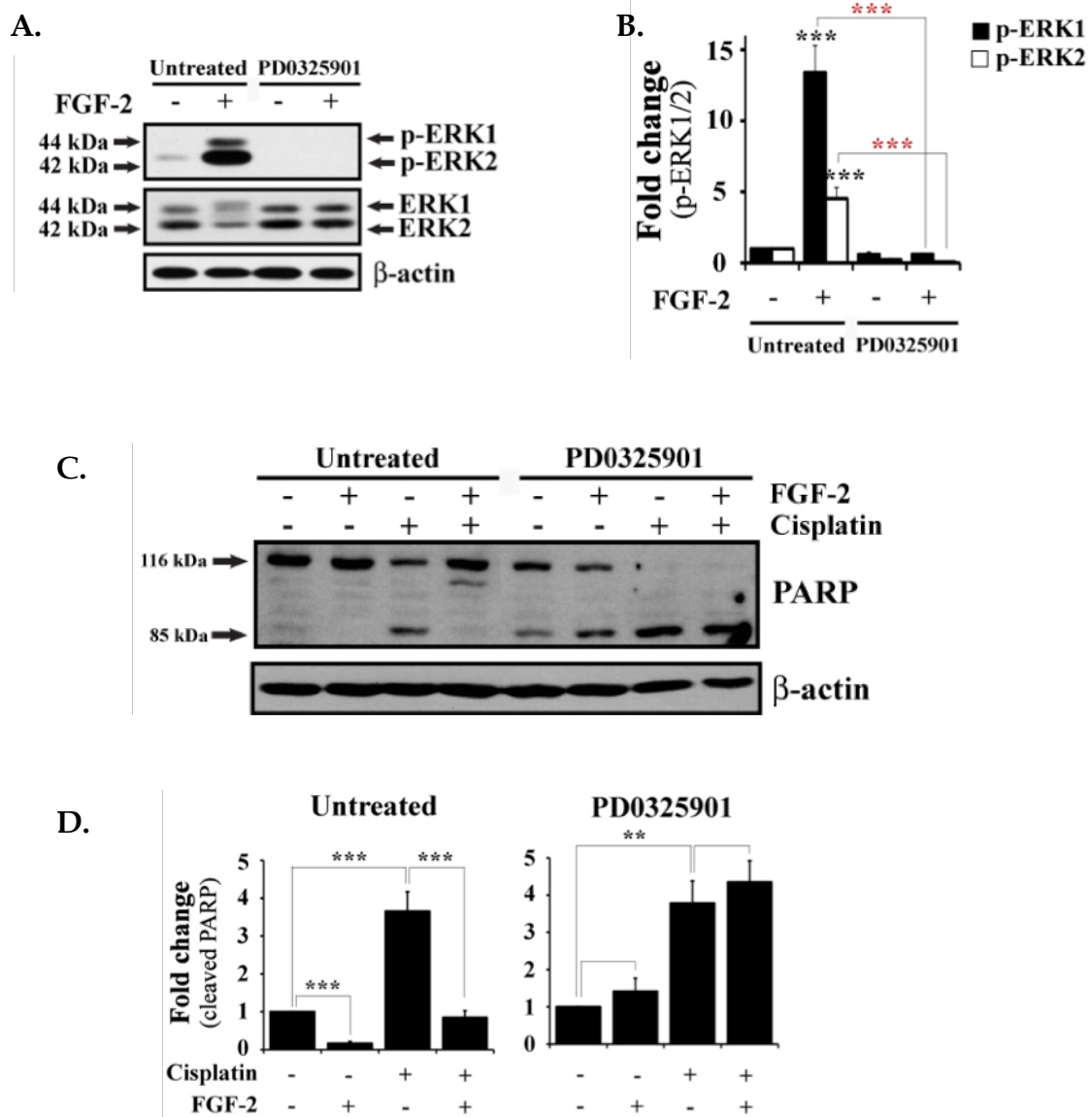


Figure 24. Inhibition of MEK/ERK signalling blocked FGF-2-mediated rescue in U2OS cells. (continued on page 114) **A.** Cells were serum-starved (0.5% FCS) overnight and incubated in serum-free media for 1 hour. Cells were pre-treated with 0.5 μ M of PD0325901 or with the drug vehicle [0.005% (v/v) of DMSO] for 30 minutes and then stimulated with 10 ng/ml of FGF-2 for 10 minutes. Proteins were extracted and separated on a 10% SDS-PAGE gel. Western blotting analysis was performed on total cell lysates using antibodies against pERK1/2-Thr²⁰²/Tyr²⁰⁴ and total ERK1/2. β -actin was used as a loading control. **B.** Mean $\pm$ SEM of densitometric values from three independent experiments are shown in a graph. Values are expressed as fold change over untreated controls (without PD0325901 and FGF-2). Statistical analysis was performed with Student's t-test (** - $p < 0.05$; *** - $p < 0.005$, black asterisks *versus* untreated control, red asterisks compare the indicated samples). **C.** and **E.** After overnight starvation in serum-free media, cells were incubated with 0.5 μ M of PD0325901 for 30 minutes and, after 4 hours pre-treatment with FGF-2 (10 ng/ml) cells were treated with the LD₅₀ of cisplatin. **C.** After 16 hours treatment of cisplatin, cells were harvested, proteins extracted and separated on 7.5% SDS-PAGE gel. Western blotting analysis was performed using total cell lysates and an antibody against PARP. β -actin was used as a loading control. **D.** Mean $\pm$ SEM of densitometric values (cleaved PARP) from three independent experiments are shown in graphs. Values are expressed as fold change over untreated controls. Statistical analysis was performed with Student's t-test (** - $p < 0.01$; *** - $p < 0.005$). **E.** After 18 hours of cisplatin treatment, cells were harvested, permeabilised and stained with PI (50 μ g/ml). Apoptotic cell death was assessed by flow cytometric analysis using the FACSCanto and the results were analysed using FlowJo. Cell cycle profiles from one experiment are shown. Numbers refer to the percentage of dead cells within the sub-G₁ population. **F.** Mean of three independent experiments $\pm$ SEM are shown in graphs. Values are expressed as fold change of sub-G₁ populations over untreated control. Statistical analysis was performed with Student's t-test (* - $p < 0.05$; *** - $p < 0.005$).

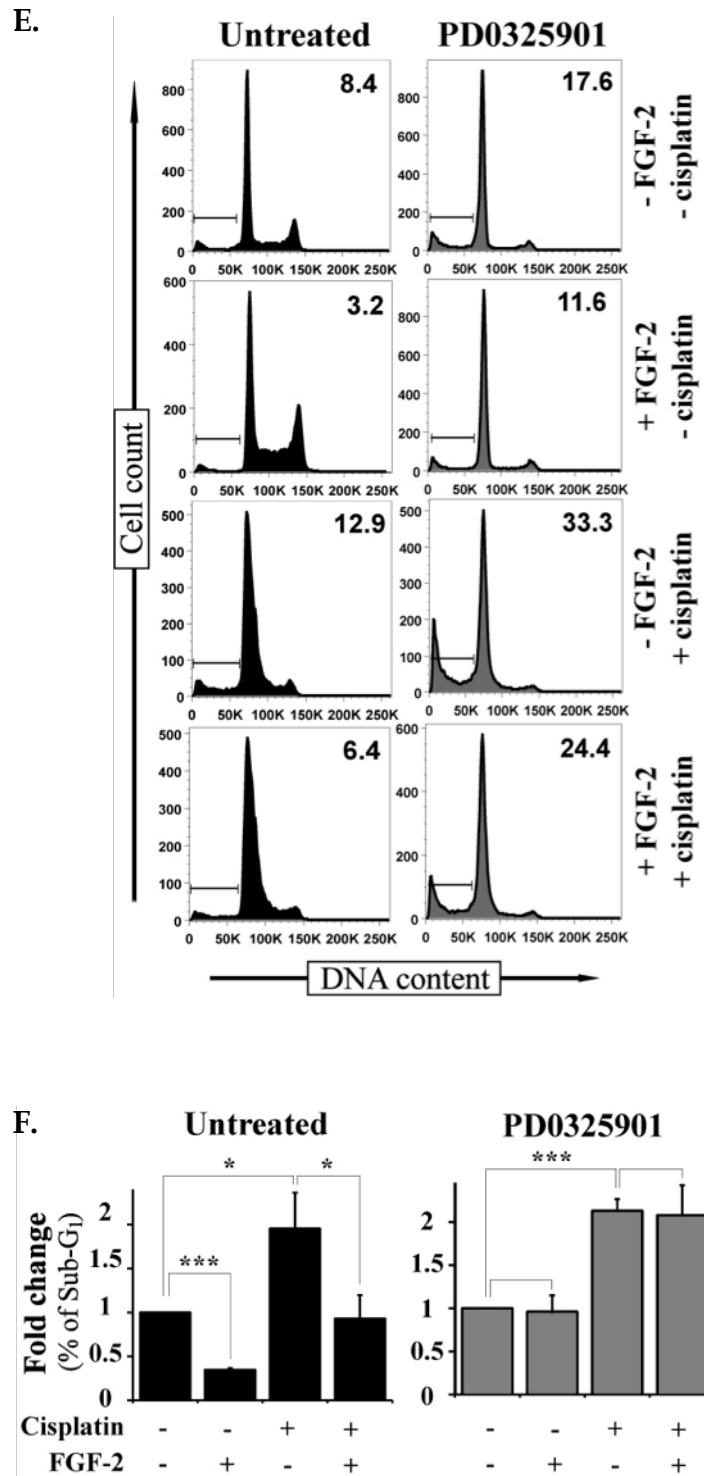


Figure 24. Inhibition of MEK/ERK signalling blocked FGF-2-mediated rescue in U2OS cells. (continued from page 113).

Similar results were obtained when cell cycle profiles of each sample were analysed (**Fig. 24 E and F**). Cells treated with the drug vehicle were rescued by FGF-2 as shown by the statistically significant reduction of the sub-G₁ peak ($p < 0.05$) in the FGF-2 pre-treated samples (**Fig. 24 E**, black coloured cell cycle profiles and **F**, 3rd versus 4th black bar). The inhibition of the MEK/ERK activity prevented FGF-2-induced rescue (**Fig. 24 C**, grey coloured cell cycle profiles, and **F**, 3rd versus 4th grey bar).

In addition, FGF-2 was not able to reduce basal cell death or cisplatin-induced killing in cells pre-incubated with PD0325901 (**Fig. 24 C**, 5th versus 6th lane, **D**, 1st versus 2nd bar, **E**, grey coloured cell cycle profiles, and **F**, 1st versus 2nd bar). These results highlight the importance of MEK/ERK activity in signalling pathways that mediate FGF-2-induced cell survival.

Pre-treatment with the inhibitor also caused an increase in the basal cell death (**Fig. 24 C and E**). This observation was not surprising as MEK-ERK signalling plays such crucial roles in survival (e.g. Ballif and Blenis *et al.*, 2001) and inhibition of its activity often has a negative impact on cell viability.

We further confirmed the crucial role of ERK1/2 for the observed FGF-2 survival effect using RNAi. Knockdown of ERK1 and ERK2 also prevented the FGF-2-induced rescue of U2OS cells (data not shown). These results indicate that MEK/ERK signalling is an essential mediator of FGF-2-induced rescue from cisplatin killing in U2OS cells, similar to what was previously observed in SCLC cells (Pardo *et al.*, 2002).

4.1.7. FGF-2 INDUCED PROTEIN-PROTEIN INTERACTIONS BETWEEN PKC ϵ , B-RAF AND S6K2 IN U2OS CELLS

It was shown in the SCLC cell line H-510 that early signalling cascades activated by FGF-2 involved the formation of a signalling complex comprising B-RAF, PKC ϵ and S6K2 (Pardo *et al.*, 2006). We, therefore, investigated if FGF-2 could also induce the interaction of these proteins in the U2OS cells. To detect potential interactions between B-RAF, PKC ϵ and S6K2 in response to FGF-2, these proteins were independently immunoprecipitated after treatment with FGF-2 for increasing periods of time. H-510 cells were treated in parallel as previously described (Pardo *et al.*, 2006) and used as a positive control for the complex formation (data not shown).

Immunoprecipitation experiments included several controls, such as immunoprecipitations using an unspecific antibody (with the same IgG as the

antibodies used for immunoprecipitation) and a sample incubated with agarose beads only. The first was used as a control for the antibody specificity and the second to determine non-specific binding to the beads. It must be noted from the onset that the S6K2 immunoprecipitations proved challenging, presumably due to the low levels of expression of this protein in U2OS cells. Importantly, B-RAF, PKC ϵ (**Fig. 25 A**) and S6K2 (**Fig. 25 B**) did not bind to the irrelevant IgG control antibodies or to the beads. In addition, cells transfected with siRNA against S6K2 were used as a further control to ascertain that the presumed S6K2 band seen on the Western blot was indeed S6K2 (**Fig. 25 B**). These data, together with additional data from other laboratory members, reassured us that the reagents available to us were trustworthy and that the data obtained was *bona fide*. In any case, immunoprecipitation with beads only was included as a control in all co-immunoprecipitation experiments.

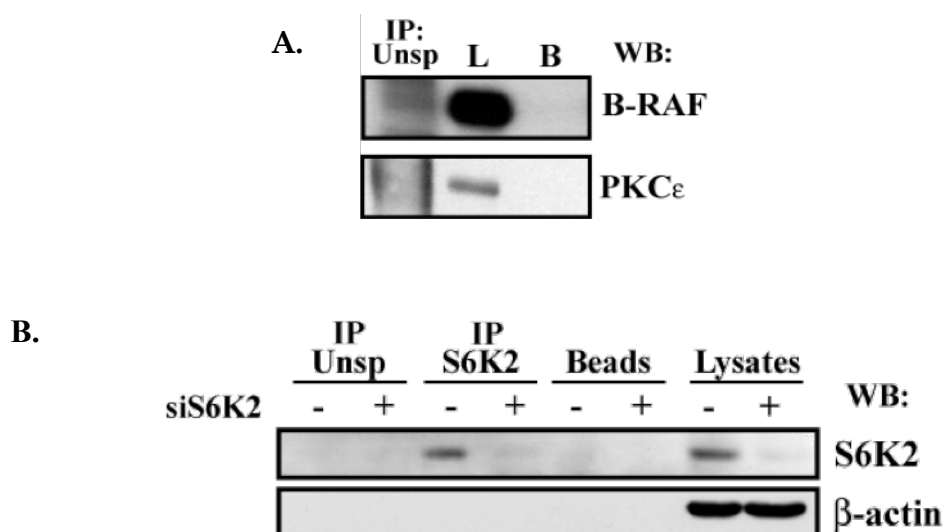


Figure 25. Controls for PKC ϵ , B-RAF and S6K2 immunoprecipitations. **A.** Cell lysates were subjected to immunoprecipitation with an unspecific antibody or with beads only, run in parallel with whole cell extracts on a 7.5% SDS-PAGE gel and analysed by Western blot. The membrane was probed for B-RAF and PKC ϵ . **B.** Cells were transfected with 75 nM siRNA against S6K2. Forty-eight hours after transfection, cell lysates were subjected to immunoprecipitation with an unspecific antibody, an antibody against S6K2 or with beads only. Immunoprecipitates were run in parallel with whole cell extracts on a 7.5% SDS-PAGE gel and analysed using Western blotting. L - lysate, B - beads only, Unsp - unspecific antibody.

U2OS cells were incubated with FGF-2 for increasing periods of time and whole cell extracts were used to immunoprecipitate B-RAF, PKC ϵ and S6K2, independently. The immunoprecipitates were run on a 7.5% SDS-PAGE gel and membranes were probed for the three proteins in order to detect co-immunoprecipitated molecules. PKC ϵ and B-RAF co-immunoprecipitated, whether the proteins were immunoprecipitated using a PKC ϵ or a B-RAF antibody (**Fig. 26 A**). Importantly, this interaction was ligand-induced. When PKC ϵ was immunoprecipitated, co-immunoprecipitated B-RAF increased 1.8-fold upon FGF-2 treatment. This reached statistical significance at all time points analysed ($p < 0.05$) (**Fig. 26 A**, 1st *versus* 2nd-4th lanes in the 1st row of the Western blot). The reverse immunoprecipitation with a B-RAF antibody co-immunoprecipitated PKC ϵ at the same time points, albeit with different kinetics. An average of 1.8-fold induction ($p < 0.05$) was detected by 10 minutes and a stronger interaction was detected at later time points (an average of 4.5-fold, $p < 0.05$) (**Fig. 26 A**, 1st *versus* 2nd-4th lanes in the 3rd row of the Western blot). By 20 and 30 minutes, the increase of B-RAF detection on PKC ϵ immunoprecipitates was weaker (1.8-fold) than the detection of PKC ϵ in B-RAF immunoprecipitates (4.5-fold) (**Fig. 26 A**, 3rd and 4th bar in the 1st and 2nd graphs). This could be related to stoichiometry or reflect differences in the efficiency of immunoprecipitation by the two different antibodies. In addition to this, some variation was observed between experiments. This can be illustrated, for example, by the Western blot showing the B-RAF immunoprecipitation, where the interaction between B-RAF and PKC ϵ peaked at 20 minutes and decreased at the 30 minutes time point (**Fig. 26 A**, 3rd row of the Western blot). This decrease at the 30 minutes time point was not seen in all the experiments performed as shown in the graph in **Figure 26 A** (4th bar in the 2nd graph). This may reflect a highly dynamic complex (that would involve a quick "kiss and run" of these molecules) or may be related to low stability of the complex that could allow some interactions to be lost in the immunoprecipitation process. In any case, and though this clearly requires additional analysis, the interaction between these two molecules was consistently observed throughout all experiments performed. Unfortunately, we were not able to detect S6K2 in the B-RAF and PKC ϵ immunoprecipitates. This does not necessarily reflect a lack of interaction between S6K2 and these proteins. Indeed, both B-RAF and PKC ϵ were detected in S6K2 immunoprecipitates. Low levels of expression of S6K2 in U2OS cells, as well as stoichiometry, may have hindered the detection of the interaction when immunoprecipitating B-RAF and PKC ϵ .

S6K2 was immunoprecipitated from the whole cell lysates, the proteins run on a 7.5% SDS-PAGE gel and analysed by Western blot. The membranes were then probed for B-RAF and PKC ϵ . Membranes were probed first for PKC ϵ (95 kDa), stripped overnight (cf. Materials and methods) and then probed for B-RAF (94 kDa). To ensure that the detection of B-RAF was not influenced by any residual unstripped PKC ϵ antibody, some membranes were probed first for B-RAF, stripped overnight, and then probed for PKC ϵ . Similar results were obtained regardless of the sequence of detection. Both PKC ϵ and B-RAF co-immunoprecipitated with S6K2 upon FGF-2 treatment for 10 minutes ($p < 0.05$) (**Fig. 26 A**, 2nd lane in the 5th and 6th rows of the Western blot and 3rd graph). Interaction of S6K2 with B-RAF and PKC ϵ appeared to be more transient than the interaction observed between B-RAF and PKC ϵ , since the interaction of S6K2 with B-RAF and with PKC ϵ decreased after 20 minutes of FGF-2 treatment.

Although these experiments establish that, upon FGF-2 treatment, S6K2 can bind to PKC ϵ and B-RAF and that PKC ϵ and B-RAF interact, they still do not categorically demonstrate that these proteins are all part of the same protein complex, as it was shown for the SCLC H-510 cells and previously shown by Seckl and colleagues (Pardo *et al.*, 2006). Conceptually, treatment of U2OS cells with FGF-2, can lead to the formation of three different dimers (B-RAF/PKC ϵ ; B-RAF/S6K2; S6K2/PKC ϵ) and/or the formation of a trimer (B-RAF/PKC ϵ /S6K2). The precise nature of the protein complexes induced by FGF-2 and the precise stoichiometry remain to be established. This will be addressed initially using RNAi: each protein will be silenced independently and the impact of each siRNA (siPKC ϵ , siB-RAF and siS6K2) on these interactions analysed. FRET could also be used to address this question but this would require tagging each protein and transfecting them into cells, which may lead to over-expression artefacts and may change the dynamics of the interactions.

In parallel with co-immunoprecipitations, whole cell extracts were also analysed by SDS-PAGE/Western blot. We monitored B-RAF, PKC ϵ and S6K2 levels and also the phosphorylation of ERK1/2 in response to FGF-2 to monitor FGF-2 functionality. Total protein levels and activation of ERK1/2 by FGF-2 are shown in **Figure 26 B**. As previously observed, FGF-2 induced the phosphorylation of ERK1/2. Interestingly, a small (1.5- to 1.7-fold) but statistically significant ($p < 0.05$ and $p < 0.005$) increase of S6K2 was detected in response to FGF-2. This increase is observed shortly after FGF-2 stimulation (10 to 30 minutes), which is too short a period for it to be the result of *de novo* protein synthesis. One interesting possibility is that FGF-2 signalling may increase the stability of this protein. This will be the subject of future studies.

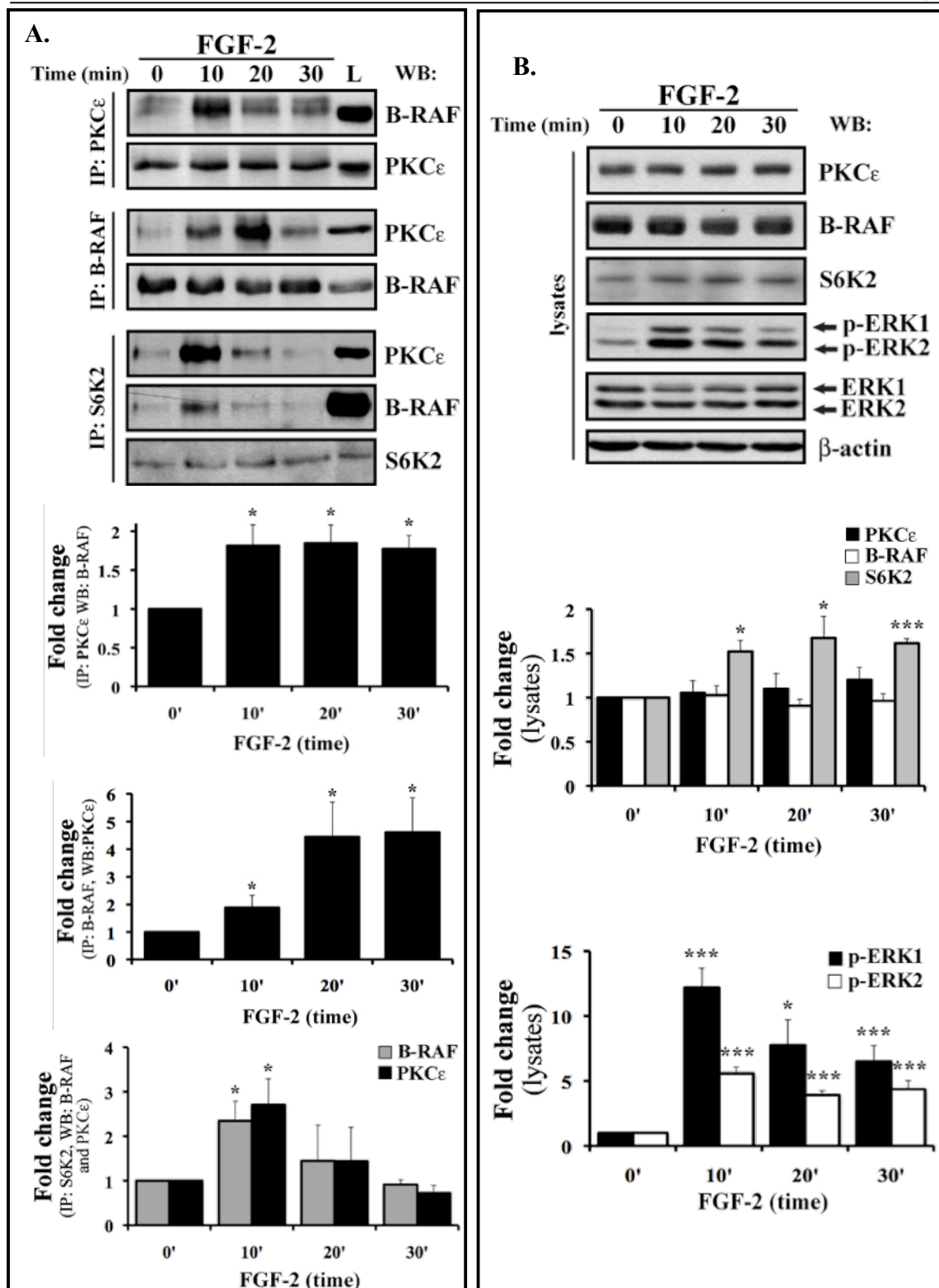


Figure 26. FGF-2 induced interactions between PKC ϵ , B-RAF and S6K2 in U2OS cells. U2OS cells were serum-starved (0.5% FCS) overnight and then incubated in serum-free media for 1 hour. After stimulation with 10 ng/ml of FGF-2 for the indicated times, proteins were extracted. **A.** Cell lysates were subjected to immunoprecipitation with a PKC ϵ , B-RAF or S6K2 antibody. Immunoprecipitates were separated on a 7.5% SDS-PAGE gel and proteins detected by Western blot. Membranes were probed for PKC ϵ , B-RAF and S6K2. **B.** Total cell lysates were separated on a 7.5% SDS-PAGE and analysed by Western blot. Membranes were probed for PKC ϵ , B-RAF, S6K2, pERK1/2-Thr²⁰²/Tyr²⁰⁴ and total ERK1/2. β -actin was used as a loading control. **A.** and **B.** Representative Western blots and mean $\pm$ SEM of densitometric values from at least three independent experiments are shown in graphs. Values are expressed as fold change over untreated controls. Statistical analysis was performed with Student's t-test (* - $p < 0.05$; *** - $p < 0.005$ versus untreated controls). L - lysate, ' - minutes.

4.1.8. INTERACTIONS BETWEEN PKC ϵ , B-RAF AND S6K2 IN HCC95 CELLS

Despite activating ERK1/2, FGF-2 was not able to protect HCC95 cells from cisplatin-mediated cell death under the conditions studied (**Section 4.1.2** and **4.1.5**). We therefore investigated whether or not FGF-2 induced interactions between B-RAF, PKC ϵ and S6K2 in this cell line as it did in SCLC (Pardo *et al.*, 2006) and U2OS (**Section 4.1.7**) cells. If FGF-2 did not induce such protein interactions this could explain why it did not provide chemoprotection in HCC95 cells.

HCC95 cells were treated with FGF-2 for increasing periods of time and whole cell extracts were used to immunoprecipitate B-RAF, PKC ϵ and S6K2 independently. The immunoprecipitates were run on a 7.5% SDS-PAGE gel and membranes were probed for the three proteins in order to detect co-immunoprecipitated molecules. We were able to detect B-RAF in PKC ϵ immunoprecipitates upon treatment with FGF-2 (**Fig. 27 A**, 1st row in the Western blot). This interaction was confirmed by performing the reverse immunoprecipitation with a B-RAF antibody (**Fig. 27 A**, 3rd row in the Western blot). The interaction between B-RAF and PKC ϵ was detected in all experiments performed, however, the time points when these two proteins interacted were not consistent between experiments, a fact reflected in the statistical analysis. Accordingly, detection of B-RAF in PKC ϵ immunoprecipitates from samples treated with FGF-2 did not reach statistical significance (**Fig. 27 A**, 1st graph) as it did in the reverse immunoprecipitation ($p < 0.05$) at the 20 minutes time-point (**Fig. 27 A**, 2nd graph). The reason why the induction of B-RAF and PKC ϵ interactions upon FGF-2 stimulation varied so much is currently unknown. This may be due to either low stability of the complex, or due to these molecules interacting 'intermittently' (on-and-off) upon FGF-2 stimulation. The variation between time points can also be related to other factors, such cell cycle phase of each cell population or other experimental factors, although the latter is unlikely as all experimental conditions had been optimised and standardised from the beginning. The data suggest that FGF-2 may induce the interaction between B-RAF and PKC ϵ in HCC95, but the kinetics of this interaction are still unclear. If the interaction(s) are highly labile and if stable complex(es) were required for FGF-2 to induce chemoresistance, this could explain why FGF-2 failed to protect these cells from cisplatin-induced cell death. Like in U2OS immunoprecipitates, we were not able to detect S6K2 in B-RAF or PKC ϵ immunoprecipitates.

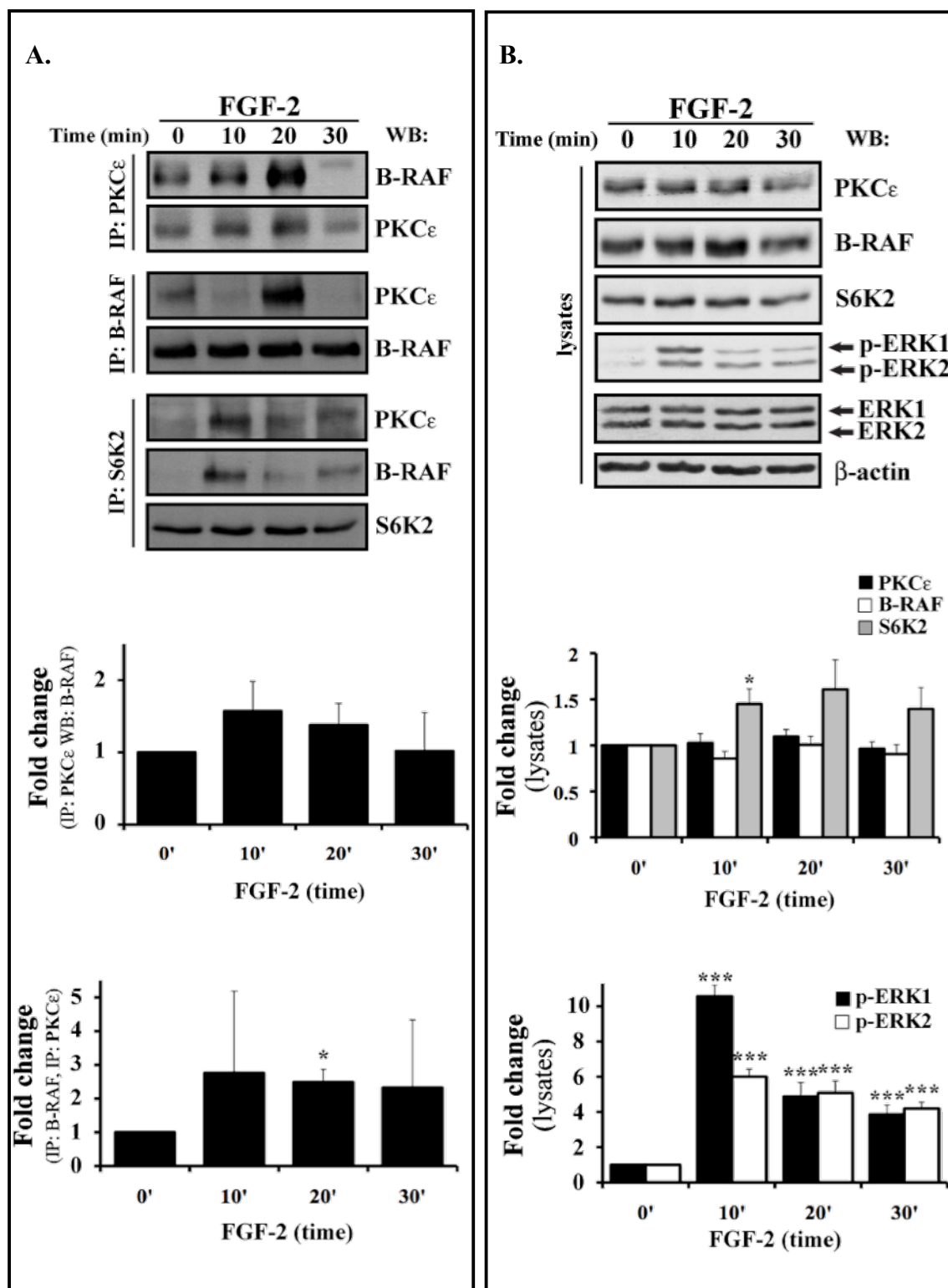


Figure 27. Interaction between B-RAF, PKC ϵ and S6K2 in HCC95 cells. HCC95 cells were serum-starved (0.5% FCS) overnight and then incubated in serum-free media for 1 hour. After stimulation with 10 ng/ml of FGF-2 at the indicated times, proteins were extracted. **A.** Cell lysates were subjected to immunoprecipitation with a PKC ϵ , B-RAF or S6K2 antibody. Immunoprecipitates were separated on a 7.5% SDS-PAGE gel and the proteins detected by Western blotting. Membranes were probed for PKC ϵ , B-RAF and S6K2. **B.** Total cell lysates were separated on a 7.5% SDS-PAGE and analysed by Western blot. Membranes were probed for PKC ϵ , B-RAF, S6K2, pERK1/2-Thr²⁰²/Tyr²⁰⁴ and total ERK1/2. β -actin was used as a loading control. **A.** and **B.** Representative Western blots and mean $\pm$ SEM of densitometric values from three independent experiments are shown in graphs. Values are expressed as fold change over untreated controls. Statistical analysis was performed with Student's t-test (* - $p < 0.05$; *** - $p < 0.005$ versus untreated controls). ' - minutes.

S6K2 appeared to bind to B-RAF and PKC ϵ after 10 minutes of FGF-2 stimulation, and this interaction was maintained with less strength for at least 30 minutes (**Fig. 27 A**, 5th and 6th row in the Western blot). As this immunoprecipitation was only performed once, this result is preliminary and needs to be further confirmed.

Whole cell extracts of samples used for co-immunoprecipitations were analysed in parallel by SDS-PAGE/Western blot. Total protein levels of B-RAF, PKC ϵ and S6K2 and the phosphorylation of ERK1/2 in response to FGF-2 were analysed (**Fig. 27 B**). ERK1/2 were phosphorylated in response to FGF-2 ($p < 0.005$) in a similar manner as previously observed (**Fig. 27 B**, 2nd graph). As seen in U2OS cells, there was a small increase of S6K2 protein levels in response to FGF-2. This increase was statistically significant at 10 minutes of FGF-2 stimulation ($p < 0.05$) (**Fig. 27 B**, grey bars in the 1st graph).

4.1.9. FGF-2 INDUCED THE UP-REGULATION OF ANTI-APOPTOTIC PROTEINS IN U2OS CELLS

FGF-2 has been shown to confer resistance to etoposide-mediated apoptosis in SCLC cells *via* a translational mechanism that results in the up-regulation of various anti-apoptotic proteins (Pardo *et al.*, 2002). FGF-2 has previously been shown to up-regulate MCL-1 in U2OS cells (Janet Lyons-Lewis and Michael Seckl, personal communication). Here we investigated which other anti-apoptotic proteins were up-regulated in response to FGF-2 in U2OS cells. For that purpose, cells were treated with FGF-2 for increasing periods of time, the proteins were extracted and, due to the small molecular sizes of the proteins of interest, separated on a 12.5% SDS-PAGE. The expression of BCL-2, BCL-X_L, XIAP and MCL-1 was subsequently monitored by Western blot with the appropriate antibodies.

The results obtained showed that, in U2OS cells, FGF-2 induced up-regulation of anti-apoptotic proteins (**Fig. 28 A**). Indeed, MCL-1 levels increased upon treatment with FGF-2 for 1 hour ($p < 0.05$) and reached a maximum (an average of 1.7-fold increase) after 2 hours of treatment ($p < 0.005$) (**Fig. 28 B**). Treatment with FGF-2 for two hours also induced the up-regulation of BCL-2 ($p < 0.005$) (**Fig. 28 A and C**), XIAP ($p < 0.05$) (**Fig. 28 A and D**) and BCL-X_L ($p < 0.05$) (**Fig. 28 A and E**).

The induction of BCL-2, BCL-X_L, XIAP and MCL-1 observed was modest (around 1.5-fold). It is possible that the protein extraction lysis buffer used here was

not ideal for the extraction of these proteins and could account for the small differences observed between untreated and FGF-treated samples. Schindler lysis buffer (cf. Material and methods) was developed to extract mainly JAKs and STATs, which are cytoplasmic proteins (Schindler *et al.*, 1992). BCL-2 family members are predominantly associated with the mitochondrial membrane (e.g. Breckenridge and Xue, 2004). Proteins associated with this organelle may be insufficiently extracted using this buffer. Extraction with a more stringent buffer that will disrupt membranes more efficiently may reveal greater differences in future experiments [e.g. radio immunoprecipitation assay (RIPA) buffer].

Whether the increase of BCL-2, BCL-X_L, XIAP and MCL-1 protein expression results from *de novo* protein synthesis, protein translation or increased protein stability remains to be established. The prominent roles that these proteins play in apoptosis raise the possibility that they may be involved in the FGF-2-mediated chemoresistance seen in the U2OS cells.

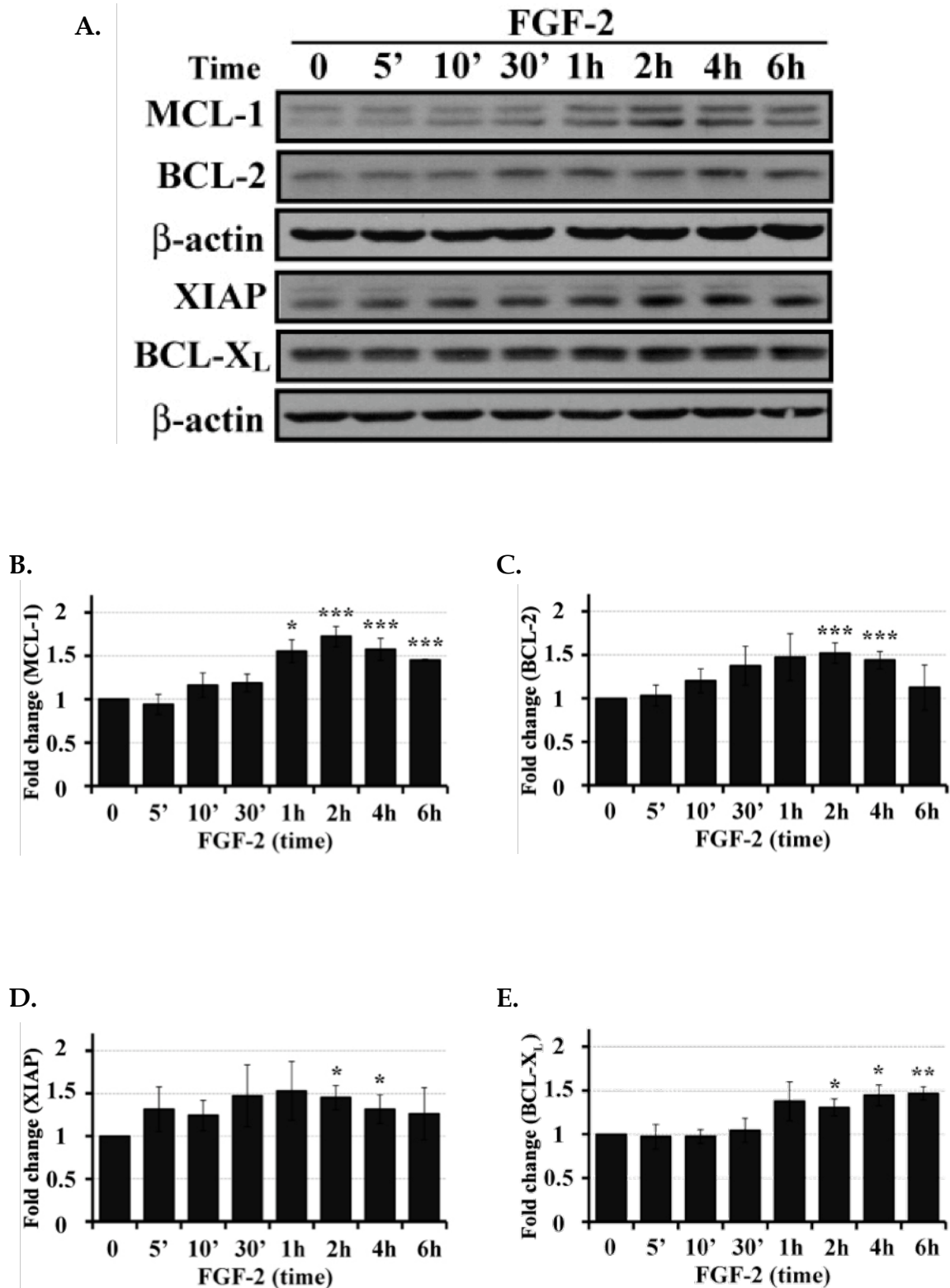


Figure 28. FGF-2 induced the up-regulation of anti-apoptotic proteins in U2OS cells. A. U2OS cells were serum-starved (0.5% FCS) overnight and then incubated in serum-free media for 1 hour. After stimulation with 10 ng/ml of FGF-2 at the indicated times, proteins were extracted and separated on a 12.5% SDS-PAGE gel. Western blotting analysis was performed using total cell lysates and antibodies against MCL-1, BCL-2, BCL-X_L and XIAP. β -actin was used as a loading control. B., C., D. and E. Mean $\pm$ SEM of densitometric values from at least three independent experiments are shown in graphs. Values are expressed as fold change over untreated controls. Statistical analysis was performed with Student's t-test (* - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.005$ versus untreated controls). ' - minutes, h - hours.

4.1.10. DISCUSSION

FGFs are known to provide prosurvival signals and to confer broad-spectrum resistance to chemotherapeutic drugs (Song *et al.*, 2000). Accordingly, aberrant FGF signalling can contribute to diverse pathologic conditions, including cancer (reviewed by Beenken and Mohammadi, 2009). There is increasing evidence in favour of an important role for FGF-2 in cancer biology and, indeed, FGF-2 has been implicated in driving chemoresistance in cancer (Song *et al.*, 2000; Pardo *et al.*, 2002; Pardo *et al.*, 2003; Vandermoere *et al.*, 2005). In addition, FGF-2 is known to play a role in cell proliferation and tumour progression in prostate and breast cancer (Giri *et al.*, 1999; Dickson *et al.*, 2000; Nurcombe *et al.*, 2000; Polnaszek *et al.*, 2003). This part of the study provides firm evidence for a role of FGF-2 in promoting chemoresistance in osteosarcoma cells by inhibiting apoptosis and stimulating cell proliferation.

FGF-2 was shown to bind preferentially to FGFRs 1 and 2 (variant IIIc) (Orniz *et al.*, 1996). Upon binding, the FGF receptors become activated and trigger well-documented early signalling events such as the activation of ERK1/2 protein kinases (e.g. Wang *et al.*, 2000; Pardo *et al.*, 2001; Kuhn *et al.*, 2006). We started by investigating the activation of ERK1/2 downstream of FGF-2 in osteosarcoma (U2OS) and NSCLC (HCC95 and HCC78) cell lines. We observed that FGF-2 significantly activated ERK1/2 in the osteosarcoma U2OS cell line and the NSCLC cell line HCC95 (**Fig. 13** and **14**). In contrast, the NSCLC cell line HCC78 was defective in the response to FGF-2 as phosphorylation of ERK1/2 was only very weakly increased in response to FGF-2 (**Fig. 15**). In fact, in HCC78 cells FGF-2 increased phosphorylation of these kinases only by 1.3-fold and, in the majority of experiments conducted, led to a reduction in the basal levels of ERK1/2 phosphorylation after 20 minutes of treatment (which due to some experimental variation that we still do not understand did not reach statistical significance). The decrease in ERK1/2 phosphorylation levels upon FGF-2 treatments observed in HCC95 (**Fig. 14**) and HCC78 (**Fig. 15**) were, presumably, due to a negative feedback control mechanism. For example, FGF was shown to induce the expression of SPRY family members (e.g. Ozaki *et al.*, 2001) that attenuate FGF signalling. In general, SPRYs inhibit the recruitment of SOS by either binding GRB2, or SOS itself, thereby

blocking MEK/ERK signalling and FGF responses (Hanafusa *et al.*, 2002; Ozaki *et al.*, 2005).

Using RT-PCR, we successfully detected FGFR1 in all the three cell lines studied here (**Fig. 16**). FGFR2IIIc, however, was amplified in U2OS and HCC95 cells but it was not detected in HCC78 cells. The modest phosphorylation of ERK1/2 in response to FGF-2 in HCC78 cells may be associated with the lack of expression of FGFR2IIIc. The observation that the constitutive phosphorylation of ERK1/2 was modestly, but significantly, increased by treatment with FGF-2 (and subsequently downregulated) suggests, however, that HCC78 cells still respond to FGF-2. It suggests that FGF-2 probably acts through receptor complex composed of FGFR1 homodimers in HCC78 cells whereas in U2OS and HCC95 cell lines there could be contributions through both FGFR1 and FGFR2. It is also possible that FGF-2 could act through another FGFR family member as FGF-2 can also signal through other receptors such as FGFR4, although it binds them with lower affinity than it binds to FGFR1 and FGFR2IIIc (Vainikka *et al.*, 1992; Ornitz *et al.*, 1996). It is also possible that ERK1/2, despite being constitutively active, can still be further phosphorylated in response to other growth factors, such as EGF. This was, however, beyond the scope of this research project and the experiment was, therefore, not conducted at the time.

The detection of FGFR1 and FGFR2IIIc by RT-PCR suggest that these receptors may be expressed in the cells and used by FGF-2. Western blot analysis would have allowed us to ascertain if the receptors were expressed at the protein level in these cell lines. In addition, flow cytometric analysis would have allowed us to determine if the receptor chains were present on the cell surface and would have given an indication of the quantity of receptor molecules available for FGF-2 signalling. Unfortunately, no suitable antibodies were available to us.

FGF-2 is known to promote proliferation of many cell types (Bikfalvi *et al.*, 1997). In this work we observed that FGF-2 induced proliferation of U2OS cells but not of the NSCLC cell lines HCC95 and HCC78 (**Fig. 17**). The inability of FGF-2 to promote proliferation of these cells suggested that FGF-2 might be unable to activate some of the pathway(s) required for FGF-2-mediated proliferation. One such pathway could be the mTOR/S6K pathway. S6K was suggested to be a major regulator of the FGF-2 growth-promoting effects, as its inhibition with rapamycin (an mTOR inhibitor) blocked FGF-2-induced proliferation of SCLC cells (Pardo *et al.*, 2001). Alternatively, FGF-2 may promote proliferation of the NSCLC cells HCC95 and HCC78 under conditions that were not tested here (such as alternative starvation settings or incubation with FGF-2 for periods longer than 96 hours).

FGF-2 has been shown to mediate chemoresistance in many cell types including HUVECs, prostate, breast and lung cancer cells (Song *et al.*, 2000; Pardo *et al.*, 2002; Alavi *et al.*, 2003; Vandermoere *et al.*, 2005). Seckl and colleagues have demonstrated that FGF-2 could prevent etoposide killing in a SCLC cell line (Pardo *et al.*, 2002). The same authors revealed key early signalling events triggered by FGF-2 that impinged on the apoptotic machinery, namely the activation of protein kinases ERK1/2, the formation of a multi-protein complex consisting of B-RAF, PKC ϵ and S6K2 (which was shown using RNAi and co-IP methodologies) and the up-regulation of various anti-apoptotic proteins (Pardo *et al.*, 2002; Pardo *et al.*, 2003; Pardo *et al.*, 2006).

Here we have investigated if FGF-2 also protected the NSCLC HCC95 and HCC78 cell lines and the osteosarcoma U2OS cell line from chemotherapeutic drug-induced apoptosis, in order to extend these observations to other cell types. In U2OS cells we observed that FGF-2 provided protection from cisplatin killing. This was demonstrated using the cell viability assay WST-1 (**Fig. 20 A**), and further confirmed by analysing cell cycle profiles (**Fig. 21 A and B**) and PARP cleavage (**Fig. 21 C and D**). FGF-2 reduced the amount of cells in the sub-G₁ peak following challenge with cisplatin and it prevented cisplatin-induced cleavage of caspase substrates such as PARP and lamin B (**Fig. 21 A, B, C, D** and data not shown). These observations suggested that FGF-2 inhibited cisplatin-mediated apoptosis rather than just promoting proliferation. Hence, in U2OS cells, FGF-2 not only induced proliferation but it also blocked apoptosis. These findings further highlighted the well-recognised pleiotropic nature of FGF-2 (Bikfalvi *et al.*, 1997). Furthermore, FGF-2-rescued cells were able to survive for extended periods once the drug was withdrawn (data not shown) as observed in the SCLC cell line H-510 (Pardo *et al.*, 2002). U2OS cells that survived cisplatin due to FGF-2 had a different morphology than unchallenged cells (they looked bigger and had a rounder shape). The reason for this altered morphology is yet unknown. Collectively, these data suggest that FGF-2 plays an important role in osteosarcoma tumour progression as it promotes cell survival in the presence of cytotoxic agents and allows the arising of new cell populations.

FGF-2 failed to induce both proliferation of the NSCLC HCC95 and HCC78 cell lines studied here and rescue these cells from cisplatin-mediated apoptosis (**Fig. 20 B and C, Fig. 22 and Fig. 23**). FGF-2 rescue experiments have been extensively repeated but, under the conditions used, no chemoprotection was ever observed in these cells. These findings do not exclude the possibility that FGF-2 can provide protection of other NSCLC cell lines. It is possible that had other cell lines been chosen we might

have observed chemoprotection. Moreover, it is possible that in HCC95 and HCC78 cells FGF-2 may prevent cell death induced by other chemotherapeutic agents.

The inability of FGF-2 to protect the NSCLC HCC78 cell line might be associated with the modest ERK1/2 activation observed (**Fig. 15**). Indeed, MEK/ERK signalling has been shown to have a key role in cell growth and drug resistance (reviewed by McCubrey *et al.*, 2007). Importantly, the activation of this pathway was also shown to be essential for the FGF-2-mediated chemoresistance observed in SCLC cells (Pardo *et al.*, 2002). The same authors subsequently showed in a series of elegant experiments that MEK/ERK was uncoupled from FGF-2 signalling in the SCLC H-69 cell line, which was refractory to the protective effect of FGF-2. In this cell line the lack of ERK activation was not due a dysfunctional MEK/ERK pathway, as it could be activated by other growth factors. The introduction of an activated version of MEK into H-69 cells induced phosphorylation of ERK1/2 and reverted the phenotype by rescuing these cells from etoposide-induced killing (Pardo *et al.*, 2002). The inability of FGF-2 to protect the NSCLC HCC95 cell line remains to be explained. In this cell line, as in H-510 (Pardo *et al.*, 2002) and U2OS cells (**Fig. 13**), FGF-2 induced the phosphorylation of ERK1/2 (**Fig. 14**). Therefore, the inability of FGF-2 to protect HCC95 cannot be attributed to a failure in MEK/ERK activation. The observed FGF-2-mediated activation of ERK1/2 in these cells was more transient than the activation observed in U2OS (**Fig. 13**). Strength and duration of ERK signalling have been shown to have an impact on the response and to influence biological outcome (Murphy *et al.*, 2004). Variations of the intensity and duration of the ERK1/2 activation signal in response to FGF-2 may have been a significant factor for the lack of protection of HCC95 by FGF-2 from apoptosis. In addition, it is conceivable that the defect lies either with signalling downstream of ERK1/2 phosphorylation, or in a parallel signalling pathway that may cross-talk with the main chemoresistance pathway identified thus far. Preliminary data suggested that FGF-2 was able to up-regulate some anti-apoptotic BCL-2 family members (data not shown). Accordingly, the reason why FGF-2 was not able to protect HCC95 cells from cisplatin-mediated cell killing remains unknown.

Having observed that FGF-2 rescued the osteosarcoma cell line U2OS from cisplatin-induced apoptosis but not the two elected NSCLC cell lines, we decided to focus our attention on the U2OS cells. We were particularly interested in the early signalling events that occur within the first four hours of treatments, which corresponded to the time of pre-incubation with FGF-2. This was because it was established previously in our laboratory that it is during this period of time that key anti-apoptotic molecules necessary to provide chemoprotection are up-regulated and

that this incubation time was sufficient to rescue cells from the noxious effects of drugs..

Many processes that lead to cell growth and drug resistance were shown to be dependent of MEK/ERK activation (reviewed by McCubrey *et al.*, 2007). Here we investigated if in U2OS cells the activation of the MEK/ERK pathway was also required for the FGF-2 chemoprotection from cisplatin. In fact, inhibition of MEK1/2, the upstream activator of ERK1/2 (Roux and Blenis, 2004), with the specific inhibitor PD0325901 (Sebolt-Leopold *et al.*, 2004) abolished the FGF-2 rescue (**Fig. 24**). This was demonstrated by analysing PARP cleavage (by Western blot) (**Fig. 24 C and D**) and loss of DNA (by flow cytometry) (**Fig. 24 E and F**). These data suggested that, similar to what was previously observed in SCLC cells (Pardo *et al.*, 2002), the activation of the MEK/ERK signalling pathway was essential for the FGF-2-induced rescue from cisplatin killing in U2OS cells. Additionally, in the absence of MEK/ERK activity, FGF-2 was not able to reduce the basal cell death seen in the cell cultures (**Fig. 24 C, D, E and F**). This observation emphasises the pivotal role of these molecules in protecting cells from various apoptotic stimuli (Bonni *et al.*, 1999; Liu *et al.*, 1999; Deng *et al.*, 2000; Jost *et al.*, 2001; Xu *et al.*, 2008).

Another early signalling event that was shown to be required for the FGF-2-mediated protection of SCLC cells from etoposide killing was the formation of a multi-protein complex consisting of B-RAF, PKC ϵ and S6K2 (Pardo *et al.*, 2006). Using co-immunoprecipitation techniques we observed that in U2OS cells FGF-2 also induced interactions between these three proteins (**Fig. 26**). The interaction between B-RAF and PKC ϵ was detected between 10 and 30 minutes upon FGF-2 stimulation (**Fig. 26 A**), which also induced the interaction of S6K2 with B-RAF and the interaction of S6K2 with PKC ϵ . These interactions appeared to be more transient than that observed between B-RAF and PKC ϵ . These two proteins also co-immunoprecipitated with S6K2 after 10 minutes of FGF-2 treatment but the Western blot signal representing the interaction was greatly reduced after 20 minutes of treatment (**Fig. 26 A**). Depending on the antibody used for co-immunoprecipitation (B-RAF or PKC ϵ), we observed some variation between experiments with regards to the kinetics of the interactions between B-RAF and PKC ϵ . It is possible that this interaction is very dynamic, involving transient interactions between these molecules, that there are multiple stoichiometric possibilities, or that this is due to an experimental artefact caused by instability of the complex under our experimental conditions. Nevertheless, and regardless of the precise kinetics, the results obtained here demonstrated that these molecules rapidly interacted with each other upon FGF-2 stimulation. Silencing B-RAF, PKC ϵ and S6K2

individually using RNAi will elucidate if, in U2OS, these molecules interact in a tripartite multi-protein complex as previously observed in SCLC cells (Pardo *et al.*, 2006). The requirement for these interactions for chemoresistance and the exact function of the complex(es) in U2OS cells is still unknown.

It was observed in SCLC cell lines that B-RAF, PKC ϵ and S6K2 were activated/phosphorylated in response to FGF-2 (Pardo *et al.*, 2001; Pardo *et al.*, 2006). Activation of B-RAF, PKC ϵ and S6K2 in response to FGF-2 will be investigated in the future. The requirement for their activation for FGF-2-mediated chemoprotection will be assessed using specific inhibitors and, as previously mentioned, siRNA molecules. It is possible that their interaction is a requirement for the downstream activation of all or some of these three molecules.

One hypothesis is that PKC ϵ is required to phosphorylate S6K2. Indeed, it was shown in another context that S6K2 was phosphorylated by several PKC isoforms, including PKC ϵ (Valovka *et al.*, 2003) and *in vitro* kinase assays showed that PKC ϵ can directly associate and phosphorylate S6K2 (Pardo *et al.*, 2006). It is also possible that PKC ϵ activity itself may be required for B-RAF activation, as it was shown that it was required for PKB-induced activity of C-RAF through a RAS-independent mechanism (Majewski *et al.*, 1999). In addition, PKC ϵ was required for ERK1/2 phosphorylation and the expression of PKC ϵ (but not of other PKC family members) correlated with the ability of FGF-2 to induce MEK/ERK activation and up-regulation of anti-apoptotic proteins in SCLC cells (Pardo *et al.*, 2006). One can thus envisage a chain of events where the activation of one kinase is required for the activation of the other kinases.

Interestingly, we observed an increase of S6K2 protein in response to FGF-2 in whole cell lysates (**Fig. 26 B**). Ten minutes of stimulation were sufficient to observe this up-regulation. In such a short period of time, it is unlikely that this increase is due to *de novo* protein synthesis. It may be that S6K2 degradation rate was decreased in response to FGF-2. Ubiquitination was shown to be important for the regulation of S6K protein levels (Wang *et al.*, 2008) and it is possible that this process may be a target of FGF-2 signalling. This obvious possibility will be the subject of future studies in our laboratory.

A transient interaction between B-RAF and PKC ϵ upon FGF-2 stimulation was also detected in the HCC95 cell line (**Fig. 27 A**). Although the intensity and kinetics of this interaction varied amongst different experiments it was detected in all experiments performed. FGF-2 also induced the interaction of S6K2 with B-RAF and PKC ϵ (**Fig. 27 A**). This interesting observation requires further confirmation as, due to technical difficulties and time restraints, it was only carried out once. Although FGF-2 chemoprotection mechanism in the HCC95 cell line needs a more thorough

investigation our data suggests that the inability of FGF-2 to protect these cells against cisplatin is likely related to the absence of particular signalling molecules other than MEK/ERK, B-RAF and PKC ϵ . Alternatively, it may be due to altered kinetics and/or signal intensity. It is now widely accepted that proteins rarely act as individual entities and that the cellular context is pivotal to a particular cellular response. It is our view that FGF-2-mediated chemoprotection is no exception to this rule. Other pathways that may also be involved and required for the FGF-2 protection against apoptosis induced by drugs such as cisplatin may be impaired in this cell line. For example, protection from apoptosis through inactivation of BAD (by phosphorylation) was seen in leukemia (Zhao *et al.*, 2004) and in response to growth factors such as EGF (She *et al.*, 2005; Sastry *et al.*, 2006) and FGF-10 (Smaragda Angelidou and Michael Seckl, personal communication). Another way by which FGF-2/FGFR could be regulating cell survival is through direct regulation of transcription. Interestingly, upon ligand binding FGF-2 is internalised and it was shown that it is transported to the nucleus together with FGFR1, thereby regulating the proliferation of astrocytes and glioma cells (Stachowiak *et al.*, 1996; Joy *et al.*, 1997; Stachowiak *et al.*, 1997). Indeed, nuclear FGFR1 induce the expression of genes, which encode proteins required for cell division and proliferation, such as c-JUN and cyclin D (Reilly and Maher, 2001).

Growth factors promote cell survival by modulating various components of the apoptotic machinery and they can regulate these components by regulating protein expression levels and/or their activation status (phosphorylation or dephosphorylation). For example, increased expression of the anti-apoptotic protein BCL-X_L was observed downstream of EGF (Jost *et al.*, 2001) and FGF-2 (Pardo *et al.*, 2003) and it was implicated in promoting cell survival. Another example is the phosphorylation of BAD in response to EGF and FGF-10 (She *et al.*, 2005; Sastry *et al.*, 2006; Smaragda Angelidou and Michael Seckl, personal communication). In our work, we observed that in U2OS cells FGF-2 induced the up-regulation of several anti-apoptotic proteins such as MCL-1, BCL-2, BCL-X_L and XIAP (**Fig. 28**). These proteins are likely to be involved in the chemoresistance provided by FGF-2. To verify the direct cause-effect of this up-regulation on FGF-2-induced chemoresistance, their up-regulation will be inhibited using RNAi. It will have to be taken into consideration that the use of siRNA against those proteins will also knockdown their endogenous levels and will probably contribute to a reduction in cell viability. The inclusion of appropriate controls will allow to take this into consideration and to normalise the data. The use of inhibitors of protein synthesis such as cycloheximide is not ideal, as they would also inhibit the synthesis of many other proteins. Proteasome inhibitors

(e.g. MG132 and lactacystein) could be used to artificially increase these anti-apoptotic proteins. The cells could then be challenged with cisplatin. If these anti-apoptotic molecules are stabilised (by blocking their degradation), and assuming that they are key inhibitors of cisplatin-induced death, then the cells should survive even in the absence of FGF-2. In any case, up-regulation of these proteins may not be due to *de novo* protein synthesis. The use of proteasome inhibitors will help to establish this assumption.

The up-regulation of anti-apoptotic proteins required in FGF-2-mediated chemoprotection of the SCLC H-510 cell line was found to occur *via* increased protein translation (Pardo *et al.*, 2002; Pardo *et al.*, 2003). Thus, another possibility is that in U2OS cells the increase in protein expression could also result from an increase in translation. This could be examined by looking at polysome fractions of cells treated with cisplatin in the presence or absence of FGF-2.

Collectively, the results from this part of the study showed that FGF-2 mediated chemoresistance in the osteosarcoma cell line U2OS, but not in the two NSCLC cell lines, HCC95 and HCC78. The failure of FGF-2 to protect these NSCLC cell lines remains to be understood. Presumably, the lack of robust ERK1/2 activation in HCC78 cells impaired FGF-2-mediated chemoprotection, as this pathway has been previously shown to require MEK/ERK activity. The inability of FGF-2 to protect HCC95 cannot, however, be attributed to this. Thus, it seems likely that other components of the rescue pathway, downstream of, or in parallel to, MEK/ERK signalling, may be missing. FGF-2 rescued U2OS cells from cisplatin-induced cell death in a MEK/ERK-dependent fashion. Furthermore, in this cell line, FGF-2 induced the interaction of proteins such as B-RAF, PKC ϵ and S6K2, and the up-regulation of anti-apoptotic proteins thought to be required to protect cells from cisplatin-induced apoptosis. Although some molecular aspects of the anti-apoptotic mechanism involved here need to be clarified, U2OS chemoprotection by FGF-2 appears to be mediated through a mechanism similar to that previously observed in SCLC cells (Pardo *et al.*, 2003). Common players in chemoresistance mechanisms between tumour types make these attractive molecular targets and potential candidates for the development of novel therapies.

Activation of JAKs and/or STATs can occur downstream of FGF-2 (Ebong *et al.*, 2004; Yang *et al.*, 2009) and these molecules have been implicated in tumourigenesis and chemoresistance (reviewed by Verma *et al.*, 2003; Yu and Jove, 2004). We next investigated, therefore, if JAKs and/or STATs also played a role in FGF-2-mediated chemoresistance pathway(s)

4.2.

THE ROLE OF JAKs AND STATs IN FGF-2-MEDIATED CHEMORESISTANCE

4.2.1. INTRODUCTION

JAKs and STATs are involved in regulating the expression of many genes that in normal cells control fundamental biological processes (Horvarth, 2000). Deregulation of JAK/STAT-mediated signalling can lead to cell transformation and oncogenesis and, indeed, aberrant activation of JAKs and/or STATs has been observed in many human cancer cells (Bowman *et al.*, 2000). JAKs have been shown to modulate the activation of signalling pathways implicated in malignant transformation and cell growth and were also shown to modulate apoptosis *via* regulation of anti-apoptotic proteins (reviewed by Verma *et al.*, 2003). Constitutive activation of STAT1, STAT3 and STAT5 has been observed in a wide variety of human cancer cell lines and primary tumours (reviewed by Yu and Jove, 2004). In particular, recent studies have suggested that STAT3 oncogenic pathways are not only involved in cellular transformation but are also associated with intrinsic drug resistance (Barre *et al.*, 2007; Grisko *et al.*, 2006; Ikuta *et al.*, 2005). Although a compelling body of evidence suggests that STAT1 has properties of a tumour-suppressor protein (Dunn *et al.*, 2004), recent findings have shown that STAT1 can also play a prominent role in the development of tumours and in mediating drug resistance (Kovacic *et al.*, 2006; Patterson *et al.*, 2006). STAT5A and STAT5B are also found constitutively activated in many cancers, particularly in leukaemias (Lewis and Ward, 2008).

Some reports have suggested that activation of JAK/STAT signalling pathways can occur downstream of FGF-2 but not much work has been done in this area. It was shown that FGF-2 can activate STAT1, STAT3 and STAT5 in murine lens cells and BMVECs and, in addition, inhibition of JAK activity was demonstrated to impair FGF-2-induced proliferation (Ebong *et al.*, 2004; Yang *et al.*, 2009). We hypothesised, therefore, that JAKs and/or STATs may play a role in FGF-2-mediated chemoresistance pathway(s).

In this part of the work, **we aimed to establish a role for JAK and/or STAT proteins in FGF-2-mediated chemoresistance pathways**. We have investigated the requirement for these molecules in this pathway by assessing the ability of FGF-2 to protect U2OS cells from cisplatin-mediated cell death when the levels of these proteins were reduced by RNAi or their activity blocked by chemical inhibition. FGF-2 rescue

experiments (**Section 4.1.5**) were performed in cells where JAK1, JAK2, TYK2, STAT1, STAT3 or STAT5 were independently silenced by RNAi using siRNA oligonucleotides molecules. Modulation of JAKs and STATs by FGF-2 was characterised in the U2OS cell line by performing extensive dose- and time-response experiments. Subsequently, having identified a role for the JAKs but seemingly not for the STATs, we investigated the requirement for the JAKs in FGF-2-mediated chemoresistance.

4.2.2. JAK1, JAK2 AND TYK2 BUT NOT STAT1, STAT3 AND STAT5 WERE REQUIRED FOR FGF-2-MEDIATED CHEMORESISTANCE IN U2OS CELLS

In the osteosarcoma cell line U2OS, FGF-2 prevented cisplatin killing by blocking death-inducing signalling pathways activated by this drug (**Section 4.1.5**). We asked, therefore, whether JAKs and/or STATs were involved in this process. To address this, the FGF-2 rescue experiment (**Section 4.1.5**) was performed in cells with reduced levels of JAK1, JAK2, TYK2, STAT1, STAT3 or STAT5A/B.

4.2.2.1. KNOCKDOWN OF JAK1, JAK2, TYK2, STAT1, STAT3 AND STAT5 IN U2OS CELLS

Knockdown of JAK1, JAK2, TYK2, STAT1, STAT3 and STAT5A/B was achieved by independently transfecting cells with siRNA oligonucleotide pools against each one of these molecules. U2OS cells were transfected with siSMART pools (provided by Dharmacon), which consist of four different oligonucleotides each targeting different regions of the same mRNA. Although siRNA oligonucleotides are designed to specifically silence specific targets, there is always the possibility that any given siRNA oligonucleotide may silence molecules other than the specific target ("off-target" effect). Exclusion of obvious "off-target" effects has been previously extensively done by members of the Costa-Pereira group. Validation of each one of the siRNA pools is routinely done in our laboratory by deconvoluting each siSMART pool into its four constituents, followed by the analysis of the phenotypes of each and by utilising siRNAs designed using a different algorithm and technology (OnTargetPlus, Dharmacon). If two out of the four individual oligonucleotides give the same

phenotype as the original siSMARTpool, then the siRNA pool is validated (Echeverri and Perrimon, 2006).

The levels of knockdown and the specificity of the siRNA pools were assessed by Western blot and RT-PCR (**Fig. 29**). Whole cell extracts from cells transfected with siRNA were run on 7.5% SDS-PAGE gels (**Fig. 29 A and E**). **Figure 29 A** shows the expression of JAK1, JAK2 and TYK2 in U2OS cells upon transfection with siRNA against each one of them. Untreated cells and cells transfected with a non-specific siRNA control (NS) were included in order to establish the basal levels of these proteins (untransfected control) and to determine the impact of transfecting cells with double-strand RNA (NS control). Because JAKs have similar molecular weights (JAK1 is 130 kDa, JAK2 is 125 kDa and TYK2 is 140 kDa), two Western blot membranes were used to ensure there was no cross-reactivity of antibodies and to avoid probing a membrane stripped twice. The Western blot membranes were probed for with antibodies against JAK1, JAK2 and TYK2. Both membranes were probed for β -actin, which was used as a loading control. siRNA against JAK1 significantly reduced the levels of JAK1 protein ($p < 0.005$) without affecting the other two JAK family members, JAK2 and TYK2 (**Fig. 29 B**). siRNA against JAK2 also caused a significant reduction of JAK2 protein levels ($p < 0.005$) and siRNA against TYK2 significantly reduced TYK2 proteins ($p < 0.005$) without affecting the other JAKs (**Fig. 29 C and D**). Because STAT1, STAT3 and STAT5 are highly homologous and have similar molecular weights (STAT1 is 91 kDa, STAT3 is 86 kDa, STAT5A is 92 kDa and STAT5B is 94 kDa), three Western blot membranes were used. The Western blot membranes were probed for with antibodies against STAT1, STAT3, STAT5A/B and β -actin, the latter used as a loading control. siRNA against STAT1, STAT3 and STAT5A/B significantly reduced the levels of STAT1 ($p < 0.005$), STAT3 ($p < 0.005$) and STAT5A and B ($p < 0.005$) proteins, respectively, without affecting other STAT family member probed here (**Fig. 29 F, G, H and I**).

Knockdown of some targets (JAK1, JAK2, STAT1 and STAT3) was also confirmed by RT-PCR. Total RNA was extracted from cells, reverse-transcribed into cDNA and fragments of JAK1, JAK2, STAT1 and STAT3 amplified by PCR. Untransfected cells and cells transfected with a non-specific siRNA were used as controls. The fragments were successfully amplified in untransfected cells and in cells transfected with the non-specific siRNA but not in cells transfected with a specific siRNA pool (**Fig. 29 J**).

Protein knockdown levels were monitored concomitantly by Western blot in all experiments involving siRNA.

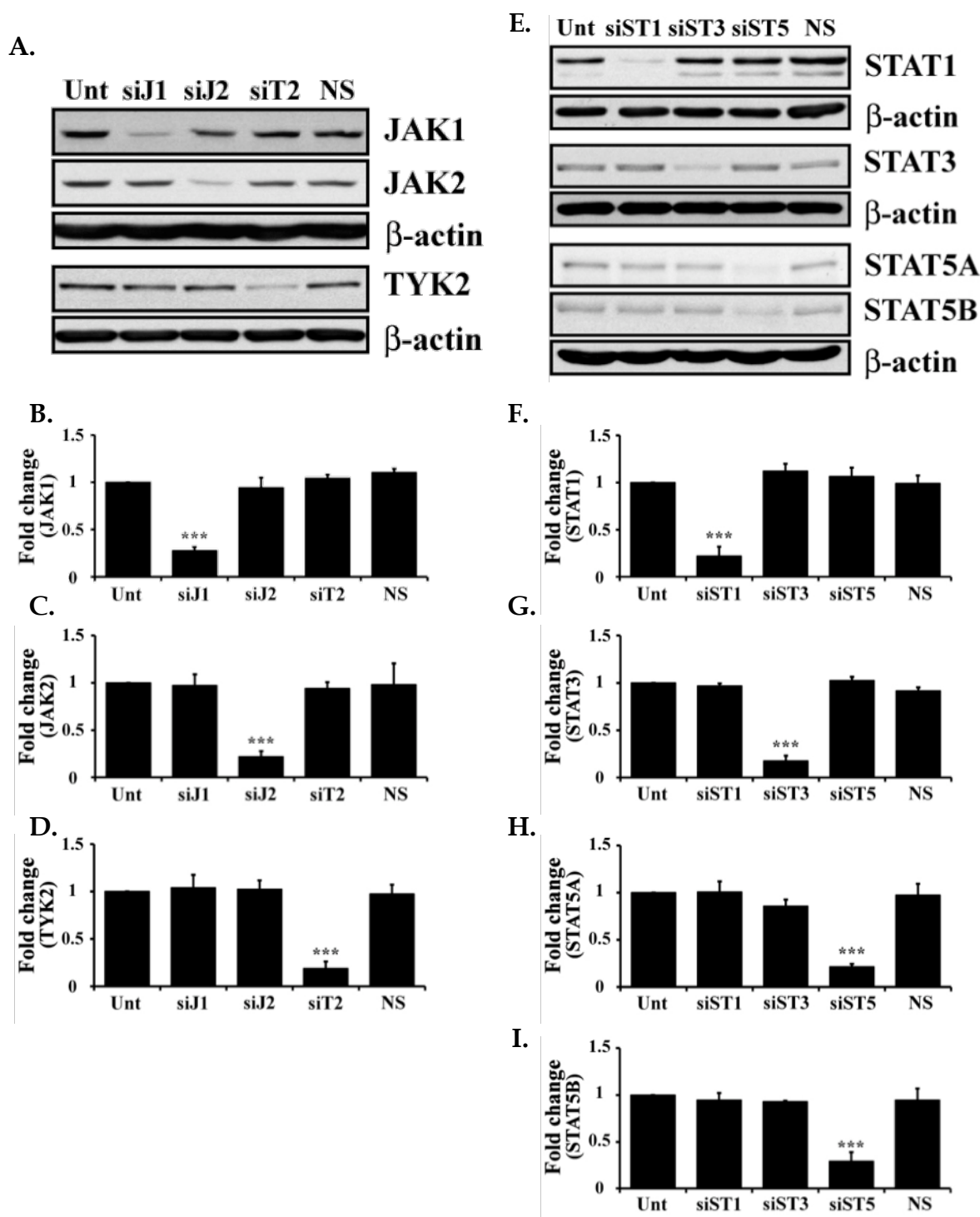


Figure 29. siRNA oligonucleotides can specifically knockdown JAK1, JAK2, TYK2, STAT1, STAT3 and STAT5A/B in U2OS cells. (Continued on page 138). U2OS cells were transfected with 75 nM siRNA using DharmaFECT II. Untransfected cells (Unt), or cells transfected with a non-specific siRNA (NS) were used as controls. **A.** and **E.** Forty-eight hours after transfection, proteins were extracted and separated on a 7.5% SDS-PAGE gel. Total cell lysates were Western blotted as indicated. **A.** Cells were transfected with siRNA against JAK1, JAK2 or TYK2. The Western blot membranes were probed for JAK1, JAK2 and TYK2. β-actin was also probed and used as a loading control. **E.** Cells were transfected with siRNA against STAT1, STAT3 or STAT5A/B. The Western blot membranes were probed for STAT1, STAT3, STAT5A and STAT5B. β-actin was also probed and used as a loading control. **B., C., D., F., G., H.** and **I.** Mean $\pm$ SEM of densitometric values of at least three independent experiments are shown in the graphs. Values are expressed as fold change relative to untransfected controls. Statistical analysis was performed with Student's t-test (***) - $p < 0.005$. **J.** RNA was isolated 48 hours post-transfection and used as a template for the amplification of fragments of JAK1, JAK2, STAT1 and STAT3. GAPDH was amplified for the same samples and used as a loading control. Unt - untransfected, NS - non-specific siRNA, J1 - JAK1, J2 - JAK2, T2 - TYK2, ST1 - STAT1, ST3 - STAT3, ST5 - STAT5A/B.

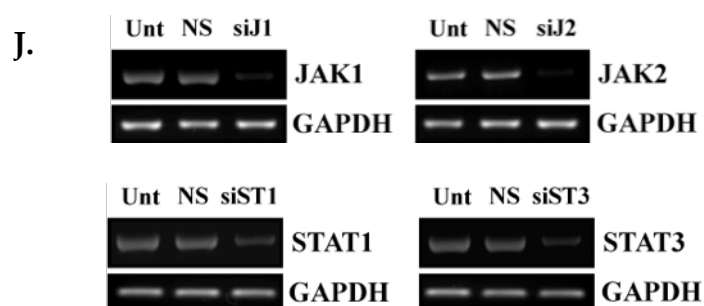


Figure 29. siRNA oligonucleotides can specifically knockdown JAK1, JAK2, TYK2, STAT1, STAT3 and STAT5A/B in U2OS cells. (Continued from page 137).

4.2.2.2. JAK1, JAK2 AND TYK2 WERE REQUIRED FOR FGF-2-MEDIATED CHEMORESISTANCE

JAKs have been shown to play a role in oncogenesis and to modulate apoptosis *via* regulation of anti-apoptotic proteins (reviewed by Verma *et al.*, 2003). Here we investigated if the JAKs were required for the FGF-2-mediated chemoresistance mechanism that was identified in U2OS cells (**Section 4.1.5**). We used RNAi to reduce protein levels of JAK1, JAK2 and TYK2, which enabled us to investigate the impact of their knockdown on FGF-2-mediated rescue from cisplatin. After verifying that JAK1, JAK2 and TYK2 could be efficiently silenced by RNAi (**Fig. 29**), FGF-2 rescue experiments were performed on these cells.

Following two days of siRNA transfection, cells were trypsinised, counted, and re-plated in 6-well plates (2×10^5 cells per well). This was important to ensure that the rescue experiment was initiated with an equal number of cells in every sample, as transfection with different siRNA pools induced different levels of cell death. Twenty-four hours later, period after which all cells should have adhered to the plates, cells were incubated in serum-free media overnight and treated as previously described: they were pre-incubated for 4 hours with FGF-2 and then treated with cisplatin. The

rescue experiment was also performed in untransfected cells and cells transfected with a non-specific siRNA (NS), both used as controls, as described before. Protein extractions were performed in parallel with all experiments involving siRNA to monitor the knockdown levels of each protein targeted. Protein extractions were performed at the moment of FGF-2 addition (72 hours after RNAi), as we were particularly interested on the impact of the absence of JAKs on early signalling cascades induced by FGF-2 occurring. One example of knockdown levels typically observed is shown in **Figure 29 A**.

Loss of DNA content was measured by flow cytometry. Cell cycle profiles were analysed after 18 hours of cisplatin treatment (**Fig. 30**). In untransfected cells pre-incubation with FGF-2 significantly blocked cisplatin-mediated cell death as previously observed (**Fig. 21, Section 4.1.5.1**). This was translated in a reduction of the sub-G₁ population of cells previously incubated with FGF-2 when compared to cells treated with cisplatin only (**Fig. 30 A**, black coloured cell cycle profiles, compare 3rd *versus* 4th histograms in the 1st column). In these cells FGF-2 also significantly reduced the basal levels of cell death ($p < 0.005$) (**Fig. 30 B**, 1st *versus* 2nd bar in the 1st graph) and treatment with cisplatin induced a significant increase of cell death ($p < 0.005$) (**Fig. 30 B**, 1st *versus* 3rd bar in the 1st graph). Pre-incubation with FGF-2 substantially blocked cisplatin-induced killing ($p < 0.05$) (**Fig. 30 B**, 3rd *versus* 4th bar in the 1st graph). Similar results were obtained in cells transfected with a non-specific siRNA (**Fig. 30 A**, brown coloured cell cycle profiles and **Fig. 30 B**, last graph).

In contrast, cells with reduced levels of JAK1 (**Fig. 30 A**, light pink coloured cell cycle profiles), JAK2 (**Fig. 30 A**, dark pink coloured cell cycle profiles), or TYK2 (**Fig. 30 A**, purple coloured cell cycle profiles) were not protected by FGF-2 from cisplatin-mediated cell death (**Fig. 30 A**, compare 3rd *versus* 4th histograms in each of the 2nd-4th columns). In these cells, the sub-G₁ population of cells treated with FGF-2 and cisplatin was not significantly different from cells treated with cisplatin only (**Fig. 30 B**, 3rd *versus* 4th bar in 2nd-4th graphs). Interestingly, in cells with decreased levels of JAK1, JAK2 and TYK2, FGF-2 significantly reduced the basal levels of cell death ($p < 0.005$, $p < 0.005$ and $p < 0.05$, respectively) (**Fig. 30 B**, 1st *versus* 2nd bar in 2nd-4th graphs) but not the cisplatin-induced cell death (**Fig. 30 B**, 3rd *versus* 4th bar in 2nd-4th graphs). This observation can have more than one explanation. It may be that JAKs are specifically required for protection from cisplatin killing, but not essential for protection from death in general. In this particular case basal cell death was probably caused by starvation and/or other experiment-related factors. Alternatively, it may be that when only a small percentage of cells died, FGF-2 could still mediate survival because the

knockdown of a particular JAK may be compensated by the presence of another JAK. In addition, although siRNA significantly reduced protein levels, the knockdown efficiency was never 100% (cf. **Fig. 29**). The presence of residual JAK molecules may be enough for FGF-2 to block basal cell death. These data suggested that JAK1, JAK2 and TYK2 were integral components of the mechanism by which FGF-2 protected cells from cisplatin-induced apoptosis.

The experiment illustrated in **Figure 30 A** is representative of several independent experiments. In this particular experiment there were differences between untransfected cells and cells transfected with siRNA molecules with regards to the percentage of apoptosis following an overnight treatment with cisplatin (**Fig. 30 A**, 3rd histogram of each column). This may suggest that the knockdown of JAKs itself protected, to a certain extent, U2OS cells from cisplatin killing. However, this was also seen for the non-specific control (NS) and it was not observed in all experiments performed as demonstrated by the mean of three independent experiments shown in **Figure 30 B** (an average of 2.9-, 2.7- and 2.8-fold for JAK1, JAK2 and TYK2, respectively compared to 2.6- and 3.0-fold for untransfected and NS, respectively).

Results from three independent experiments showed that transfection of siTYK2 rendered cells more susceptible to death even in the absence of any obvious apoptosis-inducing stimuli. This was reflected by the fact that there was more background apoptosis in these cells than in the untransfected cells ($p < 0.05$) and in cells transfected with siJAK1, siJAK2 or a non-specific siRNA (**Fig. 30 C**, 4th bar). This suggests that TYK2 may have a role in cell survival or proliferation. In siTYR2-transfected cells, cisplatin did not induce more cell death (an average of 2.8-fold) than untransfected cells or cells transfected with a non-specific siRNA (an average of 2.6- and 3.0-fold, respectively) (**Fig. 30 B**, 3rd bar, 4th *versus* 1st and 5th graphs). This suggests that siRNA against TYK2 affected the basal survival of U2OS cells, but did not confer sensitivity to cisplatin treatment.

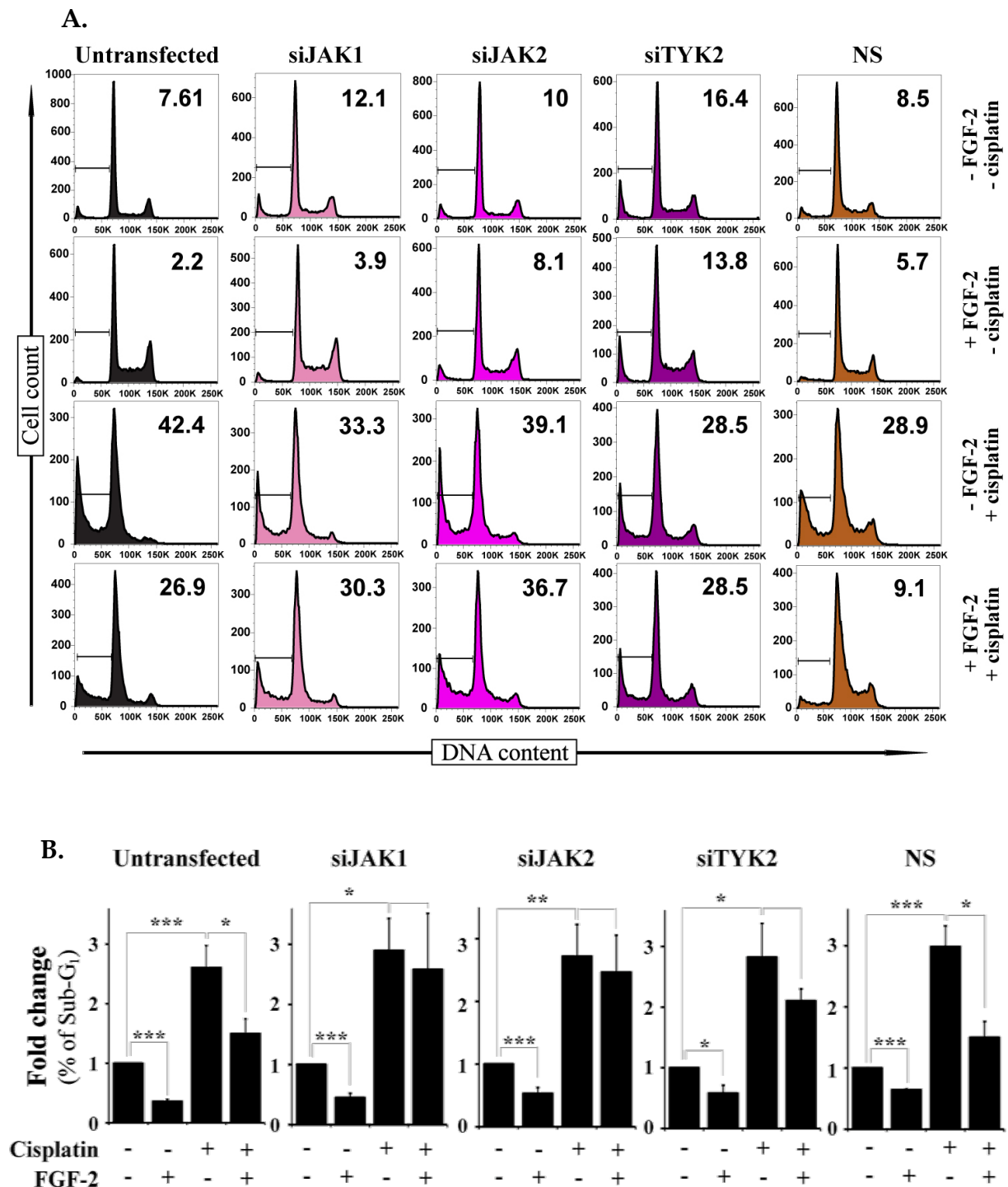


Figure 30. Knockdown of JAK1, JAK2 or TYK2 affected the ability of FGF-2 to protect U2OS cells from cisplatin-induced cell death. (Continued on page 142). U2OS cells were transfected with 75 nM of siRNA against JAK1, JAK2 or TYK2 using DharmaFECT II. After 48 hours, cells were plated in 6-well plates (2×10^5 cells/well) and incubated in serum-free media overnight. Cells were pre-treated with FGF-2 (10 ng/ml) for 4 hours and then treated with the LD₅₀ of cisplatin for 18 hours. Cells were stained with PI and apoptotic cell death was assessed by flow cytometry using the FACSCanto. Results were analysed using FlowJo. Untransfected and non-specific (NS) transfected cells were used as controls. **A.** Cell cycle profiles from one experiment are shown. Numbers refer to the percentage of dead cells represented within the sub-G₁ population. **B.** Mean $\pm$ SEM of three independent experiments are shown in the graphs. Numbers correspond to the fold change of sub-G₁ populations over untreated control. Statistical analysis was performed with Student's t-test (* - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.005$). **C.** Mean $\pm$ SEM of basal cell death from three independent experiments are shown in the graph. Numbers correspond to the fold change of sub-G₁ populations over untransfected control. Statistical analysis was performed with Student's t-test (* - $p < 0.05$ versus untransfected control).

C.

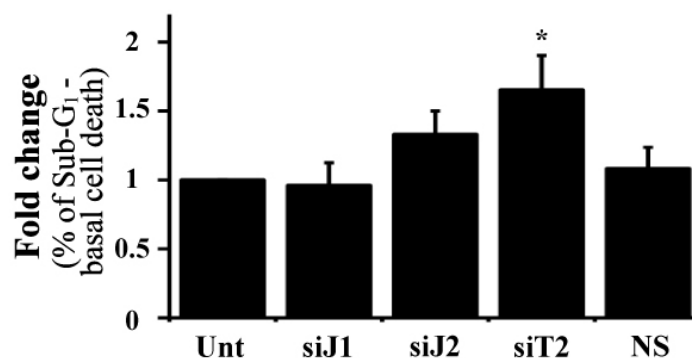


Figure 30. Knockdown of JAK1, JAK2 or TYK2 affected the ability of FGF-2 to protect U2OS cells from cisplatin-induced cell death. (Continued from page 141).

The requirement for JAK1, JAK2 and TYK2 in the mechanism by which FGF-2 protected cells from cisplatin-induced apoptosis was confirmed by analysing cleavage of PARP. FGF-2 rescue experiments were performed in cells transfected with siRNA against that JAK1, JAK2 and TYK2. As a control, the rescue experiment was performed in untransfected cells and cells transfected with a non-specific siRNA. Two days after siRNA transfection cells were trypsinised, counted and replated in 6-well plates (2×10^5 cells per well). Twenty-four hours later, cells were incubated in serum-free media overnight and pre-incubated for 4 hours with FGF-2. They were then treated with cisplatin. After 16 hours of cisplatin treatment proteins were extracted from all cells (adherent alive cells and floating “corpses”) and run on a 7.5% SDS-PAGE gel.

In untransfected cells, pre-incubation with FGF-2 significantly blocked cisplatin-mediated PARP cleavage as previously observed (**Fig. 21 C, Section 4.1.5.1**). This was shown by the reduction of cleaved PARP in cells pre-incubated with FGF-2 compared to cells treated with cisplatin only (**Fig. 31**, 1st Western blot panel, 3rd *versus* 4th lane). In these cells, FGF-2 significantly reduced the basal levels of PARP cleavage ($p < 0.005$) and treatment with cisplatin induced a significant increase PARP cleavage ($p < 0.005$). Pre-incubation with FGF-2 substantially blocked cisplatin-induced killing ($p < 0.01$). The pattern of PARP cleavage in cells transfected with a non-specific siRNA was identical to that seen in the untransfected cells (**Fig. 31**, 5th Western blot panel).

In accordance to what was observed by cell cycle analysis (**Fig. 30**), FGF-2 did not rescue cells with reduced levels of JAK1, JAK2 or TYK2 from cisplatin-mediated cell death (**Fig. 31**, 2nd-4th Western Blot panel, compare 3rd with 4th lane). In these cells treatment with cisplatin induced a significant increase of PARP cleavage ($p < 0.05$, $p < 0.005$, $p < 0.005$ for JAK1, JAK2 and TYK2, respectively), which was not prevented by pre-treatment with FGF-2 (**Fig. 31**, 3rd *versus* 4th bar in 2nd-4th graphs). In siJAK1- and siJAK2- (but not siTYK2-) transfected cells, FGF-2 significantly reduced the basal levels of PARP cleavage ($p < 0.05$) (**Fig. 31**, 1st *versus* 2nd bar in 2nd and 3rd graphs). This confirms the results obtained for JAK1 and JAK2 previously shown in **Figure 30**. The results obtained with the analysis of PARP cleavage showed that, for cells transfected with siTYK2, the difference between cell death in cells treated with FGF-2 only and untreated cells was not statistically significant (**Fig. 31**, 1st *versus* 2nd bar in 4th graph). The lack of significance was due to one particular experiment, where we did not observed a difference, which impacted on the statistical significance of the results. Further repeats should enable us to determine the truly representative result.

Together, these data suggest that the presence of JAK1, JAK2 and/or TYK2 is required for efficient FGF-2-mediated protection of cells from cisplatin-induced apoptosis.

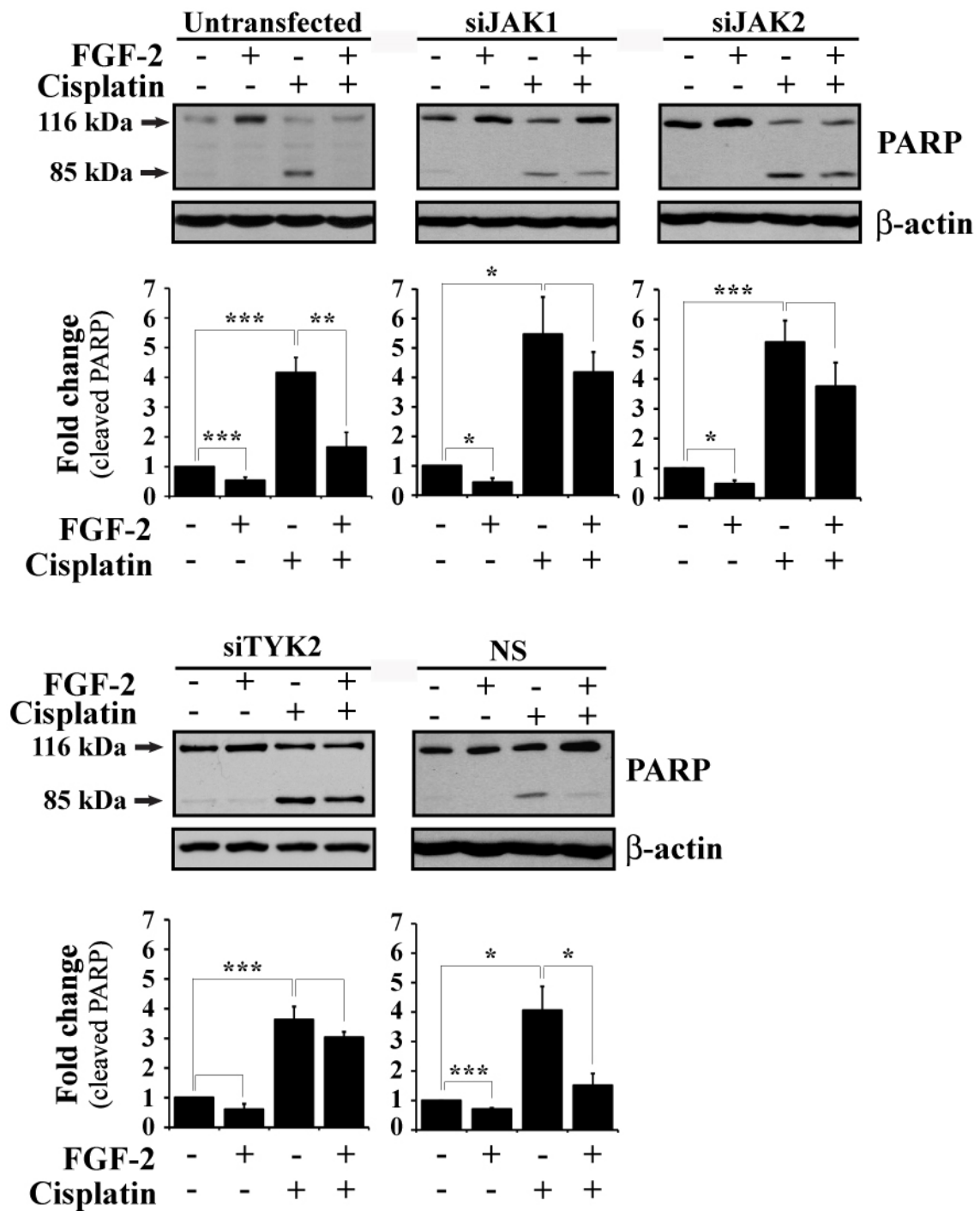


Figure 31. Knockdown of JAK1, JAK2 or TYK2 affected the ability of FGF-2 to block cisplatin-induced PARP cleavage in the U2OS cells. U2OS cells were transfected with 75 nM of siRNA against JAK1, JAK2 or TYK2 using DharmaFECT II for 48 hours. Untransfected cells and cells transfected with a non-specific siRNA were used as control. Subsequently, cells were plated in 6-well plates (2×10^5 cells/well) and incubated in serum-free media overnight. After 4 hours pre-treatment with FGF-2 (10 ng/ml), cells were treated with the LD₅₀ of cisplatin for 16 hours. Subsequently, cells were harvested; proteins were extracted and separated on 7.5% SDS-PAGE gel. Western blotting analysis was performed using total cell lysates and the membrane probed with antibodies against PARP. β -actin was also probed and used as a loading control. Representative Western blots and mean $\pm$ SEM of densitometric values (cleaved PARP) from three experiments are shown in graphs. Values are expressed as fold change over untreated controls. Statistical analysis was performed with Student's t-test (* - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.005$).

Although, to the best of our knowledge, JAK3 is not generally expressed in osteosarcoma cells, it would be interesting to investigate if it is expressed in U2OS cells and, if so, whether it plays a role in FGF-2-mediated chemoresistance. In general, constitutively activated JAK3 and mutant *JAK3* alleles can be found in lymphoproliferative disorders (Gee *et al.*, 2001; Yared *et al.*, 2005; Lai *et al.*, 2005; Tomita *et al.*, 2006; Krejsgaard *et al.*, 2006; Walters *et al.*, 2006; Marzec *et al.*, 2008). Indeed, JAK3 has been associated with tumorigenesis and since cancer cells have de-regulated gene expression, it is not inconceivable that U2OS cells may, against our expectations, express JAK3. For example, JAK3 activation was found to contribute to the oncogenesis of colon and breast cancers (Lin *et al.*, 2005; Kim *et al.*, 2009). The potential participation of JAK3 in FGF-2-mediated chemoresistance will be investigated further in the future.

4.2.2.2.1. JAK2 ACTIVITY WAS REQUIRED FOR FGF-2-MEDIATED CHEMORESISTANCE

We next investigated if JAK activity was required for FGF-2 chemoprotection. For this purpose, we used JAK2 inhibitor II (1,2,3,4,5,6-hexabromocyclohexane). This inhibitor interacts with a solvent-accessible pocket near the activation loop of JAK2 and acts as a specific and direct inhibitor of its autophosphorylation (Sandberg *et al.*, 2005). Pre-treatment with this inhibitor for 45 minutes was sufficient to block the activation of STAT3 by IL-6, which requires JAK2 activity (Stahl *et al.*, 1995), in a dose dependent manner (data not shown). The addition of 50 μ M of JAK2 inhibitor II blocked the activation of STAT3 without affecting the basal levels of activated ERK1/2.

We also tested JAK inhibitor I (Pyridone 6) that inhibits all JAKs (Thompson *et al.*, 2002). This ATP-competitive inhibitor of JAKs, however, was shown to have profound effects on ERK1/2 basal phosphorylation (phosphorylated ERK1/2 was elevated in cells treated with the inhibitor) (data not shown) and was therefore considered inappropriate for this study. The requirement of JAK2 activity for the FGF-2-mediated rescue was then investigated by performing the rescue experiment in the presence or absence of JAK2 inhibitor II.

Rescue experiments previously described were performed in cells that were incubated with 50 μ M of JAK2 inhibitor II for 45 minutes before adding FGF-2. Cisplatin was added four hours after FGF-2. The rescue experiment was also

performed in cells incubated with the drug vehicle [0.5% (v/v) DMSO] as a control. Cell cycle profiles and PARP cleavage patterns were then analysed.

Both cells pre-incubated with JAK inhibitor II and cells incubated with the drug vehicle (control) underwent significant cell death upon cisplatin treatment ($p < 0.01$ and $p < 0.005$, respectively) as measured by the increase of the sub- G_1 peak and PARP cleavage (**Fig. 32 B and D**, 1st *versus* 3rd bar in both graphs). In cells treated with the drug vehicle only, pre-incubation with FGF-2 significantly reduced cisplatin-induced cell death ($p < 0.05$) (**Fig. 32 A**, 3rd *versus* 4th black histograms and **B**, 3rd *versus* 4th black bar). This was also seen by PARP cleavage analysis (**Fig. 32 C**, 3rd *versus* 4th lane and **D**, 3rd *versus* 4th bar in the graph on the left).

Incubation with JAK inhibitor II precluded FGF-2 rescue of cisplatin-treated cells from apoptosis. Indeed neither the sub- G_1 peak population was reduced, nor was PARP cleavage prevented in FGF-2 pre-treated cells (**Fig. 32 A**, 3rd *versus* 4th grey histograms and **C**, 7th *versus* 8th lane). Accordingly, in these cells the difference between the sub- G_1 populations (or PARP cleavage) after treatment with cisplatin and FGF-2 was not statistically significant from treatment with cisplatin only (**Fig. 32 B**, 3rd *versus* 4th grey bar and **D**, 3rd *versus* 4th bars in the graph on the right).

Interestingly, in the presence of the JAK2 inhibitor, FGF-2 was also not able to significantly reduce the basal cell death (**Fig. 32 B and D**, 1st *versus* 2nd bar in the graphs on the right). Knockdown of JAK2 was previously shown to prevent the FGF-2 rescue from cisplatin but it did not impair the reduction of background cell death seen in response to FGF-2 (**Fig. 30 and 31**). This could be related to the fact that the chemical inhibitor blocks the kinase activity but does not affect JAK2 protein levels (data not shown), whereas siJAK2 reduces the amount of JAK2 in these cells without specifically affecting its potential for activation. The chemical inhibitor may thus be more efficient than siJAK2. Although JAK2 inhibitor is supposedly specific, having low impact on the activity of other family members, one cannot exclude the possibility that, in these cells and under our experimental conditions, the inhibitor may also reduce the activity of other JAKs, which may be required for FGF-2 to block background cell death. Only *in vitro* kinase assays would clarify this issue.

Together, these data suggested that in U2OS cells FGF-2 chemoprotection required JAK2 kinase activity.

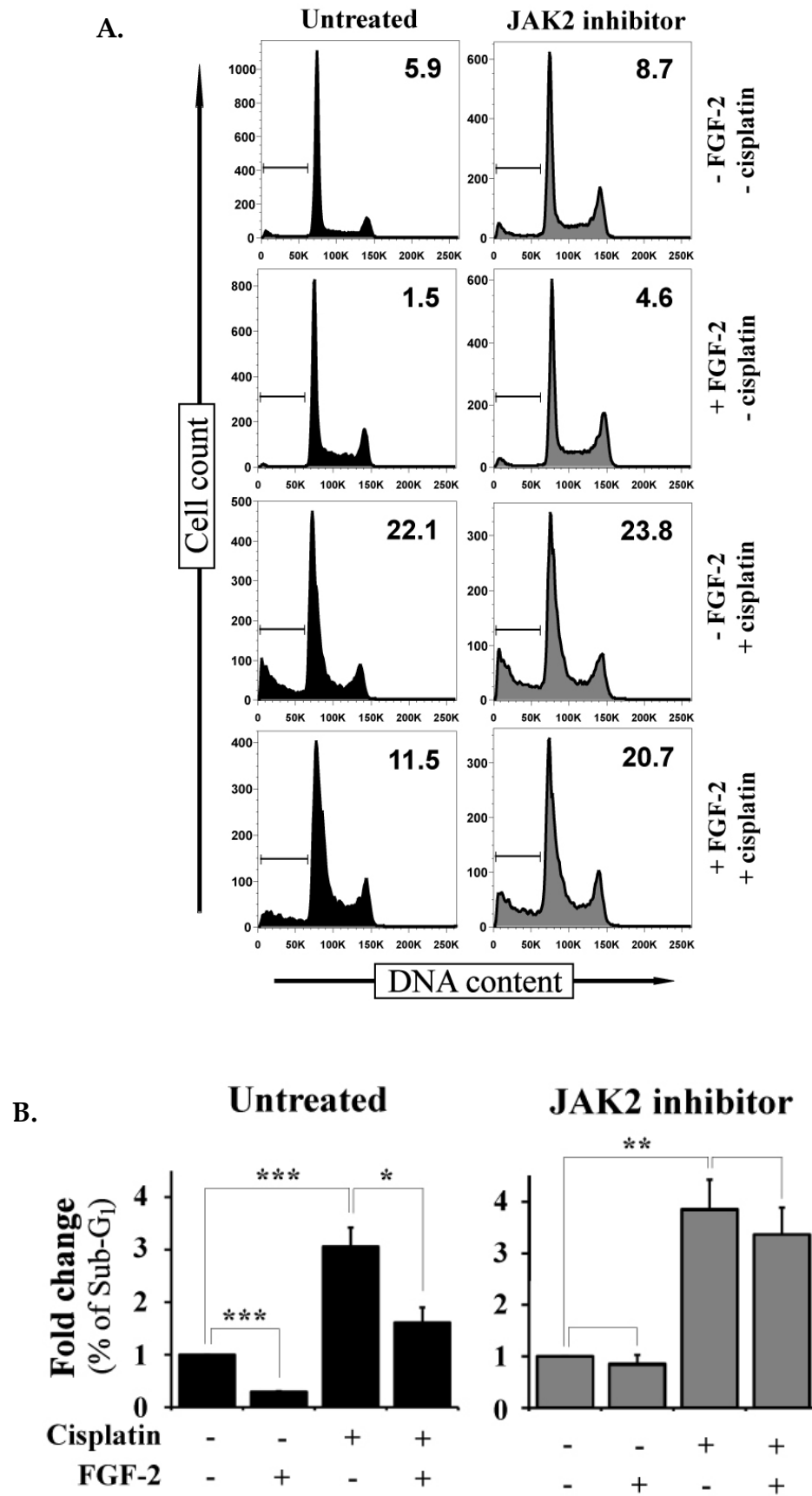


Figure 32. Inhibition of JAK2 activity impaired FGF-2-mediated rescue in U2OS cells. (Continued on page 148).

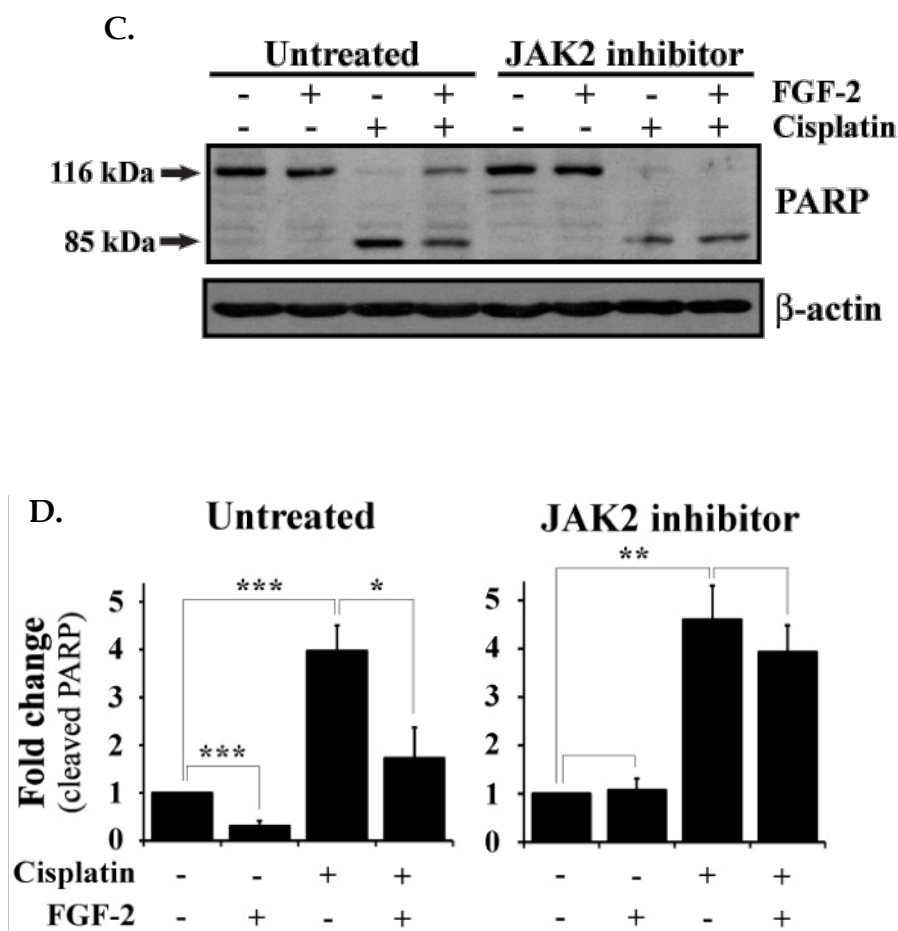


Figure 32. Inhibition of JAK2 activity impaired FGF-2-mediated rescue in U2OS cells. (Continued from page 147). U2OS cells were starved in serum-free media overnight and incubated with 50 μ M of JAK2 inhibitor II for 45 minutes, or with the drug vehicle [0.5% (v/v) DMSO] (untreated). After 4 hours of pre-treatment with FGF-2 (10 ng/ml), cells were treated with the LD₅₀ of cisplatin. **A.** After 18 hours of cisplatin treatment, cells were harvested, permeabilised and stained with PI (50 μ g/ml). Apoptotic cell death was assessed by flow cytometric analysis using the FACSCanto and the results were analysed using FlowJo. Cell cycle profiles from one representative experiment are shown. Numbers refer to the percentage of dead cells represented within the sub-G₁ population. **B.** Mean $\pm$ SEM of three independent experiments are shown in graphs. Values are expressed as fold change of sub-G₁ populations over untreated control (* - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.005$). **C.** After 16 hours of cisplatin treatment cells were harvested, proteins extracted and separated on a 7.5% SDS-PAGE gel. Western blotting analysis was performed using total cell lysates and antibodies against PARP. β -actin was used as a loading control. **D.** Mean $\pm$ SEM of densitometric values (cleaved PARP) from three experiments are shown in graphs. Values are expressed as fold change over untreated controls. Statistical analysis was performed with Student's t-test (* - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.005$).

Reduction in the levels of JAK1, JAK2 and TYK2 had a significant impact on FGF-2-mediated protection against cisplatin. The use of a pharmacological inhibitor that targets JAK2 activity suggested that its enzymatic activity was required for efficient chemoprotection. We have, therefore, investigated the impact of reducing the levels of STAT1, STAT3 and STAT5, the main signal transducers downstream of JAKs, in FGF-2-mediated chemoresistance in the U2OS cells.

4.2.2.3. STAT1, STAT3 AND STAT5A/B WERE NOT REQUIRED FOR FGF-2-MEDIATED CHEMORESISTANCE

Over-activation of STAT1, STAT3 and STAT5 has been observed in many cancers (reviewed by Yu and Jove, 2004). These three STATs were shown to contribute to oncogenesis and, in particular, STAT3, by virtue of its anti-apoptotic and growth-promoting properties, was associated with intrinsic drug resistance (Kovacic *et al.*, 2006; Patterson *et al.*, 2006; Barre *et al.*, 2007; Grisko *et al.*, 2006; Ikuta *et al.*, 2005; Lewis and Ward, 2008). As JAKs were required for FGF-2-mediated chemoresistance in U2OS cells, we investigated if within this context STATs, the main targets of JAKs, were also required to mediate FGF-2 response(s).

We used RNAi to reduce the protein levels of STAT1, STAT3 and STAT5 so that the impact of their knockdown on FGF-2-mediated rescue from cisplatin-induced cell death could be investigated. After verifying that STAT1, STAT3 and STAT5 could be efficiently silenced by using siRNA molecules, the FGF-2 rescue was attempted. The rescue experiment was also performed on untransfected cells and cells transfected with a non-specific siRNA (NS) that were used as controls, as previously explained. Two days post-transfection, cells were trypsinised, counted and replated in 6-well plates (2×10^5 cells per well). Twenty-four hours later cells were incubated in serum-free media overnight and treated as previously described with FGF-2 (4 hours) and cisplatin (18 hours). Cell cycle profiles were analysed by flow cytometry (**Fig. 33**) and the levels of knockdown for each protein targeted were monitored in parallel (e.g. **Fig. 29**).

FGF-2 significantly rescued untransfected cells from cisplatin-induced death. This was shown by a reduction in the sub-G₁ population seen in cells that were treated with FGF-2 and cisplatin when compared to cells treated with cisplatin only (**Fig. 33 A**, black coloured cell cycle profiles, 3rd versus 4th histogram). Pre-incubation with FGF-2 significantly blocked cisplatin-induced killing ($p < 0.01$) (**Fig. 33 B**, 3rd versus 4th bar in

the 1st bar graph). In these cells FGF-2 also significantly reduced the basal levels of cell death ($p < 0.005$) (**Fig. 33 B**, 1st *versus* 2nd bar in the 1st graph). Similar results were obtained in cells transfected with a non-specific siRNA (cf. **Fig. 30**).

Like in untransfected cells, treatment with cisplatin induced a significant increase ($p < 0.005$) of cell death in cells transfected with STAT1, STAT3 and STAT5A/B siRNAs (**Fig. 33 B**, 1st *versus* 3rd bars in 2nd-4th graphs). To our great surprise, despite the obvious requirement for the JAKs (**Fig. 30** and **31**), FGF-2 could rescue cells with reduced levels of STAT1, STAT3 or STAT5 (**Fig. 33 A**, yellow, orange and red coloured cell cycle profiles, 3rd *versus* 4th histograms). This was further confirmed by statistical analysis which established a significant difference between sub-G₁ populations of cells treated with FGF-2 before cisplatin and cells treated with cisplatin alone ($p < 0.005$ for siSTAT1, $p < 0.05$ for siSTAT3 and $p < 0.05$ for siSTAT5A/B) (**Fig. 33 B**, 3rd *versus* 4th bars in 2nd-4th graphs). These results suggest that, independently, each of these three proteins were not essential to the mechanism used by FGF-2 to protect cells against cisplatin. It remains to be established any possible redundancy between these three proteins and whether STAT2, STAT4 and/or STAT6 are also dispensable. In addition, in cells with decreased levels of STAT1, STAT3 and STAT5A/B FGF-2 also significantly reduced the background levels of cell death ($p < 0.05$, $p < 0.01$ and $p < 0.005$, respectively) (**Fig. 33 B**, 1st *versus* 2nd bar in 2nd-4th graphs).

In cells with reduced levels of STAT5A/B proteins, cisplatin induced less cell death (an average of 1.4-fold over the respective untreated control) compared to the untransfected cells (2.3-fold over the respective untreated control), cells transfected with siRNA against STAT1 (2.8-fold over the respective untreated control) and cells transfected with siRNA against STAT3 (2.1-fold over the respective untreated control) (**Fig. 33 B**, 3rd bar of all graphs). This was unexpected, as STAT5 is thought to have, in general, an mitogenic role (e.g. Lewis and Ward, 2008).

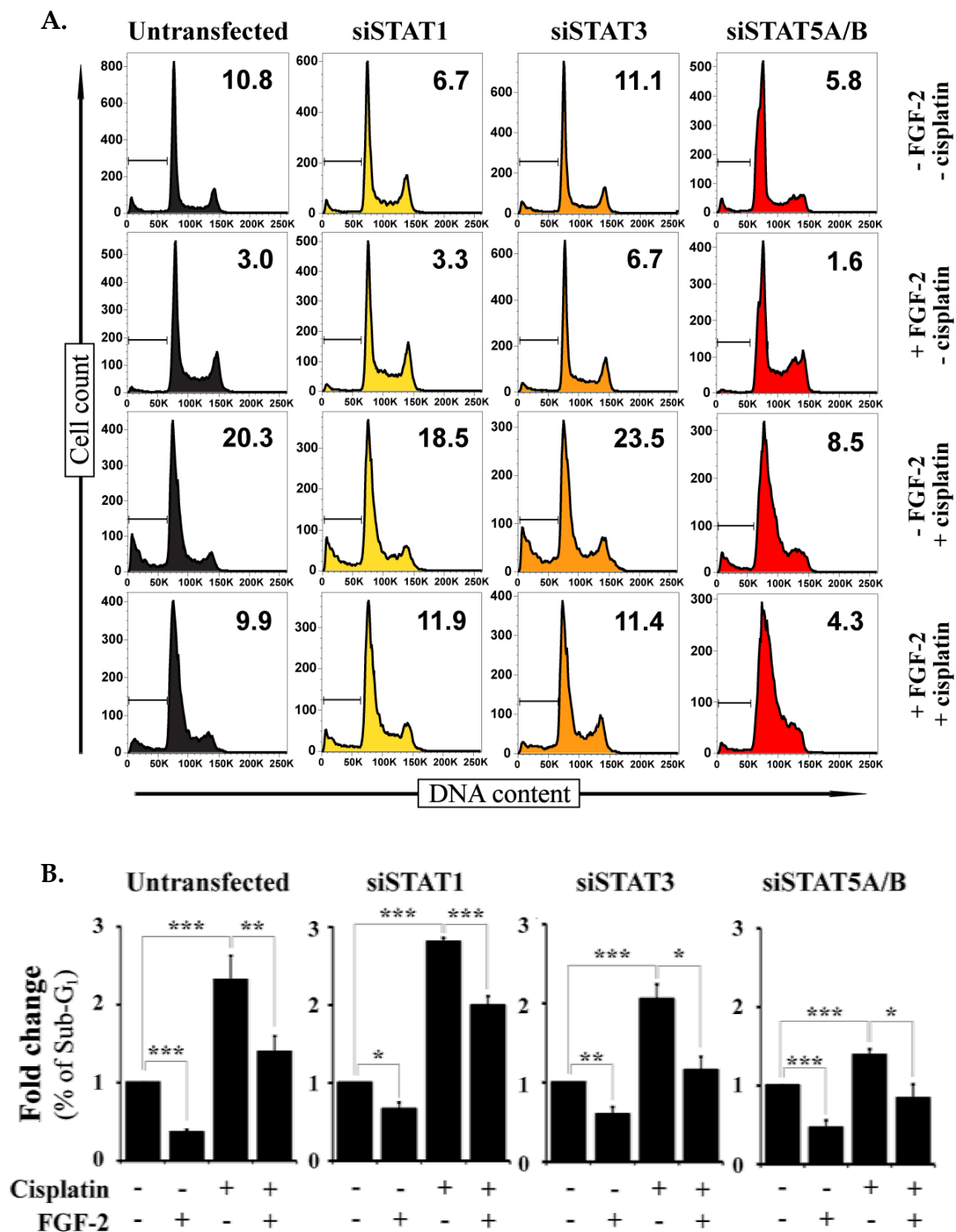


Figure 33. FGF-2 was able to rescue U2OS cells from cisplatin-induced cell death in the absence of STAT1, STAT3 or STAT5. (Continued on page 152). U2OS cells were transfected with 75 nM of siRNA against STAT1, STAT3 or STAT5A/B using DharmaFECT II. After 48 hours, cells were plated in 6-well plates (2×10^5 cells/well) and incubated in serum-free media overnight. Cells were pre-treated with FGF-2 (10 ng/ml) for 4 hours and then treated with the LD₅₀ of cisplatin for 18 hours. Cells were stained with PI and apoptotic cell death was assessed by flow cytometry using the FACSCanto. Results were analysed using FlowJo. Untransfected cells were used as controls. **A.** Cell cycle profiles from one experiment are shown. Numbers refer to the percentage of dead cells represented within the sub-G₁ population. **B.** Mean $\pm$ SEM of at least three independent experiments are shown in graphs. Numbers correspond to the fold change of sub-G₁ populations over untreated control. Statistical analysis was performed with Student's t-test (* - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.005$). **C.** Mean $\pm$ SEM of basal cell death from three independent experiments are shown in the graphs. Numbers correspond to the fold change of sub-G₁ populations over untransfected control. Statistical analysis was performed with Student's t-test (* - $p < 0.05$ versus untransfected control).

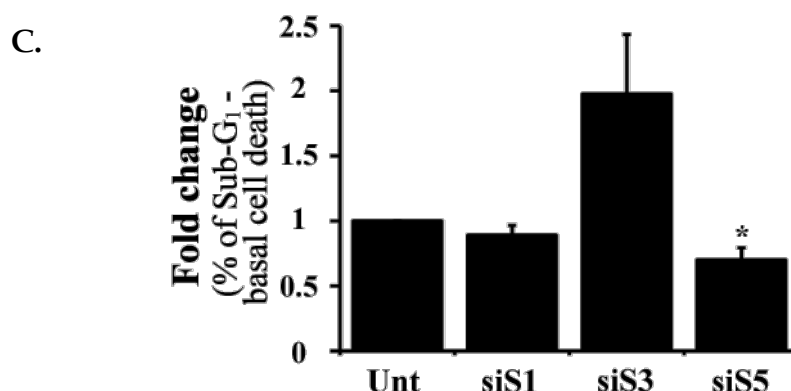


Figure 33. FGF-2 was able to rescue U2OS cells from cisplatin-induced cell death in the absence of STAT1, STAT3 or STAT5. (Continued from page 151).

In addition, results from three independent experiments showed that siSTAT5-transfected cells also had lower levels of basal death than untransfected cells ($p < 0.05$) (**Fig. 33 C**, 4th bar). It is possible that, like STAT1 (Stephanou *et al.*, 2005; Kovacic *et al.*, 2006; Patterson *et al.*, 2006), STAT5 may have both anti-apoptotic and pro-apoptotic properties according to the cell type and cellular environment. The average background cell death in siSTAT3-transfected cells was higher than untransfected cells (2-fold) (**Fig. 33 C**, 3rd bar), which is in accordance with STAT3 being required for cell growth and cell survival (Bromberg *et al.*, 2000; Yu and Jove, 2004). This observation was not, however, statistically significant.

The results obtained by cell cycle analysis were confirmed by examining PARP cleavage. The FGF-2 rescue experiment was performed in cells transfected with siRNA against STAT1, STAT3 and STAT5. Untransfected cells and cells transfected with a non-specific siRNA were once again used as controls. Following two days of siRNA transfection cells were trypsinised, counted and re-plated in 6-well plates (2×10^5 cells per well). Twenty-four hours later cells were incubated in serum-free media overnight and pre-incubated for 4 hours with FGF-2 followed by cisplatin treatment. After 16 hours of cisplatin treatment proteins were extracted from all cells (adherent alive cells and floating “corpses”) and run on a 7.5% SDS-PAGE gel.

By analysing PARP cleavage we noted that pre-incubation with FGF-2 blocked cisplatin-mediated apoptosis in untransfected cells as well as in cells transfected with

siRNA against STAT1, STAT3 and STAT5 (**Fig. 34**), confirming the results obtained while analysing the cell cycle (**Fig. 33**). This is demonstrated by the significant reduction in PARP cleavage in cells previously incubated with FGF-2 when compared to cells treated with cisplatin alone ($p < 0.005$ in untransfected, and $p < 0.05$ in siSTAT1-, siSTAT3-, si-STAT5- and NS-transfected cells) (**Fig. 34**, 3rd *versus* 4th lane in all Western blots and 3rd *versus* 4th bar in the all graphs). For all conditions, treatment with cisplatin induced a significant increase in PARP cleavage ($p < 0.005$ in untransfected, siSTAT1 and siSTAT3, and $p < 0.01$ in siSTAT5 and $p < 0.05$ in NS) (**Fig. 34**, 1st *versus* 3rd bar in all graphs).

FGF-2 also significantly reduced the basal levels of PARP cleavage, indicative of background apoptosis, in untransfected cells ($p < 0.005$) and in cells transfected with siRNA against STAT3 ($p < 0.05$), STAT5 ($p < 0.05$) and NS ($p < 0.05$) (**Fig. 34**, 1st *versus* 2nd bar in 1st and 3rd-5th graphs). However, this was not observed in siSTAT1-transfected cells (**Fig. 34**, 1st *versus* 2nd bar in the 2nd graph). This may be related to the low levels of basal cell death observed in these cells. We noted during the course of these experiments that the levels of cleaved PARP in siSTAT1-transfected cells were low and, indeed, a higher exposure of the X-ray films was needed to detect this form. This not only makes it more difficult to detect the PARP fragment by Western blot, but it also makes the quantification less robust. An alternative explanation is that STAT1 was not required for the FGF-2 rescue from cisplatin-induced cell death, but was somehow needed for the effect of FGF-2 on basal levels of apoptosis.

In most Western blots, as for example in **Figure 34**, the total (116 kDa) and cleaved (85 kDa) forms of PARP were not the only bands detected with the PARP antibody. We have noticed that these bands were detected when a freshly prepared primary antibody solution was used, suggesting that they might be unspecific. The fact that some bands appear to vary with the treatments performed suggested, however, that they might correspond to other forms of PARP. For example, PARP can be cleaved into a smaller fragment of 55 kDa that has been described in necrotic cells (Gobeil *et al.*, 2001). That fragment was never detected in our experiments though. These bands were also more evident in X-ray films that were exposed for longer periods of time in order to detect lower levels of PARP cleavage (e.g. siSTAT1 Western blot, **Fig. 34**).

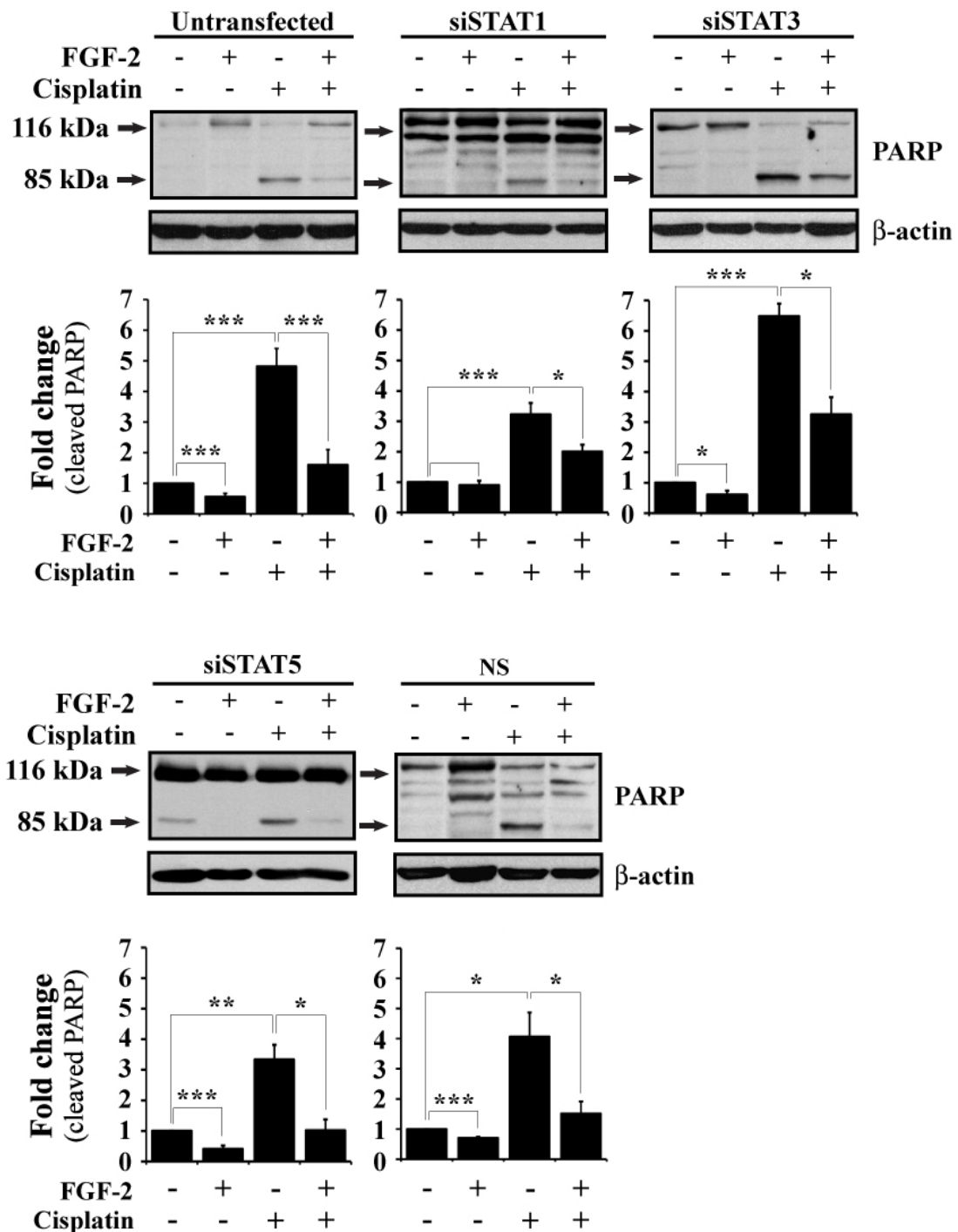


Figure 34. FGF-2 was able to reduce cisplatin-induced PARP cleavage in U2OS cells with reduced levels of STAT1, STAT3 or STAT5. U2OS cells were transfected with 75 nM of siRNA against STAT1, STAT3 or STAT5A/B using DharmaFECT II for 48 hours. Untransfected cells and cells transfected with a non-specific siRNA were used as controls. Subsequently, cells were plated in 6-well plates (2×10^5 cells/well) and incubated in serum-free media overnight. After 4 hours pre-treatment with FGF-2 (10 ng/ml), cells were treated with the LD₅₀ of cisplatin for 16 hours. Subsequently, cells were harvested, proteins were extracted and separated on 7.5% SDS-PAGE gel. Western blotting analysis was performed using total cell lysates and the membrane was probed with antibodies against PARP. β-actin was also probed and used as a loading control. Representative Western blots and mean $\pm$ SEM of densitometric values (cleaved PARP) from three independent experiments are shown in the graphs. Values are expressed as fold change over untreated controls. Statistical analysis was performed with Student's t-test (* - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.005$).

Together, these data suggested that the absence of STAT1, STAT3 or STAT5 did not impede FGF-2-mediated protection of cells from cisplatin-induced apoptosis. Nevertheless, one cannot exclude the hypothesis that each STAT may play a non-essential role in FGF-2 signalling and that the loss of a particular STAT may be compensated by another STAT.

In addition, the fact that the knockdown of STAT1, STAT3 or STAT5 did not affect the ability of FGF-2 to protect U2OS cells from cisplatin-mediated cell death does not exclude the possibility that other STATs may play a role in chemoresistance. Accordingly, the potential participation of STAT2, STAT4 and STAT6 in FGF-2-mediated chemoresistance will be investigated in the future. The role that STAT1, STAT3 and STAT5 may have in protecting cells from the cytotoxic effects of other chemotherapeutic drugs will also be addressed.

4.2.3. ACTIVATION OF STAT1, STAT3 OR STAT5A/B BY FGF-2 WAS NOT OBSERVED IN U2OS CELLS

Cells with decreased levels of STAT1, STAT3 and STAT5 were protected by FGF-2 from cisplatin-induced cell death. Although this suggested that these particular STATs were not essential for FGF-2-mediated chemoresistance, as mentioned above, there was still the possibility that they may have been involved in FGF-2 signalling.

STATs are mainly activated after stimulation of cytokine receptors but can also be activated by some growth factors and G protein-coupled receptors (Levy and Darnell, 2002; Bowman *et al.*, 2000; Mellado *et al.*, 1998; Wong and Fish, 1998; Vila-Coro *et al.*, 1999). Their activity is predominantly regulated by PTMs, such as tyrosine and serine phosphorylation of well-characterised residues (reviewed by Levy and Darnell, 2002). Ligand binding stimulates the rapid activation of STATs. In some cell lines (murine lens and BMVECs), FGF-2 induces tyrosine phosphorylation of STAT1, STAT3 and STAT5 and this was shown to occur within 5 to 15 minutes of stimulation (Ebong *et al.*, 2004; Yang *et al.*, 2009). Accordingly, we have investigated if the early signalling cascades activated by FGF-2 also involved the activation of STAT1, STAT3 or STAT5.

To determine if FGF-2 could induce STAT1, STAT3 and/or STAT5 activation, U2OS cells were treated for different periods of time within the first four hours window. This corresponded to the period of time that cells were routinely incubated with FGF-2 in order to protect them from the toxic effects inflicted by cisplatin. Levels

of phosphorylated STAT1 (Tyr⁷⁰¹), STAT3 (Tyr⁷⁰⁵) and STAT5A/B (Tyr⁶⁹⁴) were assessed by Western blot. The effectiveness of FGF-2 was determined by monitoring the phosphorylation of ERK1/2 in parallel with all the experiments (data not shown). Cells treated with IFN- γ , IL-6 and OSM were used as positive controls for the activation of STAT1, STAT3 and STAT5, respectively. The conditions for these treatments had been previously optimised in the Costa-Pereira laboratory and have been extensively described also by other groups.

Figure 35 is a representative example of several experiments performed to show that, under the conditions used, FGF-2 did not induce STAT1, STAT3 or STAT5 phosphorylation in U2OS cells. Using an antibody against STAT3 phosphorylated Tyr⁷⁰⁵, we observed that STAT3 was constitutively activated in this cell line (**Fig. 35 B**, 1st lane) and the basal levels of phosphorylated STAT3 were not significantly affected by the treatment with FGF-2 for different time points within four hours (**Fig. 35 C**). We did not detect constitutive tyrosine phosphorylation of STAT1 (Tyr⁷⁰¹) and STAT5 (Tyr⁶⁹⁴) (**Fig. 35 A and D**, 1st lane on each Western blot) and treatment with FGF-2 did not induce it either (**Fig. 35 A and D**, lanes 2-6 of each Western blot). The lack of phosphorylation on these particular residues did not reflect a failure of the antibodies, as IFN γ , IL-6 and OSM treatments induced very robust phosphorylation of STAT1, STAT3 and STAT5, respectively (**Fig. 35 A, B and D**, last lane of each Western blot). One distinct possibility is that FGF-2 induced phosphorylation of these molecules on residues other than the key tyrosines on the C-terminal, which are recognised by the phospho-specific antibodies used here. This could be addressed by immunoprecipitating each STAT and probing the Western blot membranes with the widely used and extensively characterised anti-pan-phosphotyrosine antibodies, PY20 and 4G10.

Several time points were analysed, which included the time points at which STATs are usually activated by cytokines and growth factors, including FGF-2 (Ebong *et al.*, 2004; Yang *et al.*, 2009) (**Fig. 35**). One cannot exclude that phosphorylation may have occurred transiently and was thus not easily detected here. Indeed, the duration and degree of STAT activation are under strict regulation by a series of negative regulators, which can elicit specific inhibitory signals that lead to decreased tyrosine phosphorylation (Aaronson *et al.*, 2002; Krebs and Hilton, 2001). Longer time points were not routinely analysed for two main reasons: firstly, it is known that incubations with FGF-2 for periods longer than four hours do not lead to increased chemoprotection and, secondly, any activation/phosphorylation seen past that period was likely to be a secondary, if not tertiary response, unlikely to be a key event in triggering the cell survival mechanisms that protect the U2OS cells from cisplatin.

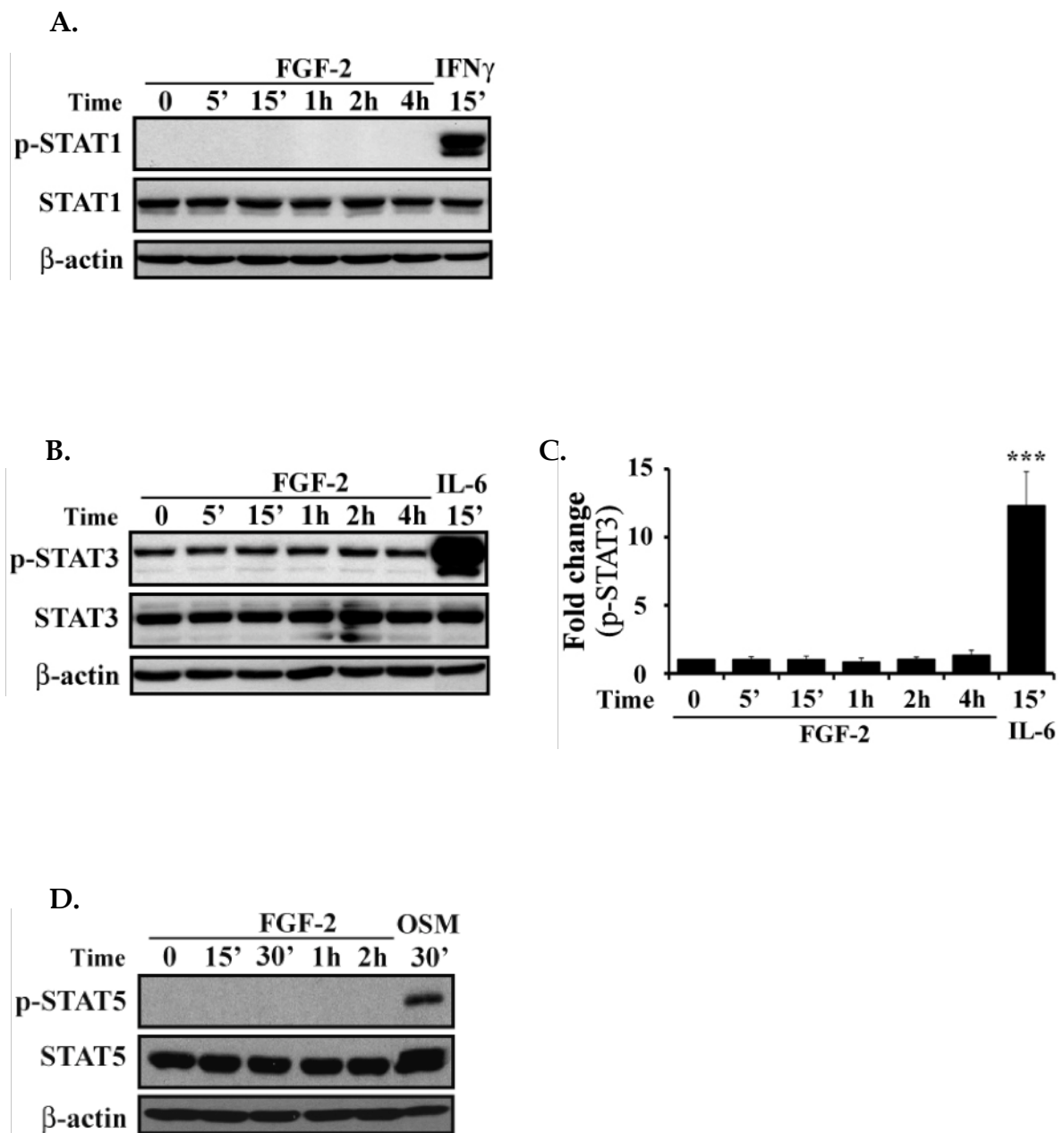


Figure 35. FGF-2 did not activate STAT1, STAT3 or STAT5A/B in U2OS cells. U2OS cells were serum-starved (0.5% FCS) overnight and, subsequently, incubated in serum-free medium for 1 hour. Cells were then stimulated with 25 ng/ml of FGF-2 for the indicated times. Cells treated with IFN- γ (500 IU/ml), IL-6 (200 ng/ml)/ sIL-6R (250 ng/ml) and OSM (50 ng/ml) were used as positive controls for activation of STAT1, STAT3 and STAT5, respectively. Proteins were extracted and separated on a 7.5% SDS-PAGE gel. Western blotting analysis was performed on total cell lysates using antibodies against (A) phosphorylated STAT1 (Tyr⁷⁰¹), (B) phosphorylated STAT3 (Tyr⁷⁰⁵) and (D) phosphorylated STAT5 (Tyr⁶⁹⁴) and antibodies against total proteins. β -actin was also probed for and used as a loading control. C. Mean $\pm$ SEM of densitometric values from three experiments are shown in graphs. Values are expressed as fold change relative to untreated control. Statistical analysis was performed with Student's t-test (***) - $p < 0.005$ over untreated control). ' - minutes, h - hours.

Importantly, recent studies have shown that unphosphorylated STATs can dimerise and be functional by inducing the transcription of a different subset of genes than that induced by phosphorylated dimers (Wenta *et al.*, 2008; Brown *et al.*, 2008). Accordingly, the participation of unphosphorylated STATs in parallel pathways downstream of FGF-2 should also not be excluded.

These data suggest that FGF-2 did not induce the activation of STAT1, STAT3 and STAT5 in U2OS cells. These observations, together with a functional rescue observed in cells transfected with siRNA against STAT1, STAT3 and STAT5 suggest that these STATs are unlikely to be involved in FGF-2-mediated chemoresistance. The fact that RNAi was not performed against combinations of these proteins, however, still precludes the firm conclusion that STAT1, STAT3 and STAT5 are dispensable for mediating FGF-2 anti-apoptotic functions.

4.2.4. FGF-2 INDUCED THE PHOSPHORYLATION OF TYK2 AND POTENTIALLY OF JAK1 AND JAK2 IN U2OS CELLS

JAK1, JAK2 and TYK2 were shown to be required for FGF-2 chemoresistance (Section 4.2.2.2). JAKs are typically the first kinases of the JAK/STAT pathway to be activated upon ligand binding (reviewed by Heinrich *et al.*, 1998). Upon cytokine binding, membrane-proximal immediate signalling is initiated through the activation of JAKs, which are associated with the receptors *via* their FERM domains (Chen *et al.*, 1997; Gauzzi *et al.*, 1997; Yamaoka *et al.*, 2004). Rapid phosphorylation and activation of JAKs has been observed in response to many cytokines and growth factors (Novak *et al.*, 1995; Leaman *et al.*, 1996; Vignais *et al.*, 1996; Gual *et al.*, 1998). For example, IFN- γ -induced activation of JAK1 and JAK2 was observed within 5-15 minutes of treatment (Silvennoinen *et al.*, 1993a; Silvennoinen *et al.*, 1993b; Watling *et al.*, 1993; Muller *et al.*, 1993). JAK2 phosphorylation downstream of FGF-2 occurred within 20 minutes of stimulation in BMVECs (Yang *et al.*, 2009). We investigated, therefore, if JAK1, JAK2 and TYK2 were activated by FGF-2 in U2OS cells at early time points.

It is generally recognised that JAKs are expressed at very low levels in human cells. Accordingly, throughout this work, detection of JAK phosphorylation in whole cells extracts by Western blot was particularly difficult to achieve. We circumvented this problem by immunoprecipitating each JAK individually. Using this approach, we investigated if FGF-2 led to JAK phosphorylation in U2OS cells. To that end, cells were

treated with 10 ng/ml of FGF-2 for increasing periods of time. Whole cell lysates were prepared using Schindler's lysis buffer and used to immunoprecipitate each JAK individually. The immunoprecipitates were run on a 7.5% SDS-PAGE gel and, following transfer, membranes were probed with two different antibodies that recognise phosphorylated tyrosine residues (PY20 and 4G10). We reasoned that this would certify we would pick up any possible tyrosine phosphorylation event regardless of the residue that was modified. In order to ensure that the antibodies were specific, protein samples from JAK-negative cells and cells transfected with siJAK1, siJAK2 or siTYK2 were also analysed in parallel (**Fig. 36, 37 and 38**, and data not shown). The functionality of FGF-2 was also monitored concomitantly by assessing the phosphorylation of ERK1/2 (data not shown).

Immunoprecipitation of endogenous JAK1 was performed in U2OS cells. Although the results presented here are preliminary (this particular experiment was only performed twice), we observed that FGF-2 induced an increase of tyrosine phosphorylation in JAK1 immunoprecipitates (**Fig. 36 A**, 3rd and 4th lanes *versus* the 2nd lane). Phosphorylation of JAK1 in response to FGF-2 peaked at 5 minutes (2.7-fold increase) and returned to basal levels at 30 minutes (**Fig. 36 B**, 2nd and 4th bars, respectively). As controls, an "immunoprecipitation" reaction was set up with beads without antibody and another with beads and an irrelevant antibody of the same isotype of anti-JAK1 (**Fig. 36 A**, 1st and 6th lanes). We did not detect any phosphotyrosine signal corresponding to JAK1 on the IgG control and, reassuringly, the lane corresponding to the beads control was totally empty (**Fig. 36 A**, 6th and 1st lanes, respectively). An additional control for the specificity of anti-JAK1 was the immunoprecipitation of JAK1 from JAK1-negative cells (U4C) (Leaman *et al.*, 1998) and cells over-expressing JAK1 (U4C.JAK1, which are U4C cells complemented with JAK1) treated with IFN- γ , a well characterised activator of this kinase (**Fig. 36 C**). As anticipated, JAK1 was not detected in U4C cells (**Fig. 36 C**, 3rd lane) and there was an increase of tyrosine phosphorylation of JAK1 in samples from the complemented U4C.JAK1 cells treated with IFN- γ (**Fig. 36 C**, 1st *versus* 2nd lane). The constitutive phosphorylation of JAK1 in the complemented cells was almost certainly due to the over-expression of JAK1: this could be trivially ascertained by including in a repeat experiment the 2C4 parental cell line, from which U4C cells were derived (Muller *et al.*, 1993; Watling *et al.*, 1993).

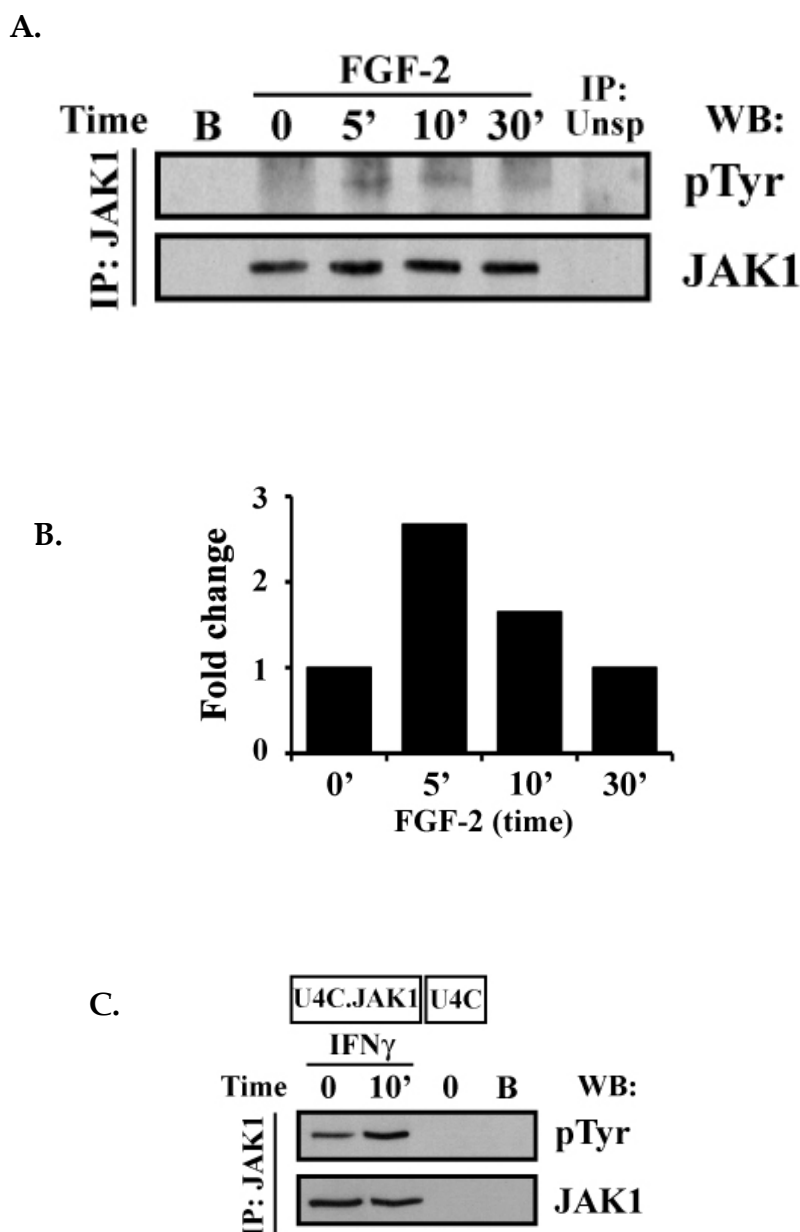


Figure 36. FGF-2 potentially activated JAK1 in U2OS cells. **A.** U2OS cells were starved (0.5% serum) overnight and incubated for one hour in serum-free media prior the experiments. Cells were incubated with FGF-2 (10 ng/ml) for the times indicated. JAK1 was immunoprecipitated from 3 mg of whole cell lysates and Western blotting analysis was performed using antibodies against phosphorylated tyrosines (PY20 and 4G10) and total JAK1. **B.** Mean of densitometric values from the experiment performed are shown in graphs. Values are expressed as fold change relative to untreated control. **C.** JAK1 was immunoprecipitated from U4C.JAK1 cells treated with IFN- γ (500 IU/ml), which were used as a positive control for the activation of JAK1. U4C (JAK1-negative) cells were used as a control for the antibodies used here. Western blotting analysis was performed as before. ' - minutes, h - hours, B - beads only, Unsp - unspecific. (I am indebted to Dr Ana Costa-Pereira for the experiment in Fig. 27 A).

In addition, in parallel, whole cell lysates corresponding to the samples used for the immunoprecipitation reactions and from siJAK1-transfected cells were run on a 7.5% SDS-PAGE gel, which was probed with antibodies raised against JAK1, pERK1/2 (to monitor FGF-2 functionality), ERK1/2 and β -actin (loading control) (data not shown). These experiments re-assured us about the suitability of 4G10 and PY20 in our studies and suggested that JAK1 is likely phosphorylated in response to FGF-2. This is currently a research priority in the Costa-Pereira group.

Numerous attempts were done to immunoprecipitate JAK2 from U2OS cells. Although we were able to immunoprecipitate JAK2 from these cells, for reasons that are not yet known, no reliable phospho-signal was detected in the positive control (i.e. cells treated with IFN- γ). Another approach was therefore taken: JAK2 was immunoprecipitated from cells over-expressing JAK2 (γ 2A.JAK2) (**Fig. 37**). γ 2A.JAK2 are JAK2-negative cells (γ 2A cells) stably transfected with a plasmid containing JAK2 (Watling *et al.*, 1993). JAK2 was immunoprecipitated from these cells upon treatment with FGF-2 for increasing periods of time. Cells treated with IFN- γ were used as positive controls for the activation of JAK2, as done before for JAK1. These preliminary results suggest that FGF-2 induced JAK2 tyrosine phosphorylation (**Fig. 37 A**, 1st lane *versus* 2nd-5th lanes). A modest increase of tyrosine-phosphorylated JAK2 (1.5-fold) was observed at 5-10 minutes of FGF-2 treatment (**Fig. 37 B**). As controls, a beads only immunoprecipitation was set up (**Fig. 37 A**, 8th lane) and JAK2 was also immunoprecipitated from γ 2A (JAK2 negative cells) (**Fig. 37 A**, 7th lane): no band was detected with the phospho-tyrosine antibodies or with a phospho-specific JAK2 antibody (pJAK2-Tyr^{1007/1008}), which indicated that the band detected by this antibody in the other samples, corresponded to phosphorylated JAK2. This suggested that FGF-2 has the potential to activate JAK2 in this cell line, as it does in BMVECs (Yang *et al.*, 2009). Optimisation of JAK2 immunoprecipitations will be carried out (initially using a different antibody and/or increasing the protein amount) so that the phosphorylation of JAK2 by FGF-2 can be further investigated in U2OS cells. This is highly relevant because enzymatic activity of JAK2 was required for FGF-2 rescue, as shown using an inhibitor of JAK2 (**Section 4.2.2.2.1**).

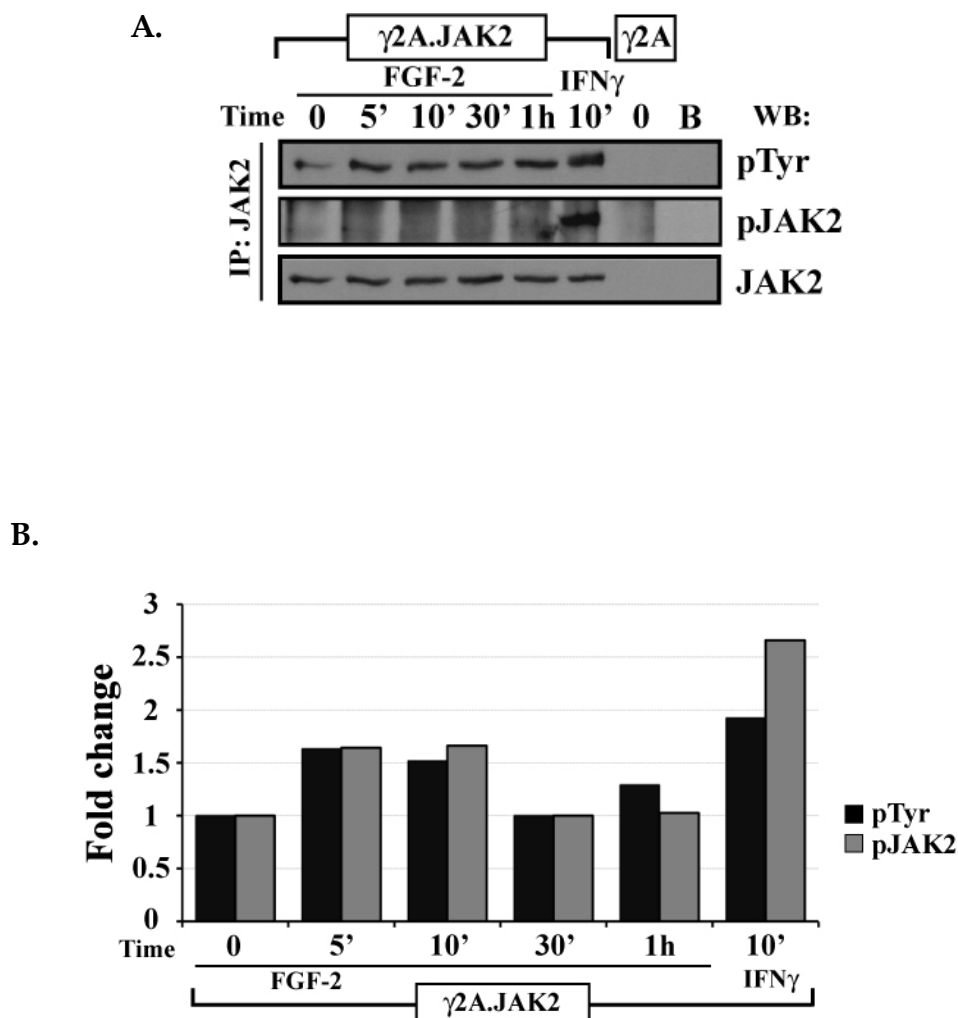


Figure 37. FGF-2 potentially activated JAK2 in γ 2A.JAK2 cells. **A.** Cells were starved (0.5% serum) overnight and incubated for one hour in serum-free media prior to experiments. Cells were incubated with FGF-2 (10 ng/ml) for the times indicated. JAK2 was immunoprecipitated from γ 2A.JAK2 cells (JAK2-negative cells (γ 2A) stably transfected with a plasmid containing JAK2). IFN- γ treatment (500 IU/ml) was used as a positive control for activation of JAK2. JAK2 was also immunoprecipitated from γ 2A cells that was used as a negative control. Western blotting analysis was performed with antibodies against phosphorylated tyrosines (PY20 and 4G10), phosphorylated JAK2 (Tyr^{1007/1008}) and total JAK2. **B.** Densitometric values of one single experiment are shown in graphs. Values are expressed as fold change relative to untreated controls. ' - minutes, h - hours, B - beads only.

Immunoprecipitation of TYK2 was also performed in U2OS cells. Cells were treated with FGF-2 for increasing periods of time and Western blotting analysis was performed using 4G10 and PY20 antibodies, as previously described. We observed that FGF-2 induced an increase of tyrosine phosphorylation of TYK2 (**Fig. 38 A**). This increase was significant after 5 minutes of incubation with FGF-2 ($p < 0.005$) (**Fig. 38 B**, 1st versus 2nd bar). The activation of TYK2 by FGF-2 in these cells seems to be transient as it returns to basal levels at the 30 minutes time point (**Fig. 38 B**, 1st versus 4th lane). As controls, immunoprecipitation with beads only and with an irrelevant antibody were performed. We did not detect any phospho-tyrosine signal or TYK2 in these samples (**Fig. 38 A**, 1st and 6th lanes). In parallel, whole cell lysates corresponding to the samples used for the immunoprecipitation reactions and from siTYK2-transfected cells were analysed by Western blot. Analysis of expression of TYK2, pERK1/2, ERK1/2 and β -actin (loading control) (data not shown), re-assured us that that FGF-2 was fully functional, that the results obtained using the 4G10 and PY20 antibodies were *bona fide* and that TYK2 was phosphorylated in response to FGF-2. Furthermore, experiments performed using TYK2-negative cells (U1A) (McKendry *et al.*, 1991) and U2OS cells treated with IFN- α (**Fig. 38 C**) also confirmed the suitability of the reagents used here. Indeed, there was an increase of tyrosine phosphorylation in samples from U2OS cells treated with IFN- α (a well characterised inducer of TYK2) (**Fig. 38 C**, 1st versus 2nd lane). No signal was detected in the TYK2-negative U1A cells. Interestingly, endogenously expressed TYK2 appears to be constitutively phosphorylated in U2OS cells and this was further inducible by ligands such as IFN- α and FGF-2. Like JAK1, this is now one of the Costa-Pereira group's research priorities.

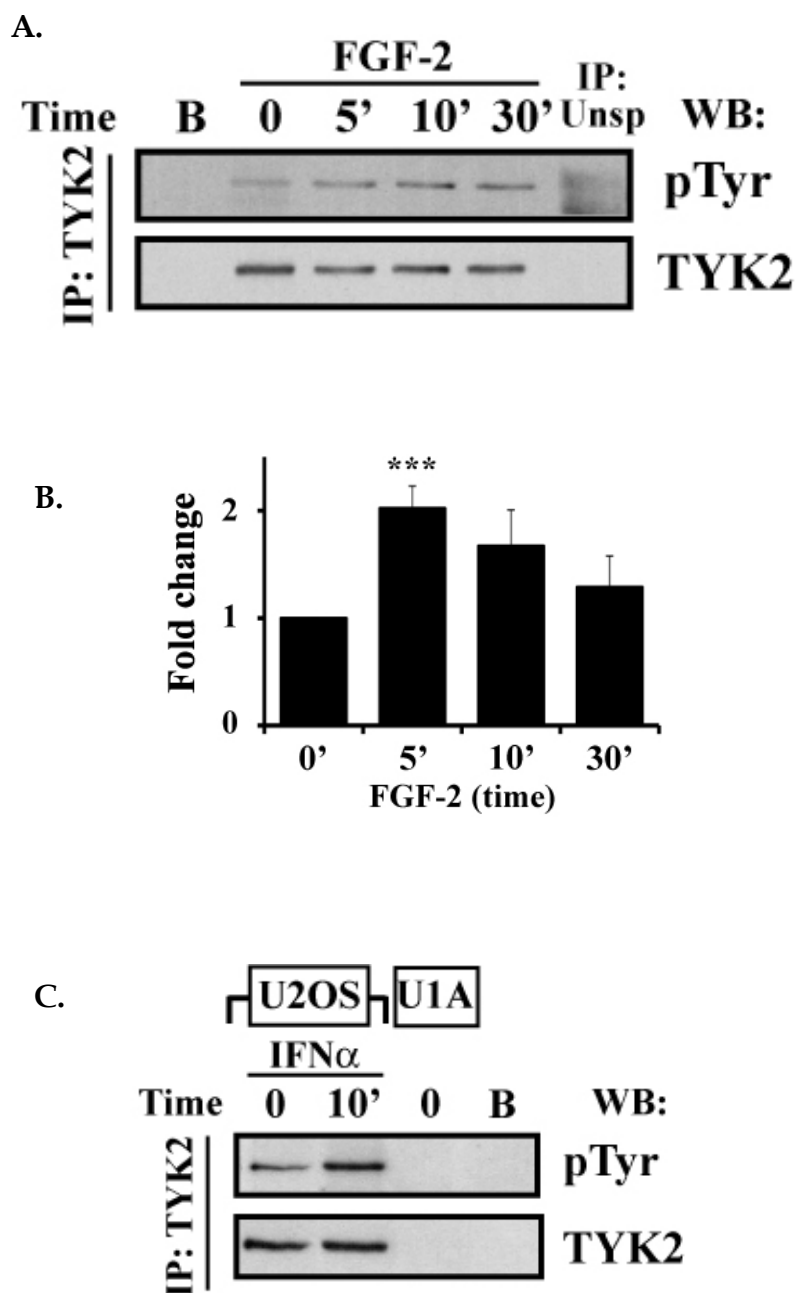


Figure 38. FGF-2 induced the phosphorylation of TYK2 in U2OS cells. **A.** U2OS cells were starved (0.5% serum) overnight and incubated for one hour in serum-free media prior the experiments. Cells were incubated with FGF-2 (10 ng/ml) for the times indicated. TYK2 was immunoprecipitated and Western blotting analysis was performed using antibodies against phosphorylated tyrosines (PY20 and 4G10) and total TYK2. **B.** Mean $\pm$ SEM of densitometric values from four independent experiments are shown in graphs. Values are expressed as fold change relative to untreated control. Statistical analysis was performed with Student's t-test (***) - $p < 0.005$ over untreated control). **C.** TYK2 was immunoprecipitated from U2OS cells treated with IFN- α (500 IU/ml) as a positive control for the activation of TYK2. U1A (TYK2-negative) cells were also used as a control for the antibodies. Western blotting analysis was performed as before. ' - minutes, h - hours, B - beads only, Unsp - unspecific. (I am indebted to Dr Ana Costa-Pereira for the experiments showed in Fig. 29 A and B).

4.2.5. A REQUIREMENT FOR JAKs IN FGF-2-MEDIATED CHEMORESISTANCE MECHANISM IN U2OS CELLS

FGF-2 was shown to protect U2OS cells from cisplatin-mediated cell death by a mechanism that also required JAK1, JAK2 and/or TYK2 (**Section 4.2.2.2**). We, therefore, investigated the requirement for these proteins in the early signalling cascades activated by FGF-2.

As ERK1/2 activity was necessary for FGF-2 rescue (**Section 4.1.6**) we investigated if JAKs were required for the activation of these proteins. Because FGF-2-mediated chemoresistance involved interactions between B-RAF, PKC ϵ and S6K2 (**Section 4.1.7**), we hypothesised that JAKs were also required for, or at least part of, these interactions. If this were the case, then it would be feasible that JAK1, JAK2 or TYK2 may be required for the up-regulation of anti-apoptotic proteins following FGF-2 treatment (**Section 4.1.9**).

4.2.5.1. KNOCKDOWN OF TYK2 AFFECTED FGF-2 INDUCED ACTIVATION OF ERK1/2

In U2OS cells, treatment with 10 ng/ml of FGF-2 for 10 minutes was shown to induce full activation of ERK1/2 (**Section 4.1.2**). To ascertain if JAKs mediated FGF-2-induced ERK1/2 phosphorylation, U2OS cells were transfected with siRNA against JAK1, JAK2 or TYK2 and subsequently treated with FGF-2. Treatments were done upon serum starvation (0.5% FCS overnight and serum-free medium for one hour prior to the experiment). ERK1/2 phosphorylation was monitored by Western blot as previously described using an antibody that recognises both phosphorylated ERK1 and ERK2. Using this approach, we established that the activation of ERK1/2 by FGF-2 was affected by the knockdown of TYK2 (**Fig. 39 A**, 8th *versus* 2nd and 10th lanes). Phosphorylation of ERK1 was reduced by an average of 40% ($p < 0.01$) and ERK2 by an average of 25% ($p < 0.005$) (**Fig. 39 B**, 4th black and grey bars, respectively). Knockdown of JAK1 also seemed to affect FGF-2 induced phosphorylation of ERK2 ($p < 0.005$), but the decrease observed was only approximately 10%. Transfection of U2OS cells with siRNA against JAK2 did not affect the induction of ERK1/2 in response to FGF-2.

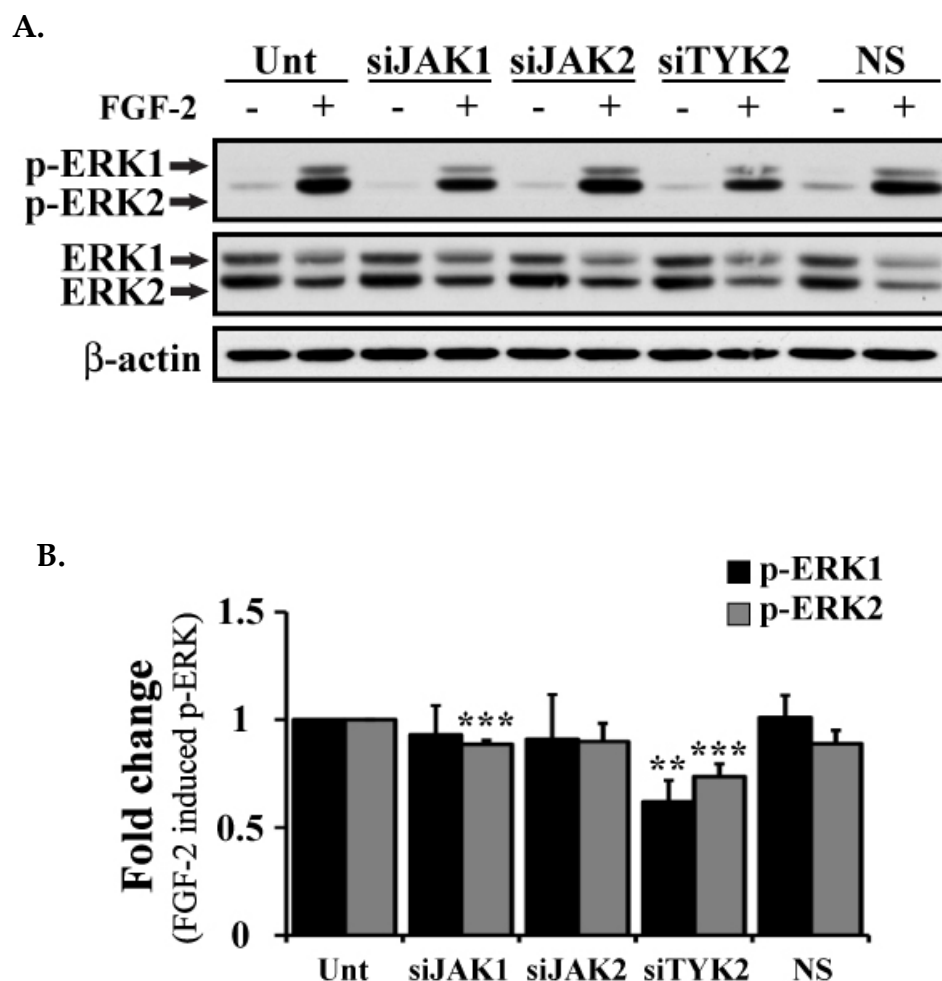


Figure 39. TYK2 knockdown in U2OS led to decreased ERK1/2 phosphorylation in response to FGF-2. **A.** U2OS cells were transfected with siRNA (75 nM) against JAK1, JAK2 or TYK2 using DharmaFECT II. Untransfected cells were used as a control. After 48 hours, cells were serum-starved (0.5% FCS) overnight, incubated in serum-free media for one hour and stimulated with 10 ng/ml of FGF-2 for 10 minutes. Proteins were extracted and separated on a 10% SDS-PAGE gel. Western blotting analysis was performed using total cell lysates and antibodies against p-ERK1/2 (Thr²⁰²/Tyr²⁰⁴) and total ERK1/2. β-actin was also probed and used as a loading control. **B.** Mean $\pm$ SEM of densitometric values of four independent experiments are shown in graphs. Values correspond to phospho-ERK1 and 2 levels and are expressed as fold change relative to untransfected controls. Statistical analysis was performed with Student's t-test (** - $p < 0.01$, *** - $p < 0.005$ versus untransfected FGF-2 treated control). Unt - untransfected.

Such small reductions warrant further investigation. Detection of phospho-ERK1/2 with alternative methods such as the Odyssey IR imaging system (LI-COR Biosciences), or flow cytometry (as in Ricciardi *et al.*, 2005) could further validate this observation. In the laboratory we have several cell lines that lack TYK2: MEFs kindly

provided by Dr Mathias Müller (Vienna, Austria) and human HT1080-derived U1A cells (McKendry *et al.*, 1991). We also have the corresponding wild-type and complemented cells. The use of these cells will be used to further confirm the effect that the lack of TYK2 has in FGF-2-induced ERK1/2 phosphorylation.

ERK1/2 Western blots showed differences between FGF-2 untreated and treated samples (**Fig. 39 A**, second row). This was also previously observed in other experiments (e.g. **Fig. 13** in **Section 4.1.2**, **Fig. 20 D** in **Section 4.1.5** and **Fig. 24 A** in **Section 4.1.6**). This difference is related to the fact that phosphorylation causes a mobility shift on the gel, which is often translated in less defined bands in FGF-2 treated samples. This is particularly noticeable in **Figure 24 A** where the band shift was not observed in samples pre-treated with a MEK inhibitor. In addition, these differences may also be due to incomplete stripping of the anti-phospho-ERK1/2 antibody. This antibody usually binds very strongly and it is particularly difficult to remove by membrane stripping. To avoid this, total ERK1/2 could have been studied in samples run on a separate parallel gel.

4.2.5.2. JAK1 AND TYK2 MAY BE REQUIRED FOR THE INTERACTION BETWEEN PKC ϵ AND B-RAF INDUCED BY FGF-2

FGF-2 induced interactions between B-RAF, PKC ϵ and S6K2 (**Section 4.1.7**). To investigate if JAKs were required for the interaction between these proteins in U2OS cells, JAK1, JAK2 and TYK2 were silenced by RNAi. Forty-eight hours later, cells were serum starved (0.5% FCS overnight and serum-free medium for one hour) and then treated with FGF-2. Proteins were isolated and B-RAF, PKC ϵ and S6K2 were independently immunoprecipitated. The immunoprecipitates were run on SDS-PAGE gels and, after Western blotting, the membranes were probed with antibodies against the three proteins. Due to time constraints, this experiment was only done once and the results are thus preliminary. In addition, additional controls should have been included in this experiment, such as cells transfected with a non-specific siRNA. In untransfected cells FGF-2 induced the interaction between B-RAF and PKC ϵ , as well as the interaction between S6K2 and B-RAF and S6K2 and PKC ϵ (**Fig. 40 A and B**). Taking it at face value, this particular experiment suggests that knockdown of JAK1, JAK2 and TYK2 impacted on the interactions between PKC ϵ , B-RAF and S6K2. It would appear that the knockdown of JAK1 induced the interaction of B-RAF with PKC ϵ in the

absence of growth factor (**Fig. 40 A**, 2nd *versus* 5th lane), that silencing JAK2 delayed the interaction upon FGF-2 stimulation (**Fig. 40 A**, 3rd *versus* 9th lane) and that reduced TYK2 levels blocked the interaction (**Fig. 40 A**, lanes 11-13). Interaction between S6K2 and B-RAF or, less convincingly, S6K2 and PKC ϵ was detected in untransfected cells and in cells transfected with siRNA molecules against JAK1, JAK2 or TYK2 (**Fig. 40 B**). Although the Western blots quality is poor it would appear that downregulation of JAK1 increased the interaction(s) between S6K2 and B-RAF and PKC ϵ (**Fig. 40 B**, 2nd *versus* 5th lane) and silencing of JAK2 and TYK2 transiently permitted interaction(s) between S6K2, B-RAF and PKC ϵ (**Fig. 40 B**, 2nd *versus* 8th and 11th lanes).

Total protein levels and activation of ERK1/2 by FGF-2 are shown in **Figure 40 C**. As previously observed (**Fig. 26 B**, **Section 4.1.7**) S6K2 increased in response to FGF-2 in untransfected cells. Variations on B-RAF, PKC ϵ and S6K2 protein levels induced by knockdown of JAK1, JAK2 or TYK2 are difficult to evaluate in a single experiment. Accordingly, this experiment will be repeated and, importantly, repeats will include samples from cells transfected with a non-specific siRNA as an additional control. In this particular experiment basal levels of phosphorylated ERK1/2 increased in cells transfected with siRNA against JAK1 (**Fig. 40 C**, 1st lane *versus* 4th lane in the 4th row). This was, however, not observed in all experiments (like the experiment shown in **Figure 39**). Such variations in basal levels of phosphorylated ERK could have an impact on the FGF-2 response. It is conceivable that increased phosphorylation of ERK1/2 could be related to the higher basal interaction of B-RAF with PKC ϵ , and of those molecules with S6K2 (**Fig. 40 A**, 5th lane and **B**, 4th lane). As observed before (**Section 4.2.5.1**), when TYK2 protein levels were reduced by RNAi, the activation of ERK1/2 in response to FGF-2 was lower compared to the untransfected cells (**Fig. 40 C**, 2nd and 3rd *versus* 11th and 12th lanes, respectively in the 4th row). In this particular experiment, siRNA against other JAKs also affected the phosphorylation of ERK1/2. However, we cannot exclude the possibility that the activation of ERK1/2 may have been affected by the siRNA transfection itself, as an unspecific control was not performed this time. These results warrant a more detailed investigation.

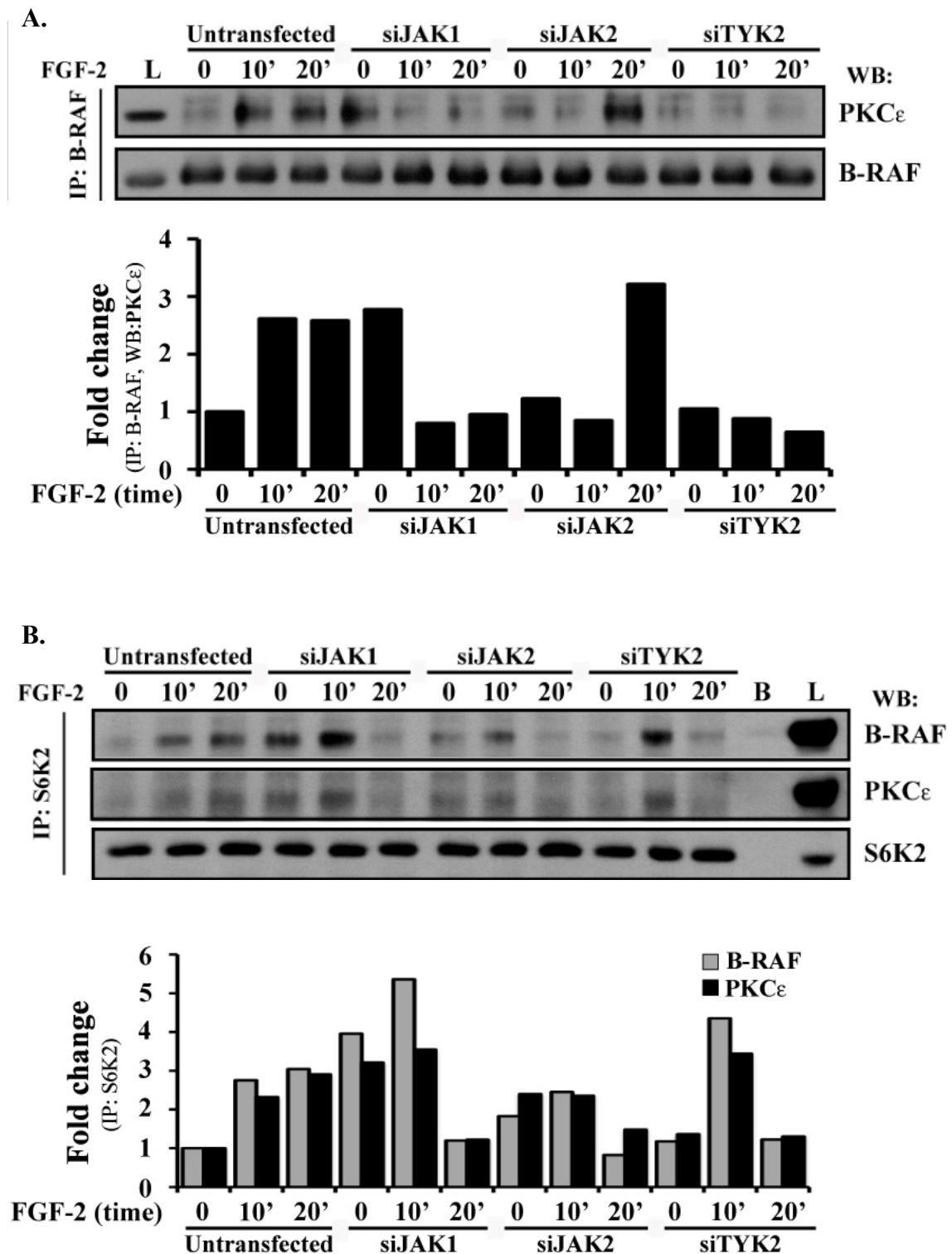


Figure 40. JAK1 and TYK2 may be required for the interaction between B-RAF and PKC ϵ induced by FGF-2 in U2OS cells. (Continued on page 170). Cells were plated in 10 cm plates and transfected with siRNA (75 nM) against JAK1, JAK2 or TYK2 for 48 hours. Subsequently, cells were plated in 15 cm plates (~4x10⁶ cells/plate), serum-starved (0.5% FCS) overnight and incubated in serum-free medium for one hour. After stimulation with 10 ng/ml of FGF-2 at the indicated times, proteins were extracted, separated on a 7.5% SDS-PAGE gel and analysed by Western blot. Untransfected cells were used as a control. Cell lysates were subjected to immunoprecipitation with antibodies against B-RAF (**A.**) or S6K2 (**B.**) and membranes were probed as indicated in the figure. **C.** Total cell lysates were Western blotted as indicated. β -actin was used as a loading control. Densitometric values from one experiment are represented in graphs. Values are expressed as fold change over untransfected untreated controls. Unt - untransfected, ' - minutes, B - beads only, L - lysate.

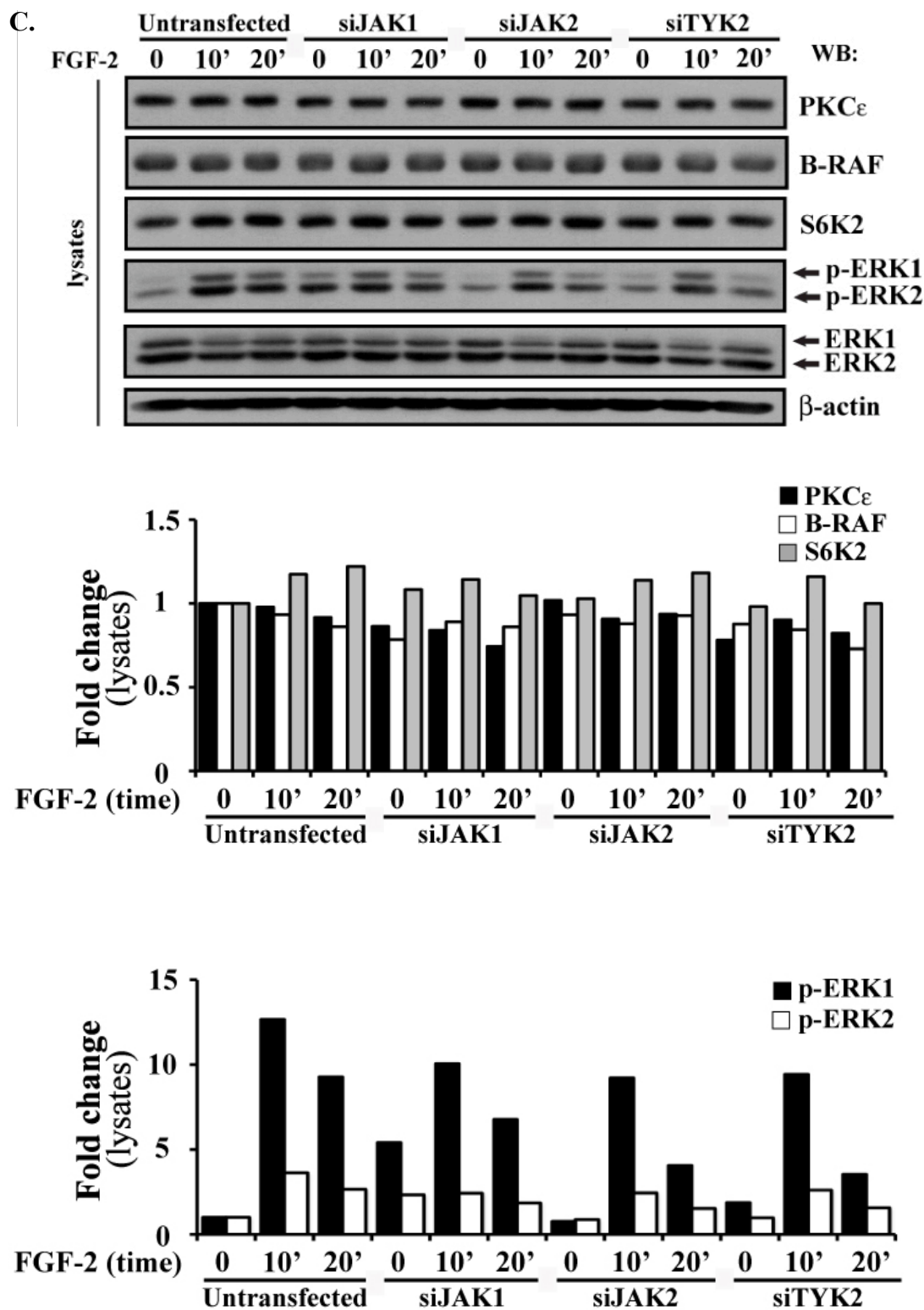


Figure 40. JAK1 and TYK2 may be required for the interaction between B-RAF and PKC ϵ induced by FGF-2 in U2OS cells. (Continued from page 169).

4.2.5.3 JAK1, JAK2 AND TYK2 INTERACTED WITH PKC ϵ AND B-RAF IN RESPONSE TO FGF-2

We sought to study the involvement of JAK1, JAK2 and TYK2 in FGF-2 mediated chemoresistance mechanism by investigating if these JAKs interacted with B-RAF and PKC ϵ or S6K2. Because JAKs are expressed at low levels in these cells, it was not trivial to detect them in the S6K2, B-RAF and PKC ϵ protein complex(es), when either of these molecules was immunoprecipitated. To address this, JAK1, JAK2 and TYK2 were independently immunoprecipitated after treatment with FGF-2 for increasing periods of time and the immunoprecipitates were run on SDS-PAGE gels. After Western blotting, membranes were probed for B-RAF, PKC ϵ and S6K2 (**Fig. 41**). Immunoprecipitated JAKs were used for normalisation. In U2OS cells, FGF-2 induced interactions of JAK1, JAK2 and TYK2 with B-RAF and PKC ϵ (**Fig. 41 A**).

In untreated cells, JAK1 was constitutively bound to PKC ϵ and B-RAF (**Fig. 41 A**, 1st lane, rows 2 and 3), as was JAK2 (**Fig. 41 A**, 1st lane, rows 5 and 6). FGF-2 induced a transient increase of the interaction between JAK1 and PKC ϵ and between JAK1 and B-RAF within 5 minutes of FGF-2 stimulation ($p < 0.005$ and $p < 0.05$, respectively), which after 10 minutes was not as evident (**Fig. 41 A**, 1st *versus* 3rd lane in rows 2 and 3, and **B**). In the experiment illustrated in **Figure 41 A**, the interaction between PKC ϵ and JAK1 was completely lost after 30 minutes of FGF-2 stimulation (**Fig. 41**, 5th lane in the 2nd row). This was not observed in one of the three experiments performed and this impacted on the statistical analysis (**Fig. 41 B**, 5th grey bar). It thus remains to be established whether this dissociation is real.

JAK2 interacted with PKC ϵ with kinetics identical to those observed for JAK1 ($p < 0.005$) (**Fig. 41 A**, 1st *versus* 2nd and 3rd lanes in the 5th row). JAK2 interacted with B-RAF more weakly than it did with PKC ϵ (an average of 1.5- *versus* 2-fold) but seemingly for a longer period, as shown by the statistical analysis at the 5, 10 and 20 minutes time points ($p < 0.05$) (**Fig. 41 A**, 2nd to 5th lanes in the 6th row, and **C**). Interestingly, no constitutive interaction(s) could be detected between TYK2 and PKC ϵ and/or B-RAF (**Fig. 41 A**, 1st lane, rows 8 and 9). Analysis of protein complex(es) induced in response to FGF-2 indicated that TYK2 interacted with PKC ϵ and B-RAF with different kinetics than those for JAK1 and JAK2 (**Fig. 41 A**). FGF-2-induced interaction between TYK2 and B-RAF was more gradual and prolonged than those observed for JAK1 and JAK2. Indeed, the interaction(s) could be observed between 5 and 20 minutes of stimulation ($p < 0.05$) (**Fig. 41 A**, 2nd to 4th lanes in the 9th row and **D**, black bars). The interaction between TYK2 and B-RAF reached a peak at 20 minutes (an

average of 2.7-fold induction) and was lost after 30 minutes incubation with FGF-2 (**Fig. 41 A**, cf. 5th lane in the 9th row, and **D**, black bars). PKC ϵ was only found to transiently interact with TYK2 at 20 minutes of FGF-2 stimulation ($p < 0.05$) (**Fig. 41 A**, 4th lane in the 8th row, and **D**, grey bars).

Although these experiments showed that JAKs interacted with B-RAF and PKC ϵ , they did not conclusively prove that JAK molecules bind B-RAF and PKC ϵ within the same complex. In addition, they did not rule out the possibility that several JAKs may bind B-RAF or PKC ϵ in the same complex. Experiments using siRNA will be conducted to further elucidate these points.

Membranes were probed first for PKC ϵ (95 kDa), after which the antibody was stripped of overnight (cf. Materials and methods) and then probed for B-RAF (94 kDa). To ascertain that the detection of B-RAF was not influenced by any residual unstripped PKC ϵ antibody some membranes were probed in reverse order. Similar results were obtained regardless the sequence of detection.

We were not able to detect S6K2 in any JAK immunoprecipitates. It is conceivable that low levels of S6K2 and JAKs, as well as stoichiometry, could have made these interactions in response to FGF-2 particularly difficult to detect. In addition, the binding of these proteins may be unstable and/or transient, in which case the interaction is likely to be 'lost' in the immunoprecipitation process, or particularly difficult to capture. The time points at which these experiments are performed may thus be crucial. Another less attractive hypothesis is that JAKs and S6K2 simply do not interact. All these possibilities are being currently investigated in the laboratory.

Routinely, in parallel with all co-immunoprecipitations, whole cell extracts were run on 7.5% SDS-PAGE gels and analysed by Western blot (**Fig. 41 E**). We monitored the levels of JAK1, JAK2, TYK2, B-RAF, PKC ϵ and S6K2 levels in samples used for the co-immunoprecipitation studies. As previously observed (**Fig. 26 B**, **Section 4.1.7**), treatment with FGF-2 increased S6K2 protein levels by an average of 1.5-fold ($p < 0.005$), which further suggests that FGF-2 can, somehow, affect S6K2 protein expression (**Fig. 41 E**, 8th row, and **G**, grey bars). JAK1, JAK2, PKC ϵ , B-RAF and TYK2 protein levels were not affected by treatment with FGF-2 (**Fig. 41 E**, 1st, 5th, 6th, 7th and 10th rows, respectively, **F** and **G**). We also monitored total and phosphorylated ERK1/2 in response to FGF-2 to ensure the functionality of the growth factor (**Fig. 41 E**, 2nd row, and **H**).

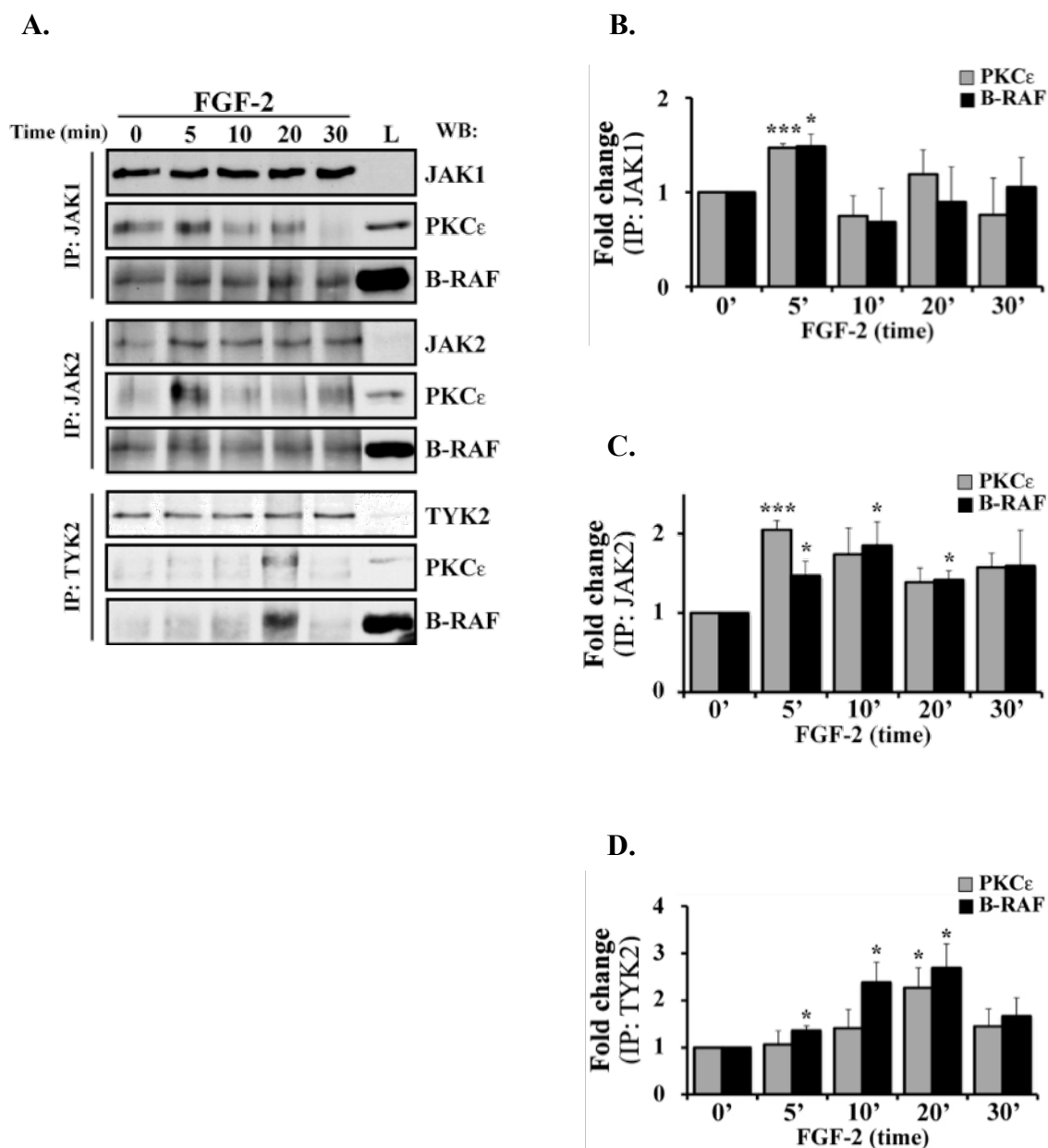
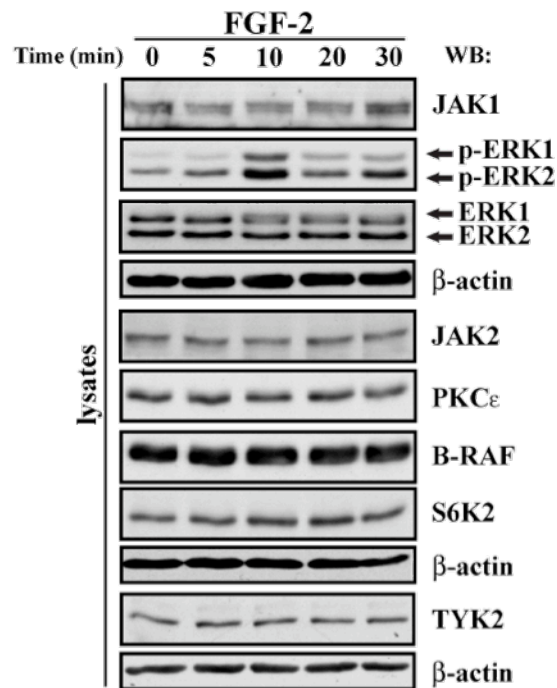
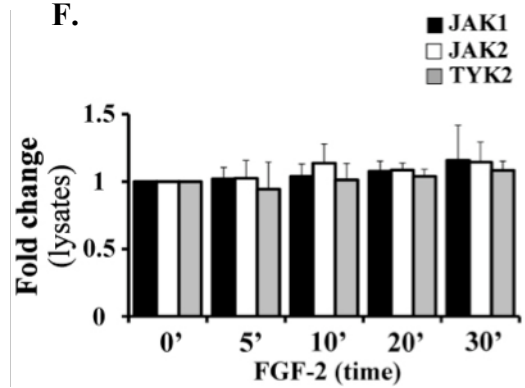


Figure 41. FGF-2 induced interaction of JAK1, JAK2 and TYK2 with PKC ϵ and B-RAF in U2OS cells. (Continued on page 174). U2OS cells were serum-starved (0.5% FCS) overnight, incubated in serum-free media for 1 hour and then stimulated with 10 ng/ml of FGF-2 at the indicated times. **A.** Cell lysates were subjected to immunoprecipitation with JAK1, JAK2 or TYK2 antibodies. Immunoprecipitates were separated on a 7.5% SDS-PAGE gel and analysed by Western blot. Membranes were probed for PKC ϵ and B-RAF. JAK1, JAK2 and TYK2 were also probed for and used as a loading controls for the respective immunoprecipitations. **E.** Total cell lysates were separated on a 7.5% SDS-PAGE gel and after Western blot, membranes were probed for PKC ϵ , B-RAF, S6K2, JAK1, JAK2, TYK2, p-ERK1/2 and ERK2. β -actin was used as a loading control. **B., C., D., F., G. and H.,** Mean $\pm$ SEM of densitometric values of three independent experiments are shown in graphs. Values are expressed as fold change over untreated controls. Statistical analysis was performed with Student's t-test (* - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.005$ *versus* control). L - lysate.

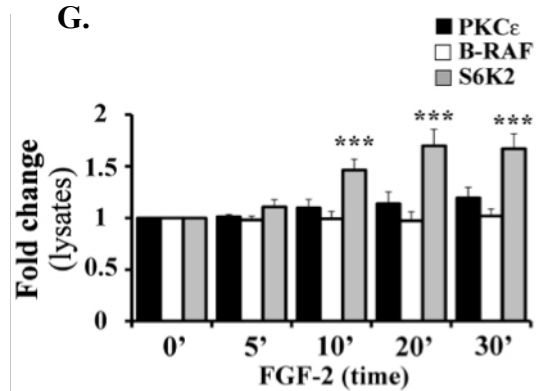
E.



F.



G.



H.

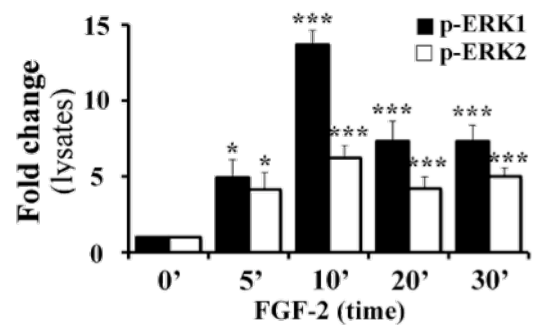


Figure 41. FGF-2 induced interaction of JAK1, JAK2 and TYK2 with PKCε and B-Raf in U2OS cells. (Continued from page 173).

4.2.5.4. TYK2 WAS REQUIRED FOR FGF-2-INDUCED UP-REGULATION OF THE ANTI-APOPTOTIC PROTEIN MCL-1

Having shown that JAKs were required for FGF-2-mediated chemoresistance in U2OS we then investigated whether up-regulation of anti-apoptotic proteins by FGF-2 was impaired upon silencing of JAKs. U2OS cells were transfected with siRNA against JAK1, JAK2 and TYK2 and subsequently treated with FGF-2 for 4 hours. Proteins were separated by SDS-PAGE and the expression of MCL-1 was monitored by Western blot. Untransfected cells and cells transfected with non-specific siRNA (NS) were used as controls. In untransfected cells, or cells transfected with a non-specific control FGF-2 induced a significant increase in MCL-1 protein levels ($p < 0.01$ and $p < 0.005$, respectively) (**Fig. 42 A**, 2nd and 12th lanes and **B**, 2nd and 12th bars). In the experiment shown in **Figure 42** this increase is not particularly evident for the NS control because the FGF-2-treated sample was under-loaded (**Fig. 42 A**, 12th lane).

Cells transfected with siRNA against ERK1/2 were also used as a control. The inhibition and knockdown of ERK1/2 impaired the FGF-2-mediated chemoprotection (**Fig. 24**, **Section 4.1.6** and data not shown), therefore, siRNA against ERK1/2 should impact on the upregulation of anti-apoptotic proteins in response to FGF-2. Reassuringly, silencing of ERK1/2 inhibited the up-regulation of MCL-1 protein (**Fig. 42 A**, 9th and 10th lanes, and **B**, 9th and 10th bars). Interestingly, JAK1 and JAK2 downregulation did not impair up-regulation of MCL-1 levels upon FGF-2 treatment ($p < 0.05$) (**Fig. 42 A**, 3rd-6th lanes and **B**, 3rd-6th bars). In cells with reduced levels of TYK2, however, MCL-1 expression did not increase in response to FGF-2 suggesting that this particular JAK is required for the up-regulation of MCL-1 (**Fig. 42 A**, 7th and 8th lanes and **B**, 7th and 8th bars). It remains to be established if this is also true for other proteins up-regulated in response to FGF-2 (**Fig. 28**, **Section 4.1.9**).

As previously observed (**Fig. 28**, **Section 4.1.9**), FGF-2-induced up-regulation of MCL-1 was modest (around 1.5-fold). This may have been due to proteins not being fully extracted from intracellular compartments with Schindler lysis buffer (cf. Material and methods). This buffer was developed mainly to extract cytoplasmatic proteins (Schindler *et al.*, 1992) and BCL-2 family members are predominantly associated with the mitochondria membrane (e.g. Breckenridge and Xue, 2004). Extraction with a more stringent buffer containing for example SDS (0.1%) and sodium deoxycholate (0.5%) (e.g. RIPA buffer) will disrupt membranes and potentially allow for a better, more efficient, protein extraction.

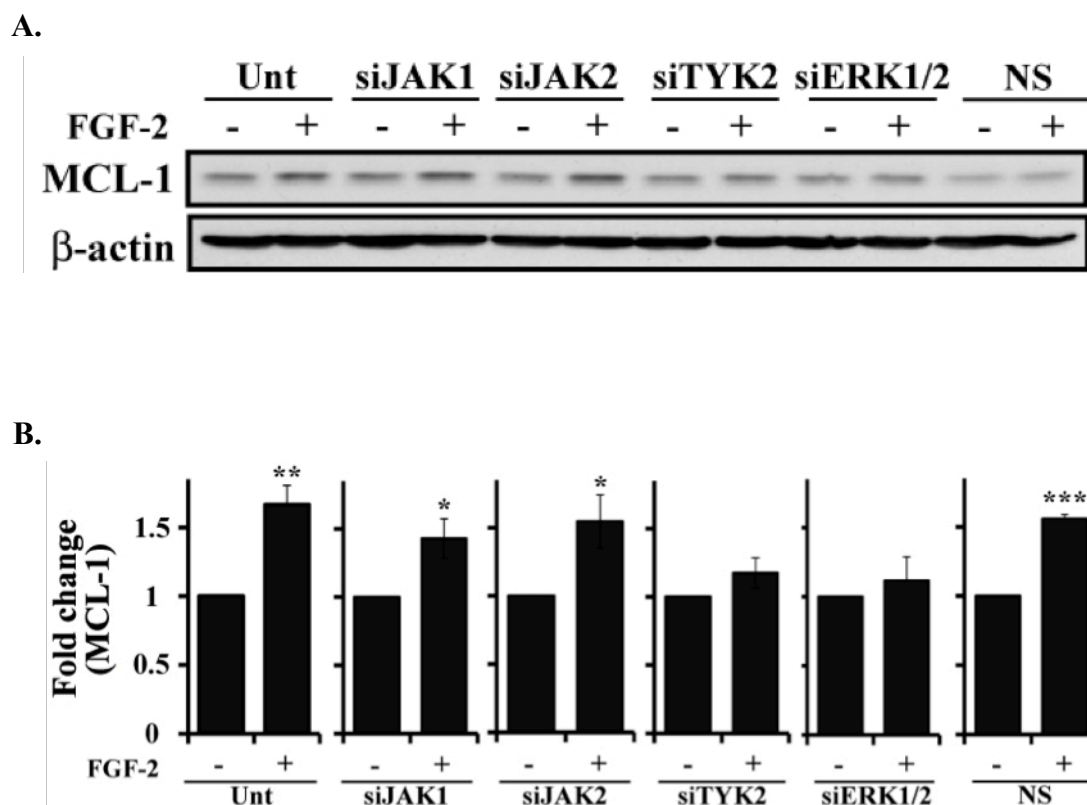


Figure 42. TYK2 is required for the up-regulation of the anti-apoptotic protein MCL-1 induced by FGF-2 in U2OS cells. **A.** U2OS cells were transfected with 75 nM of siRNA against JAK1, JAK2 or TYK2 using DharmaFECT II. After 48 hours, cells were serum-starved (0.5% FCS) overnight and incubated in serum-free media for 1 hour. Cells were stimulated with 10 ng/ml of FGF-2 for 4 hours. Proteins were extracted and separated on a 12.5% SDS-PAGE gel. Western blotting analysis was performed using total cell lysates and an antibody against MCL-1. β -actin was used as a loading control. **B.** Mean $\pm$ SEM of densitometric values of three independent experiments are shown in graphs. Values are expressed as fold change relative to untransfected controls. Statistical analysis was performed with Student's t-test (* - $p < 0.05$, *** - $p < 0.005$ versus untreated controls).

4.2.6. DISCUSSION

Results from the previous chapter showed that FGF-2 protected the osteosarcoma cell line U2OS from cisplatin-mediated apoptosis (**Section 4.1.5**) through a MEK-ERK-dependent mechanism (**Section 4.1.6**). Early signalling events downstream of FGF-2 in this cell line involved the phosphorylation of ERK1/2 (**Section 4.1.2**), the interaction between B-RAF and PKC ϵ and those with S6K2 (**Section 4.1.7**), and the up-regulation of a number of anti-apoptotic proteins (**Section 4.1.9**). Here, we investigated the requirement for JAKs and/or STATs in FGF-2 chemoprotection in U2OS cells. The requirement for JAK1, JAK2, TYK2, STAT1, STAT3 and/or STAT5 was investigated using RNAi. After verifying that these molecules were specifically silenced using siRNA (**Fig. 29**), we evaluated the impact of the knockdown each of these proteins on FGF-2-mediated chemoresistance mechanism.

Knockdown of JAK1, JAK2 or TYK2 in U2OS cells profoundly affected FGF-2-mediated protection from cisplatin killing of U2OS cells. In cells with reduced levels of JAK1, JAK2 or TYK2, FGF-2 was not able to protect cells from cisplatin's noxious effects (**Fig. 30** and **31**) suggesting that these three proteins were required for the mechanism by which FGF-2 drives chemoresistance. These results were confirmed by analysis of DNA loss by flow cytometry (cell cycle analysis) (**Fig. 30**) and analysis of caspases' cleaved substrates, such as PARP (Western blot) (**Fig. 31**). These findings further strengthen the notion that JAKs play critical roles in tumour progression (Verma *et al.*, 2003). Interestingly, in cells with decreased levels of JAK1, JAK2 and TYK2, FGF-2 reduced basal levels of cell death (**Fig. 30**). This may be related to FGF-2 being able to protect cells from reduced levels of cell death in the absence of JAKs, or JAK-dependent FGF-2 protection being specific to cisplatin-induced cell death. We confirmed these observations for siJAK1- and siJAK2-transfected cells using additional experimental approaches such as PARP cleavage analysis (**Fig. 31**). In siTYK2-transfected cells, there was no significant difference in PARP cleavage due to one particular experiment, which skewed the statistical analysis. Accordingly, this should be studied further. The use of JAK-negative cells will also be useful to confirm these observations.

JAK3, another JAK family member was not yet studied in this context. Unlike the other three JAK family members, which are ubiquitously expressed, JAK3 is preferentially expressed in leukocytes (reviewed by Schindler *et al.*, 2008). It is currently unknown if U2OS cells express JAK3. Constitutive activation of this JAK has been reported in several lymphoproliferative disorders (Gee *et al.*, 2001; Yared *et al.*, 2005; Lai *et al.*, 2005; Tomita *et al.*, 2006; Krejsgaard *et al.*, 2006; Marzec *et al.*, 2008). In addition, mutant JAK3 alleles were identified in AML patients and resulted in the constitutive activation of JAK3 and a spectrum of downstream pathways that include STAT5, PI3K/AKT pathway and RAS/MAPK pathway (Walters *et al.*, 2006). JAK3 activation was also found to contribute for the oncogenesis of other types of cells such as colon cancer (Lin *et al.*, 2005) and breast cancer (Kim *et al.*, 2009). Accordingly, we will investigate if the osteosarcoma cell line U2OS expresses JAK3 and if this JAK also participates in FGF-2-mediated chemoresistance.

Inhibition of JAK2 activity with a specific inhibitor of JAK2 autophosphorylation (Sandberg *et al.*, 2005) also blocked FGF-2-mediated chemoresistance (**Fig. 32**), indicating that the activity of this particular JAK may be required for FGF-2 signalling. Interestingly, in the presence of the inhibitor, FGF-2 did not reduce basal cell death (**Fig. 32 B and D**). In contrast, in siJAK2-transfected cells, FGF-2 significantly reduced the basal cell death (**Fig. 30 and 31**). This may be due to a more efficient inhibition of JAK2 activity by this inhibitor in comparison with the incomplete knockdown by siRNA (which was never 100%) (**Fig. 29**). In addition, although this is a specific JAK2 inhibitor, it may also block the activity of other JAKs that may be required for FGF-2 survival. Studies with additional JAK inhibitors will be useful to confirm this observation and to investigate the requirement of the activity of other JAK family members for FGF-2 responses. It would be interesting to investigate if JAKs are required in additional types of cancer cell lines in which FGF-2 was demonstrated to play a role in chemoresistance such as SCLC and breast cancer cell lines (Pardo *et al.*, 2002; Pardo *et al.*, 2003; Vandermoere *et al.*, 2005; Smaragda Angelidou and Michael Seckl, personal communication).

Activation of JAKs has been previously shown to occur downstream of growth factors. For example JAK1, JAK2 and TYK2 can be activated by PDGF (Vignais *et al.*, 1996) and JAK2 was shown to be activated by FGF-2 (Yang *et al.*, 2009). This is the first report showing that JAKs were required for FGF-2 mediated chemoresistance. In accordance with what many authors have proposed, our work provides further evidence that supports the notion that the inhibition of JAKs may be helpful for the successful treatment of cancer.

STATs, in particular STAT1, STAT3 and STAT5, were shown to contribute to oncogenesis (reviewed by Yu and Jove, 2004). STAT3 was also associated with intrinsic drug resistance (Kovacic *et al.*, 2006; Patterson *et al.*, 2006; Barre *et al.*, 2007; Grisko *et al.*, 2006; Ikuta *et al.*, 2005; Lewis and Ward, 2008). Surprisingly, we have observed that the STATs were not absolutely required for the mechanism by which FGF-2 protects cells against cisplatin (**Fig. 33** and **34**). Furthermore, under the conditions tested, FGF-2 did not induce STAT1, STAT3 or STAT5A/B phosphorylation/activation in U2OS cells (**Fig. 35**) in contrast to data shown in murine lens epithelial cell lines and BMVECs (Ebong *et al.*, 2004; Yang *et al.*, 2009). Our observations suggested that STAT1, STAT3 and STAT5A/B were not activated by FGF-2 in the osteosarcoma cell line U2OS, and therefore, are unlikely to be involved in FGF-2-mediated chemoresistance mechanism. The possibility that the loss of a particular STAT is compensated by another member of the STAT family cannot, nevertheless, be excluded at this time.

Other STAT family members (STAT2, STAT4 and STAT6) can also potentially play a role in chemoresistance. STAT2 signalling was shown to play a critical role in inflammatory responses in carcinogenesis (Clifford *et al.*, 2003; Maher *et al.*, 2008). STAT6 signalling was also implicated in tumour immuno-surveillance (Terabe *et al.*, 2000) and it was shown to play a role in the regulation of cell proliferation and apoptosis in colon cancer cells (Zhang *et al.*, 2006). However, in breast cancer cells, STAT6 was required for IL-4-mediated growth inhibition and the induction of apoptosis (Gooch *et al.*, 2002). Although these STATs do not seem to have as a prominent role in cancer biology as STAT1, STAT3 and STAT5, one cannot exclude the possibility that they may also be involved in chemoresistance. The requirement for STAT2, STAT4 and STAT6 for the FGF-2-mediated chemoresistance will be investigated further in the future.

Here we showed that FGF-2 induced the phosphorylation of TYK2 in U2OS (**Fig. 38**). This phosphorylation was rapid and transient like it is in response to many cytokines and growth factors (Novak *et al.*, 1995; Leaman *et al.*, 1996; Vignais *et al.*, 1996; Gual *et al.*, 1998). Preliminary data indicate that FGF-2 potentially induce the phosphorylation of JAK1 in this cell line and of JAK2 in another cell line (γ 2A.JAK2) (**Fig. 36** and **37**). These data suggest that TYK2, and potentially JAK1 play roles downstream of FGF-2 in U2OS cells. The activation of JAK2 by FGF-2 in U2OS cells is currently unknown and will be investigated in the future. Considering that the enzymatic activity of JAK2 seemed to be required for efficient FGF-2 chemoprotection (**Fig. 32**), we hypothesised that JAK2 may also be phosphorylated in response to FGF-2 in U2OS cells. The activation of TYK2 downstream of FGF-2 receptors may be direct or

indirect. As for cytokine receptors (Rane and Reddy, 2000), JAKs might physically interact with FGF-2 receptors. Usually, JAKs associate with membrane-proximal receptor motifs *via* the FERM domain located in their N-terminal region (Chen *et al.*, 1997; Gauzzi *et al.*, 1997; Yamaoka *et al.*, 2004). FGFRs have tyrosine residues on the tyrosine kinase domains of their cytoplasmic tail that become phosphorylated upon activation (Bellot *et al.*, 1991), which provide sites for the direct binding of secondary messenger molecules with SH2 domains (Dailey *et al.*, 2005). JAKs have a predicted SH2-related domain and they might use this region to bind to FGFRs. The possible interaction of JAKs with FGF-2 canonical receptors will be, therefore, investigated.

Activation of ERK1/2 by FGF-2 in U2OS cells was not abrogated by lack of JAK1, JAK2 or TYK2. This activation was, however, significantly reduced ($p < 0.01$ for ERK1 and $p < 0.005$ for ERK2) in cells transfected with siRNA against TYK2 (**Fig. 39**). Despite not being a dramatic reduction (approximately 40% for ERK1 and 25% for ERK2), it was significant and consistently observed. Such a reduction is likely to impact on FGF-2 response, as the strength and duration of ERK signalling can determine distinct biological outcomes (Murphy and Blenis, 2006). For example, lowering ERK signal strength and duration was shown to have an impact on the expression of genes such as *Jun* and *Myc*, and consequently on the proliferative response in mouse fibroblasts (Murphy *et al.*, 2004). Decreased phosphorylation of ERK1/2 in response to FGF-2 observed in siTYK2-transfected cells suggested that TYK2 was likely to act upstream of MEK/ERK signalling pathway and may be required for ERK1/2 full activation. Alternatively, in the absence of TYK2, ERK1/2 activation by FGF-2 may be more transient (happening at a time point earlier than 10 minutes and rapidly decreasing) or be delayed. Treatment of siTYK2-transfected cells with FGF-2 for additional periods of time will be useful to clarify this issue. TYK2 can also have a structural role in the response and may be required for stabilising FGF-2 receptor(s), as was shown for the IFN- α receptor 1 (IFNAR1) (Ragimbeau *et al.*, 2003). Indeed, cells that lack TYK2 also have reduced levels of IFNAR1, the receptor chain to which TYK2 binds. In this case, it was shown that TYK2 has a scaffolding role. The fact that knockdown of JAK1 and JAK2 had no such effect on the activation of ERK1/2 by FGF-2 suggested that these two JAKs likely play roles either downstream, or in parallel to MEK/ERK signalling pathway(s).

Preliminary data obtained here suggested that JAK1 and TYK2 might be required for the interaction between B-RAF and PKC ϵ in response to FGF-2 in U2OS cells (**Fig. 40 A**). However, the knockdown of JAK1, JAK2 and TYK2 did not affect the ability of S6K2 to interact with B-RAF or PKC ϵ in response to FGF-2 (**Fig. 40 B**). These

preliminary data suggest that these three JAKs are not required for the binding of S6K2 to B-RAF and PKC ϵ . Although these results need to be confirmed, they suggest that JAK1 and TYK2 may be required for the interaction and/or stability of interaction of B-RAF with PKC ϵ in response to FGF-2. It is also possible that direct interaction between B-RAF and PKC ϵ is essential for efficient FGF-2-mediated chemoprotection explaining why FGF-2 is not able to rescue siJAK1- and siTYK2-transfected cells. It needs to be established if B-RAF, PKC ϵ and S6K2 are in the same complex formed upon FGF-2 stimulation in U2OS cells. It may be possible that S6K2 molecules that bind B-RAF are not the same molecules that bind PKC ϵ . Accordingly, we will investigate if FGF-2 induces the interaction between B-RAF and PKC ϵ in the absence of S6K2.

It is possible that B-RAF, PKC ϵ and S6K2 are phosphorylated/activated in U2OS in response to FGF-2, as observed in SCLC cells (Pardo *et al.*, 2001; Pardo *et al.*, 2006). The activation of B-RAF requires the phosphorylation of a threonine (Thr⁵⁹⁸) and a serine (Ser⁶⁰¹) residue located within its activation segment (Zhang and Guan, 2000). PKC ϵ kinase activity correlates with the phosphorylation of a threonine residue within the activation loop of the kinase domain (Thr⁵⁶⁶) and of a serine residue (Ser⁷²⁹) within the C-terminal loop (Parekh *et al.*, 1999; Cenni *et al.*, 2002), which is critical for stabilising the kinase core in an active conformation (Newton, 2003). S6K2 activation is absolutely dependent on phosphorylation of a threonine located within the catalytic domain (Thr²²⁸) and a threonine located within the linker domain (Thr³⁸⁸) (Phin *et al.*, 2003). The phosphorylation of a serine residue located within the linker domain site (Ser³⁷⁰) also plays a role in the activity of the shorter form of S6K2 (Minami *et al.*, 2001; Phin *et al.*, 2003). Because JAKs are tyrosine kinases (Levy and Darnell, 2004; Yamaoka *et al.*, 2004) it is unlikely that they catalyse the phosphorylation of B-RAF, PKC ϵ or S6K2. However, we observed that JAK1, JAK2 and TYK2 interacted with B-RAF and PKC ϵ upon FGF-2 stimulation (**Fig. 41**). JAK1 and JAK2 interacted with these proteins after 5 minutes of stimulation with FGF-2 and TYK2 seemed to interact in a different fashion. Indeed, B-RAF interacted with TYK2 after 5 minutes of incubation with FGF-2 and this interaction was still evident at 20 minutes, whereas PKC ϵ significantly bound to TYK2 only after 20 minutes of FGF-2 stimulation. Interaction of JAK1 and JAK2 with B-RAF and PKC ϵ at a different time point than that of TYK2 suggested that different JAKs (at least TYK2 compared to JAK1 and JAK2) do not play redundant roles in the early signalling events triggered by FGF-2 in U2OS cells. It would be interesting to know if JAKs also interact with S6K2, a protein that we were not able to detect in JAK immunoprecipitates. Low levels of S6K2 and JAKs, as well as stoichiometry of the

interaction, may have hindered the detection of these interactions in response to FGF-2. Another possibility is that JAKs and S6K2 do not interact.

JAKs may be required for bringing at least B-RAF and PKC ϵ together, functioning as chaperone proteins. These interactions may facilitate their activation and/or function or establishment of their intracellular localisation. Alternatively, JAKs may be required for the interaction of B-RAF and PKC ϵ (and possibly S6K2) with the translation machinery that led to up-regulation of anti-apoptotic proteins. It will be crucial to determine if JAKs are required for the activity of B-RAF, PKC ϵ and S6K2, and consequently for their function. The hypothetic roles that JAKs could have in this mechanism will be studied further in the future.

FGF-2 was shown to up-regulate MCL-1 in U2OS cells (Janet Lyons-Lewis and Michael Seckl, personal communication and **Fig. 28, Section 4.1.9**). This may be of particular importance, as MCL-1 was found highly expressed in osteosarcoma patients and its down-regulation induced apoptosis in osteosarcoma cell lines (Pignochino *et al.*, 2009). In cells with reduced levels of TYK2 FGF-2 did not significantly increase the expression of MCL-1 (**Fig. 42**). This may be related to reduced ERK1/2 activation by FGF-2 in siTYK2-transfected cells. Indeed, inhibition of ERK activity by MEK inhibitors was shown to result in down-regulation of MCL-1 in H-510 and U2OS cells (Janet Lyons-Lewis and Michael Seckl, personal communication and data not shown), human pancreatic cell lines (Boucher *et al.*, 2000), and melanoma cell lines (Wang *et al.*, 2007b). It remains to be established if this is also true for other proteins up-regulated in response to FGF-2. The knockdown of JAK1 and JAK2 did not affect the up-regulation of MCL-1 by FGF-2 (**Fig. 42**). Interestingly, JAK2 and JAK3 were reported to be involved in the activity of members of the BCL-2 family such as BCL-2 and BCL-X_L, in some cases through STAT-independent pathways (Packham *et al.*, 1998; Wen *et al.*, 2001). Accordingly, the knockdown of JAK1 and JAK2 may affect other anti-apoptotic proteins that might also be required for the FGF-2-mediated mechanism.

The results obtained thus far suggest that JAK1, JAK2 and TYK2 are required for the FGF-2-mediated chemoresistance. These three JAKs are likely involved in the regulation of proteins such ERK1/2, B-RAF, PKC ϵ and S6K2 and may thus be important modulators of the FGF-2 response. More importantly, our study has provided evidence for a key role of JAKs in FGF-2 anti-apoptotic pathway(s).

Whilst analysing a negative control for an RNAi experiment, we observed that the ERK2 knockdown caused a concomitant reduction in STAT3 protein levels, a fact that, to the best of our knowledge, has not been previously described. This was an

unexpected result, but one that due its novelty and relevance to cancer was further pursued (**Section 4.3**).

4.3.

REGULATION OF STAT3 BY ERK2

4.3.1. INTRODUCTION

In U2OS cells FGF-2 was shown to mediate chemoresistance to cisplatin by a MEK/ERK-dependent mechanism. We observed that STAT1, STAT3 and STAT5 were not absolutely required for the mechanism by which FGF-2 protects cells against cisplatin because in their absence FGF-2 was able to protect cells from cisplatin-mediated cell death (**Fig. 33 and 34, Section 4.2.2.3**). While analysing these data, we identified a novel interplay between STAT3 and ERK2. Curiously, ERK2 (but not ERK1) knockdown in U2OS cells caused a concomitant reduction in STAT3 levels. This suggested that ERK2 might be involved in the regulation of STAT3 protein levels, a fact that, to the best of our knowledge, had not been described yet. Our finding brings potentially new perspectives to the regulation of STAT3, a STAT family member with a prominent role on oncogenic signalling pathways (Yu and Jove, 2004). In addition, during the course of investigation, we observed that the knockdown of ERK2 also affected IL-6 signalling. Our hypothesis is that the ERK2 is a regulator of STAT3 and gp130, the later the signal transducing unit of IL-6-type cytokines receptors. ERK2 may be acting at the level of transcription, translation or post-translational by, for example, stabilising these proteins. In this part of the work **we aimed to further characterise the impact of ERK2 knockdown on STAT3, gp130 and IL-6 signalling and to determine the nature of the requirement for ERK2 in the control of STAT3 and gp130 expression.**

One of the regulators of the *STAT3* gene is STAT3 protein itself (Ichiba *et al.*, 1998). Indeed, in response to IL-6, STAT3 activity is required for transcription of the *STAT3* gene and subsequent STAT3 protein synthesis, thereby generating a positive signalling loop. Although ERK2 has not been previously shown to transcriptionally regulate the *STAT3* gene *per se*, it is known that ERK1/2 can regulate STAT3 transcriptional activity (Jain *et al.*, 1998; Tian and An, 2004). STAT3 phosphorylation on residue Ser⁷²⁷ was shown to be ERK-dependent when induced by EGF (Chung *et al.*, 1997) and, in this context, negatively modulated its tyrosine phosphorylation, which resulted in the inhibition of STAT3 DNA binding and transcriptional activity in COS cells. Serine phosphorylation of STAT3 induced by IL-6, however, was shown to be ERK-independent (Chung *et al.*, 1997). In addition to MAPK, many other kinases have

now been shown to phosphorylate STAT3 Ser⁷²⁷, and it is generally accepted that the kinase involved is highly cell type- and context-dependent (Decker and Kovarik, 2000).

ERK1 and ERK2 are very similar proteins, with a sequence homology of 83% (Chen *et al.*, 2001). They are activated by the same upstream kinases (MEK1 and MEK2), display identical kinetics of action and seem to have the same substrate specificities (for example RSK family members) (reviewed by Roux and Blenis, 2004). The observation that ERK2 but not ERK1 affected STAT3 protein levels strongly suggest that ERK1 and ERK2 have, in fact, non-overlapping functions. This further strengthens reports that indicate that ERK1 and ERK2 do not have exclusively cellular redundant roles. Importantly, mutation of the *erk2* gene in mice leads to early embryonic lethality (Saba-El-Leil *et al.*, 2003), while *erk1*^{-/-} mice are viable and reproduce normally, presenting just small defects such as a deficit in thymocyte maturation (Pages *et al.*, 1999). It has been suggested that *erk2* can compensate for most of the functions of *erk1*, but the contrasting phenotypes could in fact indicate that the two *erk* isoforms may also have distinct functions (Aouadi *et al.*, 2006).

Here we have established a role for ERK2 (but not ERK1) in the regulation of STAT3. We excluded the possibility of a siRNA off-target effect by deconvolution of the ERK2 siRNA pool, and extended this observation to other cell lines other than U2OS cells. We then investigated if the kinase activity of ERK2 was required for STAT3 expression. We also studied how IL-6/gp130 signalling was affected by reduced ERK2 cellular levels.

4.3.2. KNOCKDOWN OF ERK2 BY siRNA REDUCED STAT3 PROTEIN LEVELS IN U2OS CELLS

The knockdown of ERK2 by siRNA used as an experimental control in this study revealed a very interesting and intriguing result: ERK2 knockdown appeared to affect STAT3 protein levels. Cells were transfected with siRNA oligonucleotides against ERK2, and protein levels were assessed by SDS-PAGE/Western blot. **Figure 43 A** shows the expression of STAT3 and STAT1 in U2OS cells upon transfection with siERK2. As a control for the STAT3 antibody, cells were also transfected with siSTAT3 oligonucleotides (**Fig. 43 A**, 2nd lane and **B**, 3rd lane). Because STAT3 and STAT1 are highly homologous and have the similar molecular weights (both are 91 kDa), two parallel SDS-PAGE gels were run. The Western blot membranes were probed for with

antibodies against STAT3, STAT1 and ERK1/2, the latter as a read-out for silencing efficiency of siERK2. Both membranes were probed for β -actin, which was used as a loading control. Cells with reduced levels of ERK2 showed a concomitant 50% decrease in STAT3 protein ($p < 0.005$) (**Fig. 43 D**, 5th bar) without any apparent effect on STAT1 levels (**Fig. 43 C**, 3rd bar).

Having observed this, the initial experiment (**Fig. 43 A**) was repeated and, from this point on, cells were also transfected with siERK1 so that the impact of silencing ERK1 on STAT3 levels could also be investigated (**Fig. 43 B**, 4th lane). ERK1 silencing was highly efficient ($p < 0.005$) (**Fig. 43 F**, 4th bar), but without effect on STAT3 and ERK2 protein levels (**Fig. 43 D and E**, 4th bars). In contrast, siERK2-transfected cells had approximately 50% less STAT3 than untransfected cells, which was highly statistically significant ($p < 0.005$) (**Fig. 43 D**, 5th bar). ERK2 was very efficiently silenced by siERK2 ($p < 0.005$) and ERK2 expression was not affected by transfection with siERK1, or siSTAT3 as compared to the untransfected and mock-transfected controls (**Fig. 43 E**, 5th bar *versus* 1-4th bars). Interestingly, there was a statistically significant increase ($p < 0.05$) in ERK1 levels upon downregulation of ERK2 (**Fig. 43 F**, 5th bar). Controls used included untransfected and mock-transfected cells (cells transfected with the transfection reagent DharmaFECT II only). Ideally, a non-specific siRNA control should also have been included. Indeed, at the time these experiments were conducted, the controls provided by Dharmacon affected a variety of signalling pathways and cell systems. They were thus deemed inappropriate. We now have siRNA against luciferase, which has proven to be a more appropriate 'non-specific' siRNA control and is routinely used by the Costa-Pereira Group in all RNAi experiments.

It would be interesting to investigate the impact of the knockdown of ERK2 (and, indeed, ERK1) on the expression of other STAT family members. This is currently being addressed by Ms Nair Bonito, a new PhD student, who is continuing work on this aspect of my PhD research work.

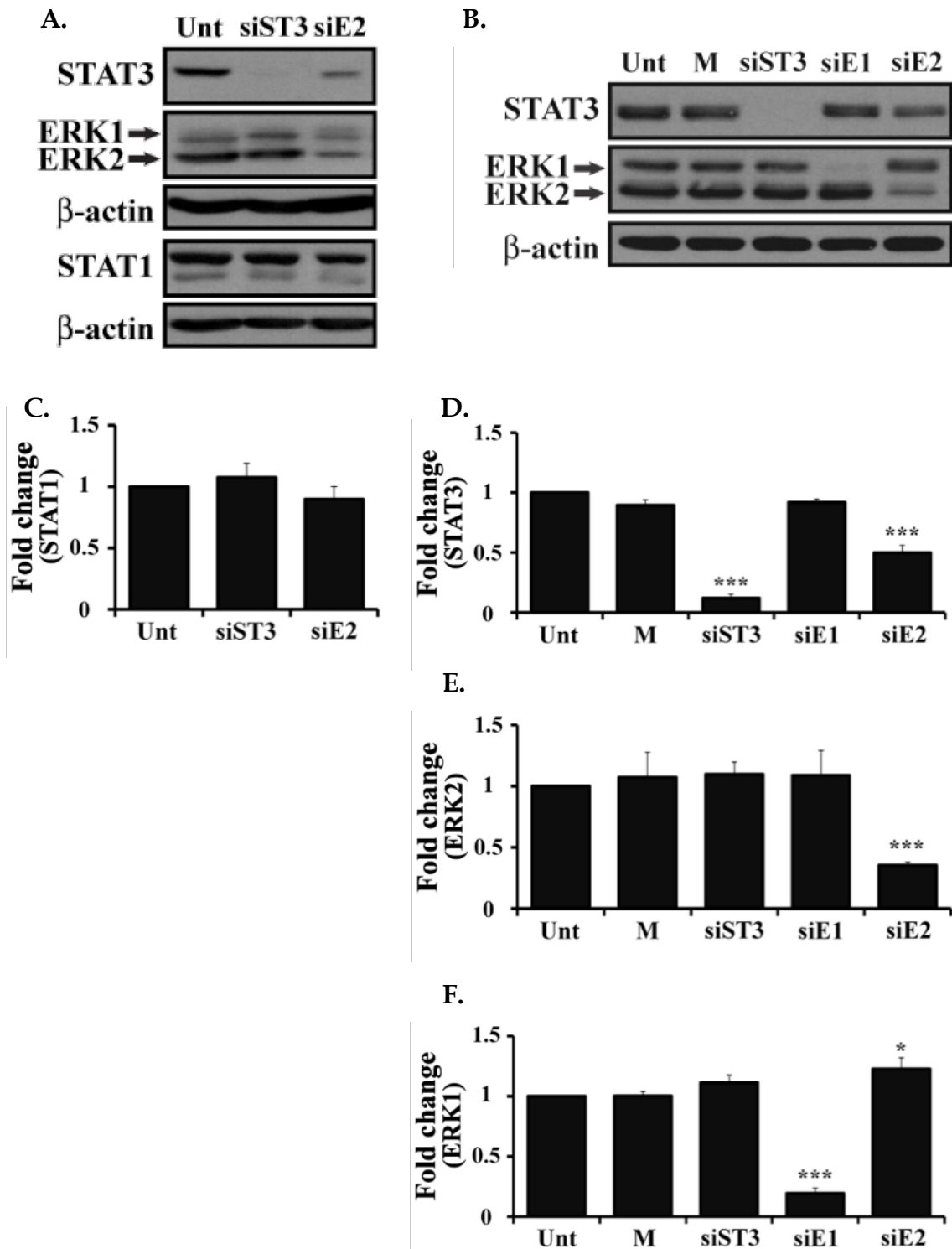


Figure 43. siRNA against ERK2 (but not ERK1) reduced STAT3 protein levels in U2OS cells. U2OS cells were transfected with 75 nM of siRNA using DharmaFECT II. After 48 hours, proteins were extracted and separated on a 7.5% SDS-PAGE gel. Total cell lysates were Western blotted as indicated. **A.** Cells were initially transfected with siRNA against STAT3 and ERK2. The Western blot membranes were probed for ERK1/2, STAT1 and STAT3. β-actin was also probed and used as a loading control. **B.** Cells were transfected with siRNA against STAT3, ERK1 or ERK2 as described previously. Untransfected and mock-transfected cells were used as base-line controls. β-actin was used as a loading control. The Western blot membranes were probed for ERK1/2 and STAT3. β-actin was also probed and used as a loading control. **C., D., E., F.** Mean ± SEM of densitometric values of four independent experiments are shown in graphs. Values are expressed as fold change relative to untransfected controls. Statistical analysis was performed with Student's t-test (* - $p < 0.05$, *** - $p < 0.005$ versus untransfected control). Unt - untransfected, M - mock transfected, ST3 - STAT3, E1 - ERK1, E2 - ERK2.

To rule out the possibility of off-target effects elicited by the siRNA oligonucleotides against ERK2, we deconvoluted the ERK2 siRNA pool and analysed the phenotype induced by the transfection with each oligonucleotide independently. Out of the four oligonucleotides, two had an identical effect to that induced by the siRNA pool, and these correlated to ERK2 protein knockdown levels (**Fig. 44 A**, 2-5th lanes). Transfection with oligonucleotides number 1 and 9 provided the best ERK2 knockdown (at similar levels to the knockdown in cells transfected with the siRNA pool) (**Fig. 44 C**, 2nd and 5th bars), and in these cells we observed a significant reduction of STAT3 protein levels (**Fig. 44 B**, 2nd and 5th bars). siRNA against ERK2 was also carried out with a different pool of oligonucleotides (ON-TARGETplus from Dharmacon) and identical results were obtained (data not shown).

Overall, the results strongly suggested that the diminished STAT3 protein levels were a consequence of a decrease in ERK2 and not the result of an off-target effect.

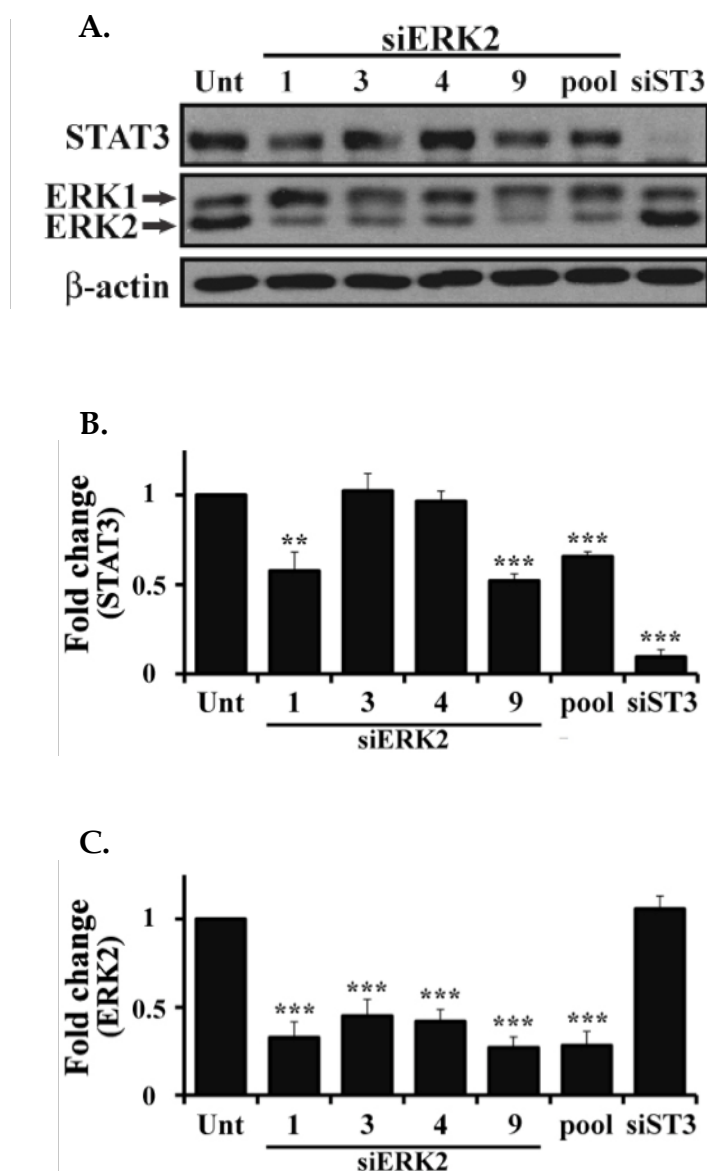


Figure 44. Decrease of STAT3 protein levels in cells transfected with individual siRNA oligonucleotides against ERK2 correlated with the degree of ERK2 knockdown. **A.** U2OS cells were transfected with 75 nM of siRNA against ERK2 (oligonucleotides 1, 3, 4 and 9, or a pool of these four) and STAT3 using DharmaFECT II. After 48 hours, proteins were extracted and separated on a 7.5% SDS-PAGE gel. Western blotting analysis was performed using total cell lysates and the membrane was probed with antibodies against ERK1/2 and STAT3. β -actin was also probed and used as a loading control. **B.** and **C.** Mean $\pm$ SEM of densitometric values of four independent experiments are shown in graphs. Values are expressed as fold change relative to untransfected controls. Statistical analysis was performed with Student's t-test (** - $p < 0.01$, *** - $p < 0.005$ *versus* untransfected control). Unt - untransfected, ST3 - STAT3.

4.3.2.1 KNOCKDOWN OF ERK2 ALSO REDUCED STAT3 PROTEIN LEVELS IN OTHER CELL LINES

In order to determine whether this was a cell type-specific phenomenon we investigated if the effect of ERK2 knockdown on the levels of STAT3 protein observed in U2OS could also be seen in other cell lines. Here we used two NSCLC cell lines (A549 and HCC193). These NSCLC cell lines were different from the ones used in the first part of this study (**Section 4.1**) because when performing siRNA experiments we observed that HCC95 cells were not efficiently transfected, and the transfection of HCC78 cells led to the up-regulation of STAT1. Accordingly, HCC95 and HCC78 were considered unsuitable for these experiments.

A549 and HCC193 cells were transfected with siRNA against ERK2 and STAT3. The levels of STAT3 were analysed by SDS-PAGE/Western blot (**Fig. 45 A**). Similarly to what was observed in U2OS cells, decreased levels of ERK2 (**Fig. 45 C and E**, 3rd bars) caused a concomitant decrease in STAT3 protein levels in these cell lines (**Fig. 45 A**, 3rd and 6th lanes in the 1st row). We observed an average 33% reduction ($p < 0.01$) in STAT3 protein levels in A549 cells transfected with siRNA against ERK2 (**Fig. 45 B**, 3rd bar), and an average of 46% reduction ($p < 0.05$) in STAT3 protein levels in HCC193 cells transfected with siRNA against ERK2 (**Fig. 45 D**, 3rd bar). siSTAT3-transfected cells were used as an antibody control (**Fig. 45 B and D**, 2nd bars).

This suggests that the decrease in STAT3 levels in siERK2-transfected cells was a general phenomenon, not exclusively observed in the U2OS cell line, and may thus have far-reaching biological implications. It would be interesting to analyse the levels of STAT3 in ERK2-negative cells. This would be useful to further confirm the results obtained with siRNA (upon which the knockdown efficiency was never 100%).

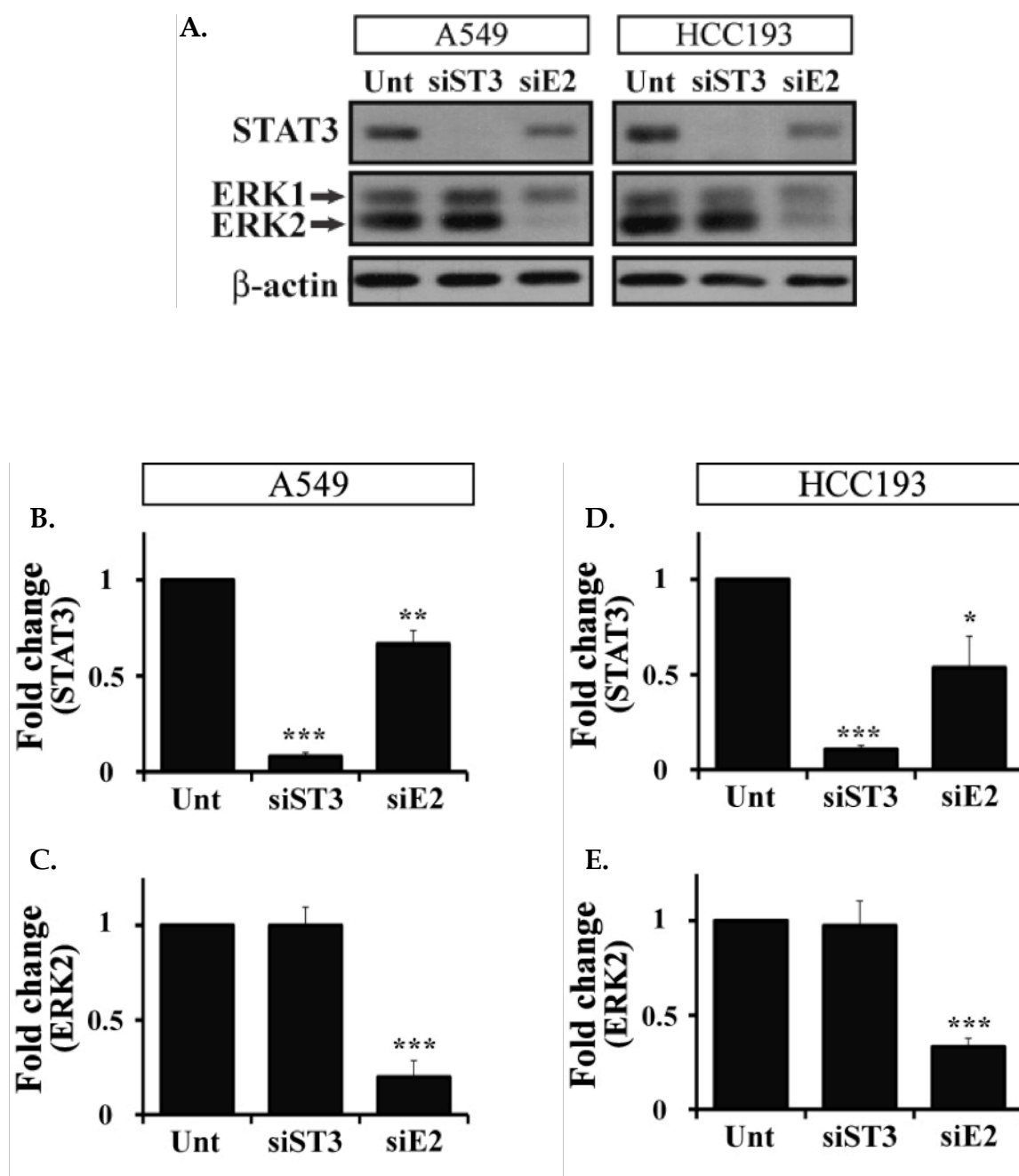


Figure 45. siRNA against ERK2 reduced STAT3 protein levels in lung cancer A549 and HCC193 cells. **A.** U2OS cells were transfected with 75 nM siRNA against STAT3 and ERK2 using DharmaFECT II. After 48 hours, proteins were extracted and separated on a 7.5% SDS-PAGE gel. Western blotting analysis was performed using total cell lysates, and the membrane was probed with antibodies against ERK1/2 and STAT3. β-actin was also probed and used as a loading control. **B., C., D. and E.** Mean ± SEM of densitometric values of three independent experiments are shown in graphs. Values are expressed as fold change relative to untransfected controls. Statistical analysis was performed with Student's t-test (* - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.005$ versus untransfected control). Unt - untransfected, ST3 - STAT3, E2 - ERK2.

4.3.3. THE INHIBITION OF ERK1/2 ACTIVITY BY MEK INHIBITORS DID NOT AFFECT STAT3 PROTEIN LEVELS IN U2OS CELLS

Since the knockdown of ERK2 caused a decrease in STAT3 levels we next asked whether this was due to the lack of ERK2 enzymatic activity or due to reduced levels of ERK2 protein itself.

Accordingly, U2OS cells were treated with PD0325901, a MEK1/2 specific inhibitor, and the levels of STAT3 measured by SDS-PAGE/Western blot (**Fig. 46**). Treatment of U2OS cells with this inhibitor for 6, 18 or 24 hours blocked the basal activation of ERK1/2 (**Fig. 46 C and D**). Under the conditions used here, inhibition of MEK-ERK activity did not affect STAT3 protein levels (**Fig. 46 B**). This suggests that the requirement for ERK2 in maintaining STAT3 protein levels was independent of its enzymatic activity.

ERK2 has been previously shown to regulate several transcription factors and consequently gene expression (e.g. Whitmarsh *et al.*, 1995). It is thus possible that it may regulate STAT3 transcription. Although transcription normally requires ERK enzymatic activity (e.g. Chen *et al.*, 2001) we cannot exclude the possibility that ERK2 may do it independently of its activation status as seen in the yeast *Saccharomyces cerevisiae* (Bardwell *et al.*, 1998). STAT3 protein levels may also be regulated by ERK2 in other ways. For example, ERK2 may regulate STAT3 translation or may have a scaffolding role, stabilising it or protecting it from degradation.

The transfection of cells with a negative-dominant ERK2 plasmid will be an additional approach to address this question and further confirm this observation. If the activation of ERK2 is not needed for the maintenance of STAT3 protein levels, we would expect that the transfection with such a plasmid would not affect STAT3 expression levels. An inactive ERK2 plasmid is currently available in the Costa-Pereira Group together with additional plasmids containing wild type ERK2, inactive ERK1 and wild type ERK1, which will be use as controls.

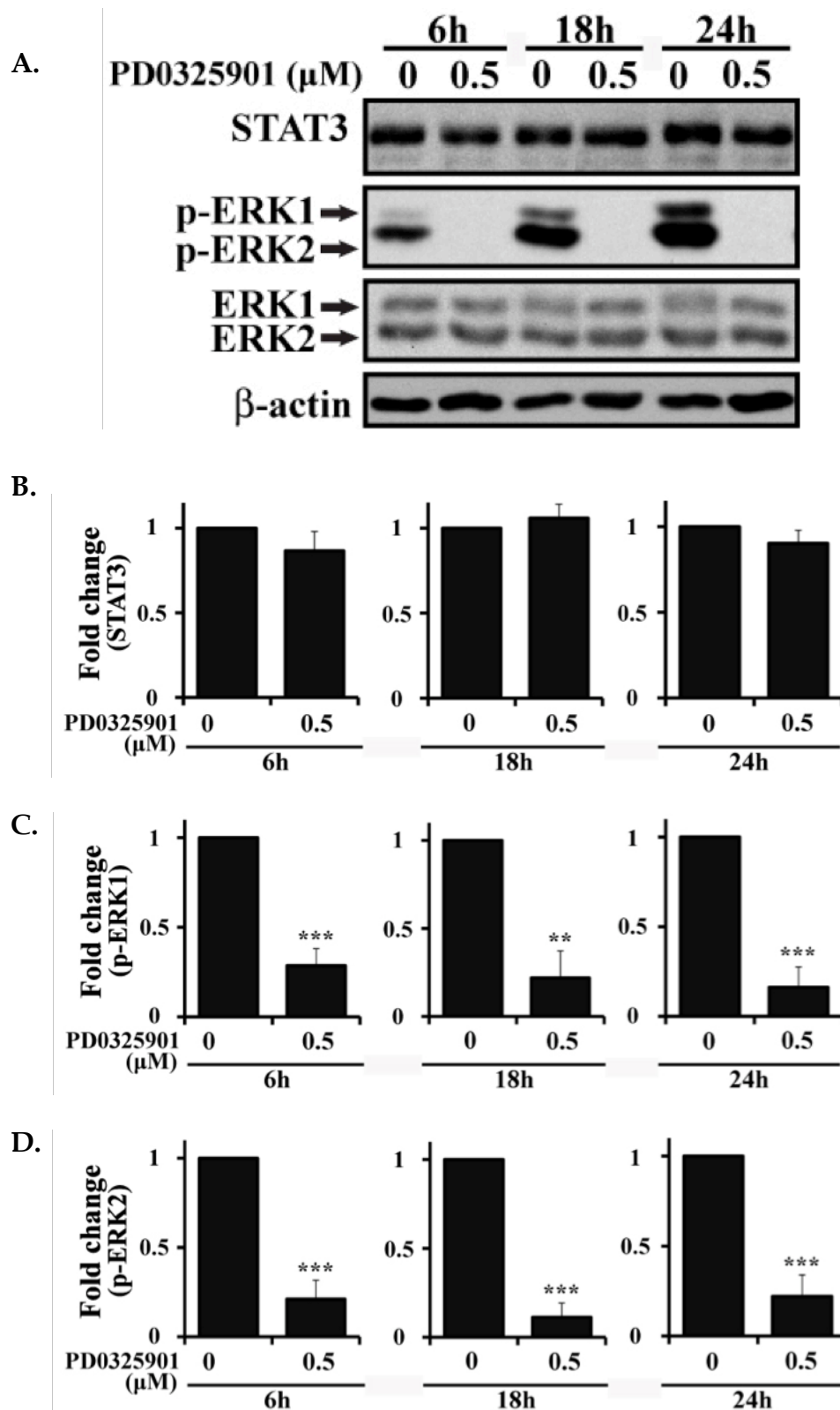


Figure 46. Inhibition of MEK/ERK did not affect STAT3 protein levels in U2OS cells. U2OS cells were pre-treated with the indicated doses of PD0325901 for 6, 18 or 24 hours. Proteins were extracted and separated on a 10% SDS-PAGE gel. **A.** Western blotting analysis of STAT3 levels was performed using total cell lysates and the membrane was probed with antibodies against STAT3, pERK1/2 (Thr²⁰²/Tyr²⁰⁴) and total ERK1/2. β -actin was used as a loading control. **B., C., D.** Mean $\pm$ SEM of densitometric values of three independent experiments are shown in graphs. Values are expressed as fold change relative to untransfected controls. Statistical analysis was performed with Student's t-test (** - $p < 0.01$, *** - $p < 0.005$ *versus* control). h - hours.

4.3.4. KNOCKDOWN OF ERK2 AFFECTED IL-6 SIGNALLING IN U2OS CELLS

STAT3 is the main transcription factor in the IL-6 pathway (Heinrich *et al.*, 2003). IL-6 can also activate STAT1 but only transiently and to a much lesser extent than it activates STAT3 (Stahl *et al.*, 1995). We have hypothesised, therefore, that if STAT3 protein levels were sufficiently diminished as a consequence of the reduced levels of ERK2 then the IL-6 pathway should also be affected. It was shown previously that in the absence of STAT3, IL-6 can mediate a completely different cytokine response (Costa-Pereira *et al.*, 2002). Indeed, in cells that lack STAT3 IL-6 mediates an IFN- γ -like response, including the prolonged activation of STAT1 and the induction of multiple ISG γ . We thus investigated whether the IL-6-induced STAT1 phosphorylation was in any way affected by the ERK2 knockdown.

U2OS cells were transfected with siRNA against ERK2 as described before. Cells transfected with siRNA against ERK5, another ERK family member and cells transfected with siRNA against STAT3 were used as controls (ERK5 as a 'non-specific' siRNA control and siSTAT3 as an antibody control). After incubation with siRNA for 48 hours cells were treated with 200 ng/ml of IL-6 together with 250 ng/ml of sIL-6R for 15 minutes. sIL-6R is a receptor subunit that together with gp130 form the receptor complex by which IL-6 binds and signals in cells devoid of the sIL-6R chain (e.g. Heinrich *et al.*, 1998). This subunit is normally found in human plasma and is mainly expressed by hepatocytes, monocytes and some lymphocytes (Honda *et al.*, 1992; Muller-Newen *et al.*, 1996). sIL-6R can also be secreted by some cancer cells, such as myeloma (Gaillard *et al.*, 1993). In this experiment, sIL-6R was added together with IL-6 because U2OS cells do not express it (Ana Costa-Pereira, personal communication). Cells treated with 500 IU/ml of IFN- γ were used as a control for a robust, prolonged, STAT1 activation. Proteins were extracted, run on a SDS-PAGE gel and analysed by Western blotting.

We observed that in untransfected cells, STAT1 was activated differently by IFN- γ and IL-6, being stronger in response to IFN- γ than in response to IL-6 (**Fig. 47 A**, 2nd and 3rd lanes in the 1st row and **47 D**, 2nd and 3rd bars). In accordance with what was observed by other authors (Qing and Stack, 2004) IFN- γ also induced a weak but significant activation of STAT3 ($p < 0.05$) (**Fig. 47 A**, 2nd lane in 3rd row and **47 D**, 2nd bar). In contrast, in siSTAT3-transfected cells the activation of STAT1 by IL-6 was stronger ($p < 0.05$) and almost comparable to that induced by IFN- γ (**Fig. 47 B**, 3rd *versus* 6th bar). This observation was expected and is in agreement with previously published data by our laboratory (Costa-Pereira *et al.*, 2002). The knockdown of ERK5 did not affect the ability of IL-6 to activate STAT1 or STAT3 and these cells behaved effectively

as if they were untransfected (**Fig. 47 B and D**, 9th bars). Surprisingly, phosphorylation of STAT1 in response to IL-6 in siERK2-transfected cells was significantly lower than IL-6-induced phosphorylation in untransfected cells ($p < 0.01$) (**Fig. 47 B**, 3rd *versus* 12th bar) or in cells transfected with siSTAT3 ($p < 0.01$) or siERK5 ($p < 0.05$) (**Fig. 47 B**, 6th and 9th *versus* 12th bar). It was, in fact, undetectable by the naked eye (**Fig. 47 A**, 12th lane in the first row). STAT1 protein levels were not affected by siRNA against STAT3, ERK5 and ERK2 (**Fig. 47 C**). STAT3 phosphorylation by IL-6 in cells with reduced ERK2 was also significantly reduced comparing to IL-6-induced STAT3 phosphorylation in untransfected ($p < 0.005$) or siERK5-transfected cells ($p < 0.01$) (**Fig. 47 D**, 3rd and 9th *versus* 12th bar). This was presumably because total protein levels of STAT3 were decreased in these cells (**Fig. 47 E**, 12th bar). ERK2 protein levels were significantly decreased by the siERK2 transfection ($p < 0.005$) but were not affected by siRNA against STAT3 and ERK5 (**Fig. 47 F**, 10-12th *versus* 1-9th bar).

ERK5 total protein levels seemed to be decreased in cells transfected with siRNA against STAT3 (**Fig. 47 G**). This was significant in cells subsequently treated with cytokines ($p < 0.05$) (**Fig. 47 G**, 5th and 6th bars). ERK5, also called big MAPK (BMK) for being more than twice the size of the other MAPK family members is one of the less well-characterised MAPKs (Wang and Tournier, 2006). To the best of our knowledge, there are no reports suggesting that STAT3 regulates, directly or indirectly, ERK5 protein levels. This observation could represent an unknown role for STAT3, a fact that merit future investigation. The specificity of STAT3 siRNA oligonucleotides has been extensively investigated in the laboratory (Ana Costa-Pereira and Diane Watling, personal communication) by deconvolution and use of additional siRNA oligonucleotides and it is unlikely that the impact on ERK5 levels by siSTAT3 transfection was due to off-target effects. This needs, nevertheless to be fully established in this particular context. Alternatively, the immunodetection of ERK5 could have been hindered by a change of protein mobility (a band shift) caused by possible PTMs induced by IFN- γ and IL-6. In particular, IL-6 was shown to activate ERK5 in myeloma cells (Carvajal-Vergara *et al.*, 2005). Preliminary data from our group established that IL-6 activated ERK5 in the absence of STAT3 (data not shown). Although the ERK5 Western blot shown here is not very “clean”, it is possible to see a band shift in siSTAT3-transfected cells treated with IFN- γ and IL-6 (**Fig. 47 A**, 5th and 6th lanes in the 6th row). These may correspond to ERK5 PTMs but further work is necessary to confirm this assumption.

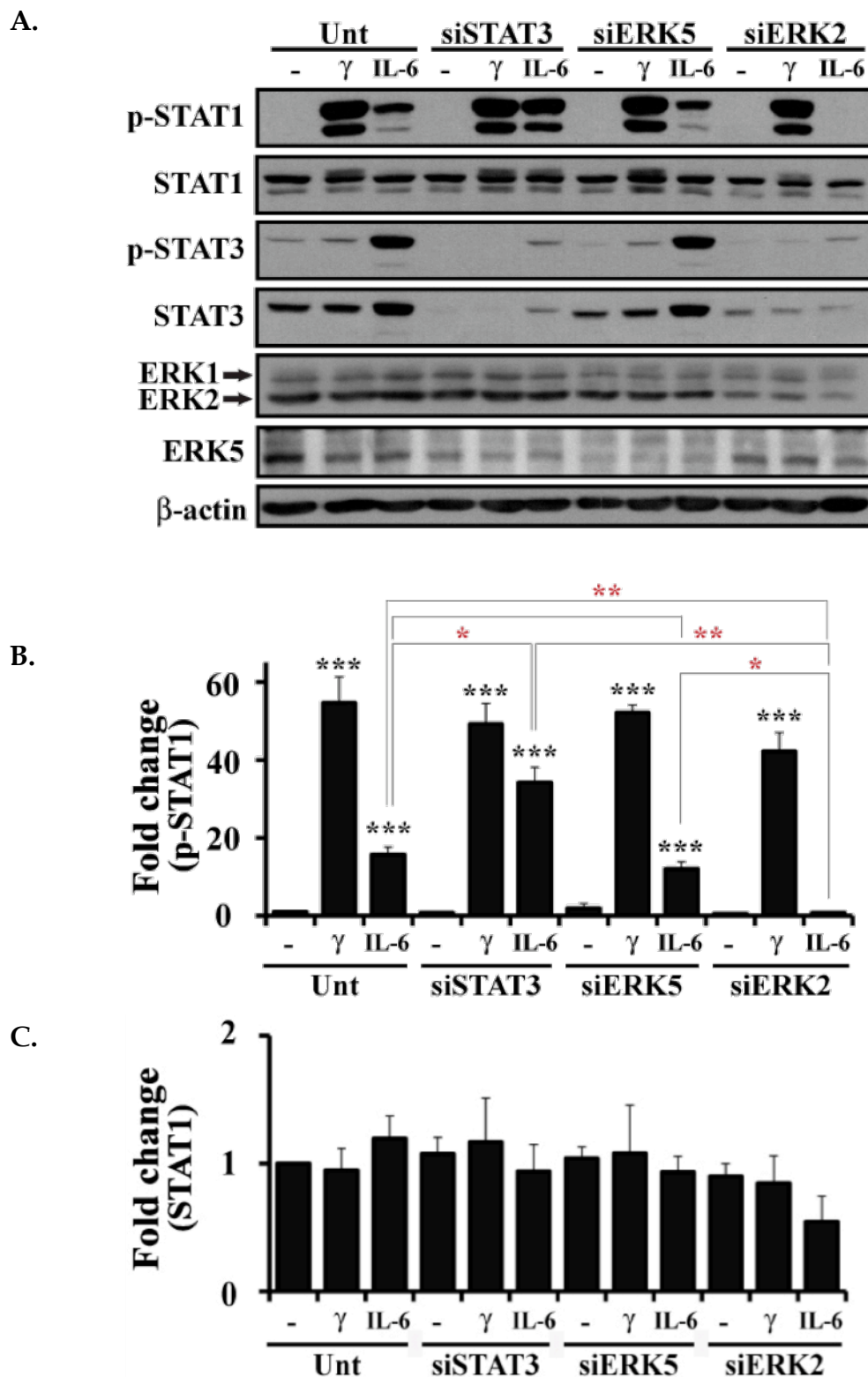


Figure 47. IL-6-induced activation of STAT1 and STAT3 was impaired in U2OS cells with reduced levels of ERK2. (continued on page 198). U2OS cells were transfected with 75 nM of siRNA targeting STAT3, ERK5 or ERK2 using DharmaFECT II. After 48 hours, cells were stimulated with IFN- γ (500 IU/ml) or IL-6 (200 ng/ml) together with siL-6R (250 ng/ml) for 15 minutes. Proteins were extracted and separated on a 7.5% SDS-PAGE gel. **A.** Western blotting analysis was performed using total cell lysates and the membrane was probed with antibodies against ERK1/2, ERK5, phosphorylated STAT1 (Tyr⁷⁰¹), STAT1, phosphorylated STAT3 (Tyr⁷⁰⁵) and STAT3. β -actin was used as a loading control. **B., C., D., E., F., G.** Means $\pm$ SEM of densitometric values from three independent experiments are represented in graphs. Values are expressed as fold change relative to untransfected controls. Statistical analysis was performed with Student's t-test (* - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.005$, black asterisks *versus* untransfected untreated control, red asterisks compare the indicated samples). Unt - untransfected cells, γ - IFN- γ .

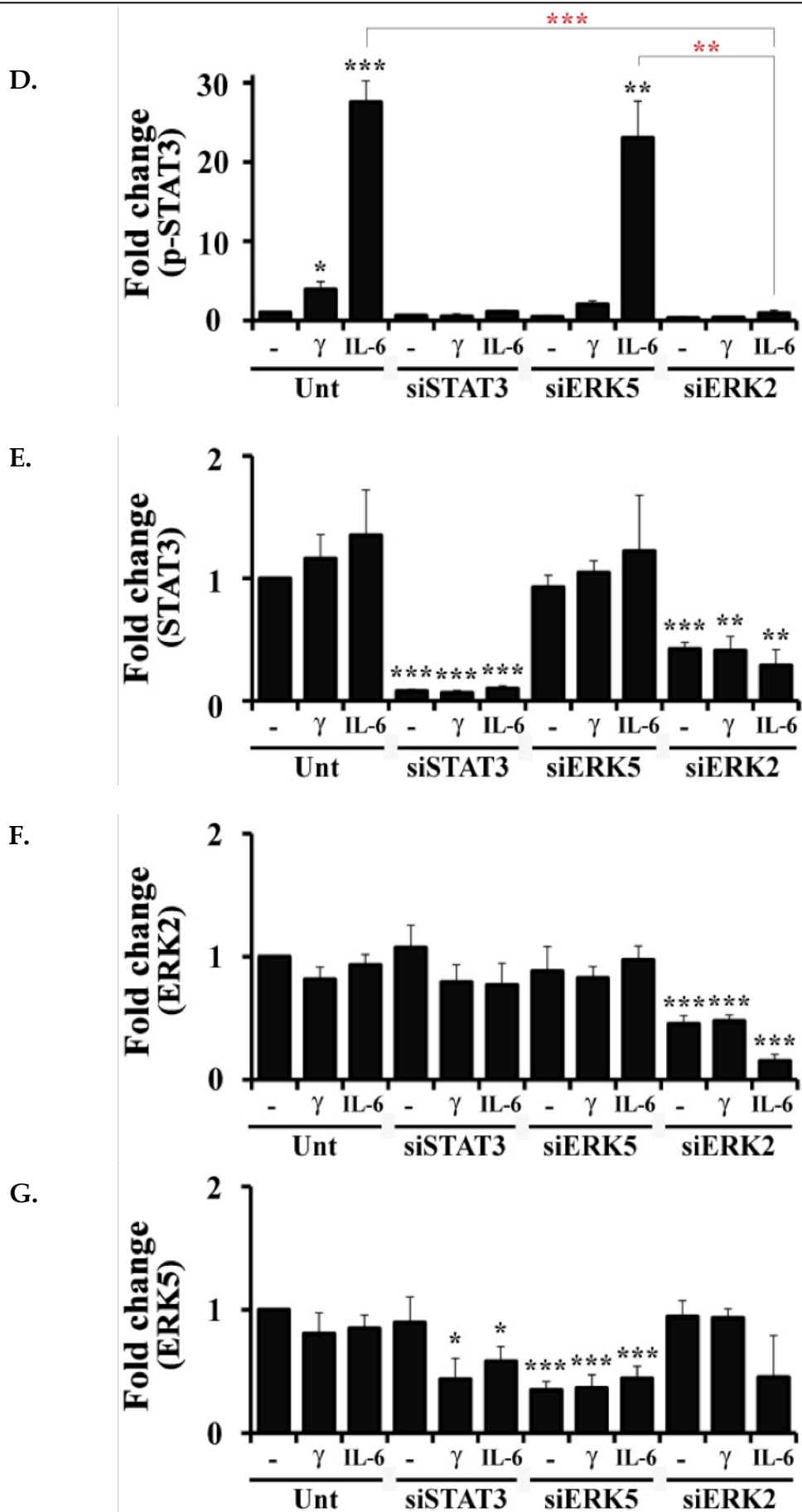


Figure 47. IL-6-induced activation of STAT1 and STAT3 was impaired in U2OS cells with reduced levels of ERK2. (continued from page 197).

The inability of IL-6 to activate STAT1 in cells transfected with siRNA against ERK2 was an unexpected and surprising observation. One plausible hypothesis to explain this observation might be that ERK2 knockdown might affect the expression or localisation gp130, the signal transducing receptor chain that all IL-6-type cytokines utilise. We therefore next examined if ERK2 silencing affected this receptor.

4.3.4.1. KNOCKDOWN OF ERK2 REDUCED GP130 RECEPTOR EXPRESSION

To determine if the inability of IL-6 to activate STAT1 was due to reduced levels of the receptor gp130 on the cell surface, cells were transfected with siRNA against ERK2 and cell surface levels of gp130 measured by flow cytometry. Controls used here included untransfected cells, siSTAT3-transfected cells and cells transfected with a non-specific siRNA (NS). After 48 hours, cells were stained for gp130 (cf. Materials and methods) and analysed using a FACSCanto. siRNA oligonucleotides specificity and protein knockdown levels were assessed by SDS-PAGE/Western blotting, as previously described (data not shown). The amount of gp130 present on the cell surface was determined by comparing cells stained with an anti-gp130 antibody to cells stained with the secondary antibody only (shadowed coloured peaks) (**Fig. 48 A**).

We detected similar levels of gp130 on the cell surface of untransfected and cells transfected with siRNA against STAT3, or with a non-specific siRNA pool (**Fig. 48 A**, 1st, 4th and 5th histograms and **B**, 1st, 4th and 5th bars). In siERK2-transfected cells detection of gp130 on the cell surface was significantly reduced to around 20% ($p < 0.005$) (**Fig. 48 B**, 2nd bar). The absence of gp130 on the cell surface could certainly explain the lack of signalling in response to IL-6 observed upon knocking down ERK2 using RNAi (**Fig. 47**). Interestingly, in cells transfected with siRNA against ERK1 a significant reduction ($p < 0.005$) of around 25% of gp130 in the cell surface was also observed (**Fig. 48 B**, 3rd bar).

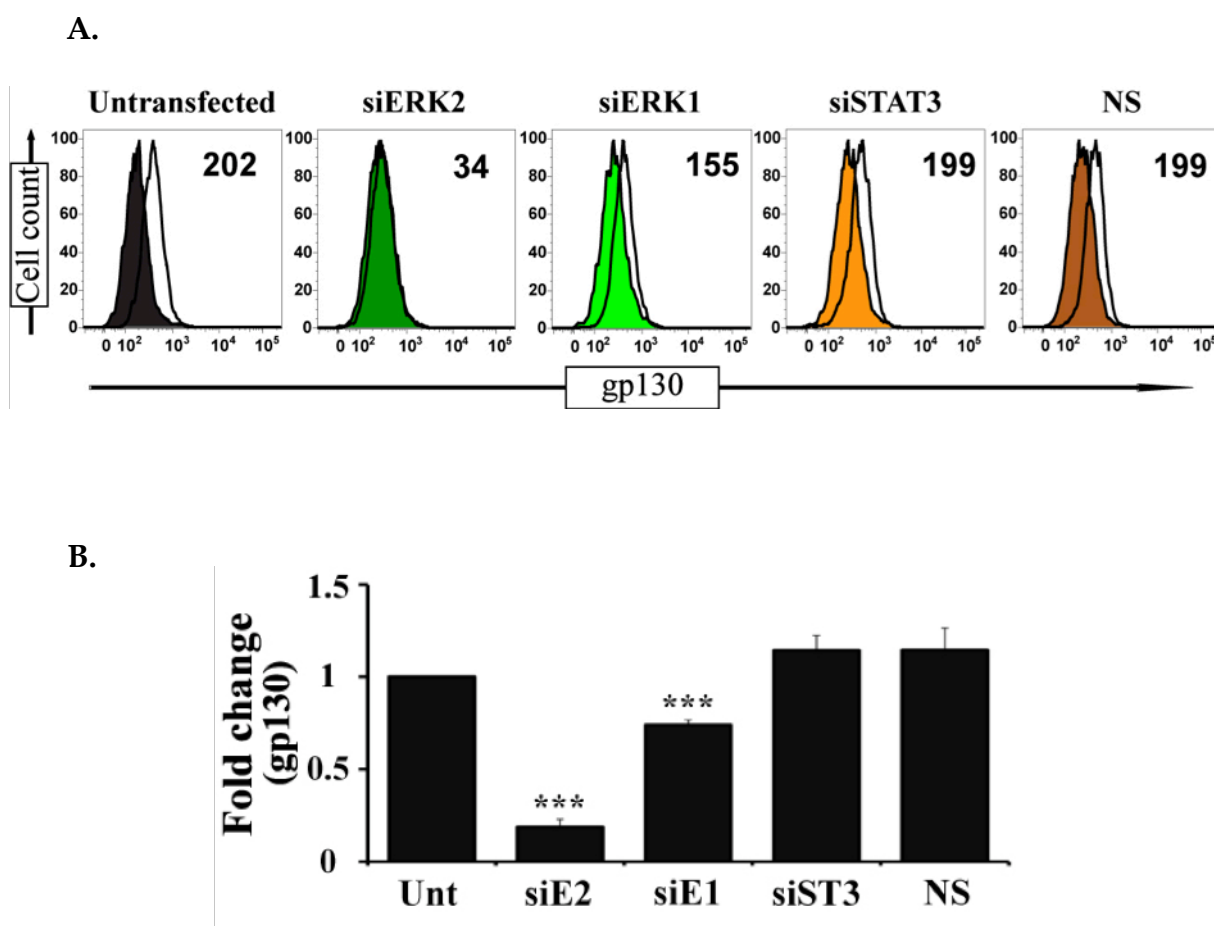


Figure 48. siRNA against ERK2 reduced the levels of the gp130 receptor on the surface of U2OS cells. **A.** U2OS cells were transfected with 75 nM siRNA against ERK2, ERK1, STAT3 or a non-specific target using DharmaFECT II, and 48 hours later, were stained for gp130. Levels of gp130 in the cell surface were assessed by flow cytometric analysis using a FACSCanto. Results were analysed using FlowJo. Shadowed coloured peaks represent cells stained with the secondary antibody only and transparent peaks represent cells stained with the anti-gp130 antibody together with the secondary antibody. The different colours correspond to different transfections: black: untransfected, dark green: ERK2, light green: ERK1, orange: STAT3 and brown: non-specific. Numbers correspond to the geometric means of fluorescence in stained samples. **B.** Mean $\pm$ SEM values of three independent experiments are shown in graphs. Values are expressed as fold change relative to untransfected controls. Statistical analysis was performed with Student's t-test (***) - $p < 0.005$ versus untransfected control). Unt - untransfected cells, E2 - ERK2, ST3 - STAT3, E1 - ERK1, NS - non-specific.

The decrease of gp130 levels on the cell surface following siRNA against ERK1 or ERK2 could be due to **i)** a reduction of protein levels in the cell, or **ii)** a defect on cell surface presentation (transport to the plasma membrane), or **iii)** increased endocytosis. Therefore, gp130 total protein levels in cells were assessed by SDS-PAGE/Western blotting after transfection with siRNA against ERK2.

Using an antibody that recognises total gp130 we were able to detect two bands (approximately 150 and 130 kDa) in untransfected and siSTAT3-transfected cells (**Fig. 49 A**, 1st and 3rd lanes in the 1st row). These two bands presumably correspond to the gp130 precursor form (130 kDa) and the processed form (150 kDa) that contains complex-type oligosaccharides (Gerhartz *et al.*, 1994). The expression of gp130 receptor was significantly reduced in siERK2-transfected cells ($p < 0.005$) (**Fig. 49 B**, 2nd bar). The hypothesis of an off-target effect elicited by the siERK2 oligonucleotides on gp130 was excluded by performing RNAi against ERK2 using a different pool of oligonucleotides (ON-TARGETplus from Dharmacon), which gave similar results to those described above (data not shown).

The defect in gp130 expression in siERK2-transfected cells might thus be due to either shorter protein half-life, or to a defect in transcription or translation. Or perhaps the gp130 receptor has a different conformation (due to incorrect protein folding) in siERK2-transfected cells. This could impair its recognition by the anti-gp130 antibody used for Western blotting.

Interestingly, ERK1 silencing also affected gp130 and this effect was distinct from ERK2 knockdown. In siERK1-transfected cells, this receptor was detected (**Fig. 49 B**, 4th bar). Instead of reducing total gp130 levels, ERK1 silencing resulted in a selective reduction in the 150 kDa processed form of gp130 ($p < 0.05$) (**Fig. 49 C**, 4th bar) and the predominant form was the lower molecular weight form (130 kDa) (**Fig. 49 D**, 4th bar), which may correspond to the immature receptor. These results suggest that the knockdown of ERK1 may impair the maturation of gp130 thereby affecting its cellular localisation. This is currently being investigated.

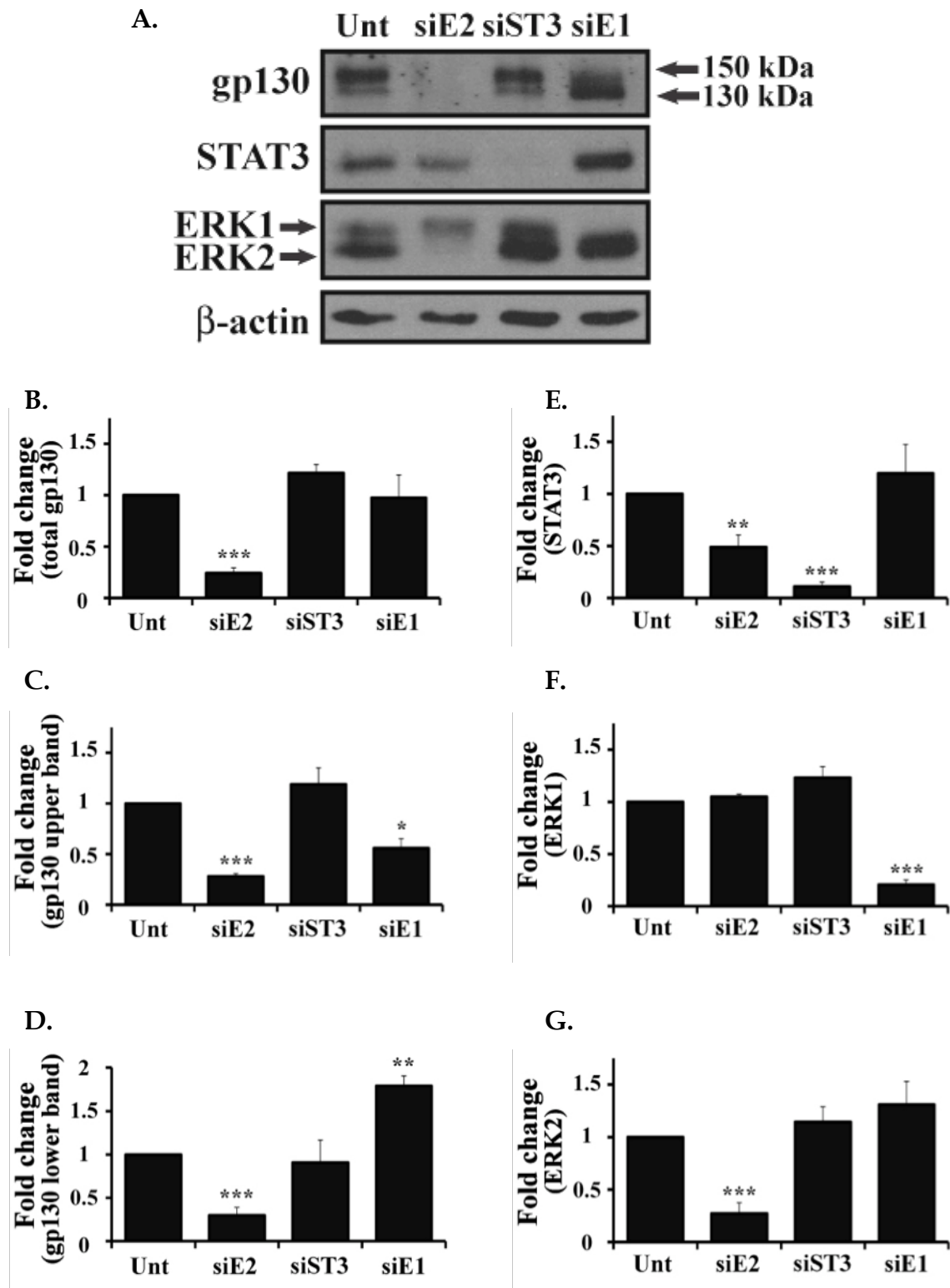


Figure 49. siRNA against ERK2 and ERK1 altered gp130 protein expression pattern in U2OS cells. U2OS cells were transfected with 75 nM siRNA against ERK2, STAT3 and ERK1 using DharmaFECT II. After 48 hours, proteins were extracted and separated on a 7.5% SDS-PAGE gel. **A.** Western blotting analysis was performed using total cell lysates and the membrane was probed with antibodies against ERK1/2, gp130 and STAT3. β-actin was also probed and used as a loading control. **B., C., D., E., F., G.** Means ± SEM of densitometric values from three independent experiments are represented in graphs. Values are expressed as fold change relative to untransfected controls. Statistical analysis was performed with Student's t-test (* - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.005$ versus untransfected control). Unt - untransfected cells, E2 - ERK2, ST3 - STAT3, E1 - ERK1.

4.3.5. DISCUSSION

The inhibition of ERK2, used in this work initially as a control, has revealed a fascinating result. ERK2 knockdown reduced the levels of STAT3 protein levels by 50% (**Fig. 43**). In contrast, cells transfected with siRNA against ERK1 express STAT3 protein to similar levels of untransfected cells. STAT1 levels remained unaffected by the knockdown of ERK2 (**Fig. 43**). The reduction of STAT3 protein expression was not due to an off-target effect elicited by the siRNA oligonucleotides against ERK2, since deconvolution of the siRNA pool yielded a similar phenotype to those observed with the siRNA pool in two out of four oligonucleotides (**Fig. 44**). Cells transfected with the two oligonucleotides that gave the best ERK2 knockdown were the cells that showed the most reduction in STAT3 protein levels. In addition, siRNA against ERK2 carried out with a different pool of oligonucleotides showed identical results. Overall, the results strongly suggest that the diminished STAT3 protein levels were a consequence of decreased ERK2 and not the result of an off-target effect. This observation suggests a novel interplay between ERK2 and STAT3 and maybe a new mechanism for the regulation of STAT3, the member of a STAT family believed to play a major role in cancer (Yu and Jove, 2004).

The reduction of STAT3 protein levels upon the silencing of ERK2 was initially observed in the osteosarcoma cell line U2OS but this observation was further extended to other cell lines, the NSCLC A549 and HCC193 lines (**Fig. 45**). These results suggest that ERK2 plays a role in the regulation of STAT3 in different cell lines, suggesting that this is a more generalised phenomenon.

ERK2 can be regulating STAT3 expression in diverse ways. ERK2 may be involved in the regulation of *STAT3* transcription. This MAPK is known to induce the expression of many genes by directly activating gene promoter-bound transcription factors (Whitmarsh *et al.*, 1995), which suggest that ERK2 could be acting as a transcription factor for STAT3. ERK signalling was also shown to be involved in translational machinery (Roux *et al.*, 2007). In fact, ERKs can regulate the translation of various proteins through MKs (reviewed by Roux and Blenis, 2004). It is plausible that ERK2 may thus be a modulator of mRNA translation of *STAT3*. ERK kinase activity was, however, required for the induction of translation, which makes this a less likely

explanation for the result described here. STAT3 protein levels reduction in the absence of ERK2 may also be related to lack of STAT3 activity. Indeed, STAT3 protein regulates the *STAT3* gene, a process that requires STAT3 activity (Ichiba *et al.*, 1998). It is possible that ERK2 is required for STAT3 activity (for example, by virtue of being an inhibitor of PIAS): knockdown of ERK2 would thus reduce STAT3 protein activity, which would then result in reduced STAT3 expression. Lastly it is also a possibility that ERK2 is not involved in *de novo* STAT3 protein synthesis *per se*, but is instead required for the stabilisation and protection of STAT3 from protein degradation and/or cleavage. Indeed, activation of MEK/ERK2 signalling pathway is associated with inhibition of caspase activation (Erhardt *et al.*, 1999; Rytomaa *et al.*, 2000; Tashker *et al.*, 2002). It was shown that ERK1/2 can inhibit caspase-9 activity by directly phosphorylating and subsequently inhibiting caspase-3 (Allan *et al.*, 2003). Under certain circumstances, STAT3 is known to undergo proteolytic processing by caspases (Darnowski *et al.*, 2006). Perhaps ERK2 inhibit such caspases and the knockdown of ERK2 may lead to STAT3 cleavage and degradation.

We observed that inhibition of MEK activity did not affect STAT3 expression (**Fig. 46**), suggesting that enzymatic activity was not required for the stabilisation of STAT3. Although the activation of ERKs was shown to be required for the transcription and translation of many genes (e.g. Chen *et al.*, 2001) we cannot exclude the possibility that ERK2 may also regulate transcription or translation independently of its enzymatic activity. A kinase inactive form of the yeast *Saccharomyces cerevisiae* MAPK, the kinase suppressor of Sst2 (Kss1), was shown to negatively regulate gene expression by directly binding to the transcription factor Ste12 thereby inhibiting invasive growth (Bardwell *et al.*, 1998). Moreover, MAPKs were shown to be able to translocate into the nucleus independently of their phosphorylation state (Gonzalez *et al.*, 1993; Lenormand *et al.*, 1993; Meili and Ballmer-Hofer, 1996; Matsubayashi *et al.*, 2001). Accordingly, it is possible that ERK2 may be regulating STAT3 expression through a mechanism independent of its kinase activity.

The reduction of STAT3 protein levels in the absence of ERK2 was expected to affect the response to IL-6. This cytokine activates STAT3 (Heinrich *et al.*, 2003), and STAT1 transiently (Stahl *et al.*, 1995). In cells that lack STAT3, IL-6 can mediate an IFN- γ -like response that involves the induction of a strong and prolonged STAT1 phosphorylation (Costa-Pereira *et al.*, 2002). Accordingly, because transfection with siRNA against ERK2 caused a decrease in the STAT3 protein levels, a stronger STAT1 phosphorylation was expected to be seen in siERK2-transfected cells. This, however, was not observed (**Fig. 47**). Instead of increased IL-6-induced STAT1 phosphorylation

we observed that IL-6 was unable to activate STAT1, which was unexpected. STAT3 phosphorylation by IL-6 in cells with reduced ERK2 was also significantly reduced compared to IL-6-induced STAT3 phosphorylation in untransfected or siERK5-transfected cells, but this was no surprise as STAT3 total protein levels were also reduced. The most straightforward explanation would be that, somehow, reduced ERK2 led to reduced levels of the receptor gp130, or to reduced binding of STAT1 to the recruitment motifs in gp130 molecule.

Indeed, transfection of U2OS cells with siRNA against ERK2 resulted in the near obliteration of gp130 on the cell surface of these cells (**Fig. 48**). This receptor is required for STAT1 and STAT3 recruitment to the IL-6 receptor complex, whereupon these molecules become phosphorylated and activated (Stahl *et al.*, 1995; Heinrich *et al.*, 1998). Interestingly, in cells transfected with siRNA against ERK1 gp130 was also reduced on the cell surface, although this reduction was not as dramatic as that observed in siERK2-transfected cells (25% *versus* 80%). The decrease of gp130 on the cell surface could be due to a reduction in protein levels in the cell, due to a defect in cell surface presentation caused by deficient transport to the membrane and/or defects in the maturation of the receptor. Increased gp130 endocytosis could also affect the levels of gp130 at the plasma membrane.

By Western blot analysis of total cell lysates we were able to detect gp130 in untransfected cells but not cells transfected with siRNA against ERK2 (**Fig. 49**). This explained the dramatic reduction of gp130 on the cells surface and suggested that ERK2 was regulating transcription, translation, or had a scaffolding role that protected gp130 from degradation.

Similarly to what was mentioned for STAT3, ERK2 may be a transcription factor for gp130 or it may be required for mRNA translation of *gp130*. Alternatively, ERK2 may be a protein that protects gp130 from degradation. Lysosomal degradation of gp130 upon IL-6 binding has been previously reported, leading to a rapid and critical translocation of the receptor from the cell surface to endosomal compartments (Dittrich *et al.*, 1994; Blanchard *et al.*, 2001). Like for many other receptors, ubiquitination plays a key role in gp130 endosomal trafficking (Tanaka *et al.*, 2008). Down-regulation of the receptor was suggested to play an important role as a means of protecting cells against over-stimulation. A di-leucine motif (Leu⁷⁸⁶ and Leu⁷⁸⁷) located in the intracellular domain of gp130 was shown to be crucial for efficient receptor endocytosis (Dittrich *et al.*, 1994). Interaction of the adaptor protein AP2 with this motif was observed to enable the transfer of receptors to clathrin-coated pits, and intracellular targeting to lysosomal degradation (Dittrich *et al.*, 1996; Thiel *et al.*, 1998).

In addition, phosphorylation of the Ser⁷⁸² residue located immediately next to the dileucine motif of gp130 regulates its turnover (Dittrich *et al.*, 1996; Gibson *et al.*, 2000) and is a requisite for receptor degradation *via* ubiquitin-proteasome pathway (Mitsuhashi *et al.*, 2005). CamKs, such as CaMKII, which phosphorylate gp130, can phosphorylate this particular residue (Gibson *et al.*, 2005). ERK2 may be a repressor of a kinase such as CamKII and regulate gp130 trafficking and degradation. The requirement for ERK2 kinase activity for the maintenance of gp130 protein levels is still unknown. This will be investigated using MEK inhibitors. Another possibility is that, in the absence of ERK2, gp130 is cleaved. A previous report showed that gp130 can be cleaved by caspase 3 at position 800-806 (Graf *et al.*, 2008). Cleavage in this region would impair gp130 recognition by the antibody used here for Western blotting since it recognises the region 713-918. The use of other antibodies against gp130 that recognise other residues will be useful to detect cleaved forms of the receptor, if this is the case. If this proves true, the involvement of caspases in this process will be investigated using specific caspase inhibitors.

A connection between the decrease of STAT3 and gp130 cannot be excluded and will be taken into consideration in future studies. On one hand, the reduction in STAT3 protein levels caused by the knockdown of ERK2 could be the reason why there is decreased expression of gp130. The 5'-flanking region of the human *gp130* gene functions as a constitutively active transcriptional promoter and can be stimulated by IL-6-type cytokines (O'Brien and Manolagas, 1997). These authors showed that STAT3 and STAT1 homo and heterodimers bind STAT-binding elements (SBEs) identified in this region. This was suggested to stimulate the production of new gp130 molecules to replenish receptors consumed upon ligand activation. This is unlikely to be the case here as we observed that knockdown of STAT3 by siRNA did not affect gp130 expression (**Fig. 48** and **49**). On the other hand, lack of gp130 protein may affect STAT3 protein levels. One possibility is that the reduction of gp130 protein may cause a decrease STAT3 signalling and this could have an impact on STAT3 itself because, as previously mentioned, one of the regulators of the *STAT3* gene is STAT3 itself (Ichiba *et al.*, 1998). The main point thus pertains to how ERK2 levels can influence both STAT3 and gp130 expression and, presumably, regulate signalling pathways involving these molecules.

Total levels of gp130 protein in the cells were not significantly affected by the knockdown of ERK1 (**Fig. 49 B**). However, we observed a reduction (of around 25%) of this receptor on the cell surface of siERK1-transfected cells. The anti-gp130 antibody (Upstate, against residues 713-918) used on Western blots detected two bands in

untransfected and siSTAT3-transfected cells (**Fig. 49**). These two bands presumably correspond to the gp130 precursor form (130 kDa) and the processed form (150 kDa) (Gerhartz *et al.*, 1994; Wang and Fuller, 1995). gp130 molecules are produced as precursors of 130 kDa and are processed within 45-60 minutes in the endoplasmic reticulum (ER) into mature forms that weight 150 kDa (Gerhartz *et al.*, 1994). This receptor was shown to be N-glycosylated at 9 out of 11 asparagine potential residues for glycosilation (Moritz *et al.*, 2001). N-glycosylation of gp130 is essential for the stability and localisation of the receptor (Waetzig *et al.*, 2009). By mutating the asparagines of all 9 functional N-glycosylation sites of gp130, these authors showed that gp130 molecules without N-glycans are inherently unstable and are mostly degraded in the proteosome before reaching the cell surface (Waetzig *et al.*, 2009). In siERK1-transfected cells the gp130 processed (150 kDa) form decreased and the lower molecular weight (130 kDa) was the predominant form. This raises the intriguing possibility that ERK1 may be required to efficiently process gp130. This could be addressed using endoglycosidase H, which would allow us to confirm that the accumulation of the 130 kDa form in siERK1-transfected cells is indeed due to a lack of glycosylation. If this proves to be the case then the effects of ERK1 knockdown on the maturation and/or function of gp130 will be studied in great detail. First, the requirement for ERK1 in the maturation of gp130 will be investigated. The requirement for ERK kinase activity will be determined using MEK inhibitors. Furthermore, it will be investigated if ERK1 can function as a chaperone required for the transport of gp130. It will be important to investigate if the gp130 receptor is functional in the absence of ERK1. This could be addressed by knocking down ERK1 using siRNA and monitoring the phosphorylation of STAT1 and STAT3 in response to IL-6. Glycosylation of gp130 was shown to be required for receptor function in rat hepatocytes (Wang and Fuller, 1995). N-glycosylation was, however, suggested to be dispensable for ligand binding as the small quantities of non-glycosylated gp130, which do reach the cell surface, were are still able to induce stat3 activity upon IL-6 binding in BAF3 (murine) cells (Waetzig *et al.*, 2009). In mouse neuroepithelial cells N-glycans are required for the activation but not for the localisation of gp130 (Yanagisawa and Yu, 2009). If ERK1 is required for the maturation of gp130 it is almost certain that the knockdown of ERK1 will somehow affect the function receptor.

Although these results warrant a more detailed investigation the data establish a novel role for ERK1 that is not shared by ERK2. Besides ERK1 and ERK2 being very similar proteins with analogous activities, kinetics and substrates (Chen *et al.*, 2001; Roux and Blenis, 2004), our findings corroborate the idea that ERK1 and ERK2 have, in

fact, non-overlapping functions (Aouadi *et al.*, 2006; Vantaggiato *et al.*, 2006; Bessard *et al.*, 2008; Fremin *et al.*, 2009). Here we have established a role for ERK2 in the regulation of STAT3 and gp130 levels. Our data also suggests that whilst gp130 expression or stability is dependent on ERK2, its maturation is dependent on ERK1. These are totally novel observations.

Together these findings further strengthen the relevance of ERK1/2 in cancer, as these molecules regulate the expression of gp130 and STAT3, two important players in pathways relevant to cancer cells, in addition to their already well described and recognised mitogenic role.

5. GENERAL DISCUSSION AND FUTURE DIRECTIONS

5.1. GENERAL DISCUSSION

Chemoresistance is probably the main obstacle for the successful treatment of many tumour types. As a prime example, lung cancer has an extremely low cure rate mainly due to innate or acquired resistance to chemotherapy (Spiro and Silvestri, 2005). Activation of various survival signalling pathways has been related to the induction of chemoresistance. This work has focused on a signalling pathway induced by FGF-2 which has been shown to play an important role in driving chemoresistance in lung, prostate and breast cancer cells (Song *et al.*, 2000; Pardo *et al.*, 2002; Pardo *et al.*, 2003; Vandermoere *et al.*, 2005). Here, we have extended these findings to another type of tumour, namely bone cancer. In the osteosarcoma U2OS cell line, FGF-2 induced cell proliferation and provided protection from cell death induced by the chemotherapeutic agent cisplatin, refelecting well-recognised multiple functions of FGF-2 (Bikfalvi *et al.*, 1997). Using a variety of assays and read-outs we have confirmed that rescue from cisplatin-induced death by FGF-2 reflected a *bona fide* inhibition of apoptosis rather than just an increase in cell proliferation. In U2OS cells, FGF-2 induced the expression of anti-apoptotic proteins (MCL-1, BCL-2, BCL-X_L and XIAP) that are likely to be involved in the chemoprotection mechanism. Given that FGF signalling has an important role in bone growth and cancer (Muenke and Schell, 1995; Coffin *et al.*, 1995; Deng *et al.*, 1996; Bodo *et al.*, 2002) our findings might help to develop additional ways of circumventing common medical problems arising from chemoresistance in osteosarcoma. This is particularly relevant for this type of cancer (and potentially for many other cancers) in which the development of resistance to chemotherapy is associated with a lower survival rate (Bacci *et al.*, 2000).

The RAS/RAF/MEK/ERK signalling pathway has been demonstrated to control several fundamental cellular processes that lead to cell growth, cell survival and drug resistance (reviewed by Kolch, 2000; Ballif and Blenis, 2001; McCubrey *et al.*, 2007). In particular, MEK/ERK activation was shown to be indispensable for FGF-2-mediated protection from etoposide in SCLC cells (Pardo *et al.*, 2002). Here, we observed that FGF-2 rescues U2OS cells through a mechanism that also requires MEK/ERK activation. Indeed, in this cell line, FGF-2 was unable to rescue cells from cisplatin-induced cell death in the presence of a MEK chemical inhibitor, or siRNA molecules that target ERK1 or ERK2. Accordingly, this finding adds further evidence for the notion that this pathway plays a

central role in chemoresistance in different cancer types. Moreover, it is possible that additional pathways may be activated in this context. For example, the PI3K pathway was shown to mediate FGF-2-induced survival in breast cancer cells and osteoblasts (Debiais *et al.*, 2004; Vandermoere *et al.*, 2005). PI3K was, however, dispensable in FGF-2-mediated chemoresistance in SCLC cells (Pardo *et al.*, 2002). Future work focusing on other pathways downstream of FGF-2 in U2OS cells will help to elucidate further the mechanisms by which this growth factor confers protection from apoptosis in this cell line.

We have also investigated the FGF-2-mediated chemoresistance in NSCLC cells, the most common lung cancer cell type (Spiro and Porter, 2005). Under the conditions used here, FGF-2 failed to induce proliferation in the two NSCLC cell lines studied (HCC95 and HCC78) and was also unable to rescue them from cisplatin-mediated cell death. In HCC78, this may be due to a defective ERK1/2 activation in response to FGF-2. While maximum induction of ERK1/2 phosphorylation was on average 13.2-/4.3- and 7.9-/5.1-fold for U2OS and HCC95 cells, respectively, FGF-2 increased the phosphorylation of ERK1/2 only by 1.4-/1.3-fold in HCC78 cells. One possible reason why ERK1/2 is only modestly activated in response to FGF-2 is that HCC78, in contrast to U2OS and HCC95 cells, lack the expression of FGFR2IIIc, one of the two main canonical FGF-2 receptors (FGFR1 and FGFR2IIIc) (Ornitz *et al.*, 1996). The reason for the inability of FGF-2 to protect the NSCLC cell line HCC95 from cisplatin-induced killing is less obvious and remains to be explained. It is possible that other pathways potentially activated downstream of FGF-2 may be impaired in HCC95 cells. Nevertheless, FGF-2 may provide chemoprotection in other NSCLC cell lines, which have not been studied here. Therefore, analysing additional NSCLC cell lines will be crucial to establish if FGF-2 is able to mediate chemoprotection in this type of lung cancer.

Furthermore, our work not only provides additional evidence for the crucial role played by ERKs in chemoresistance (reviewed by McCubrey *et al.*, 2007), but also unravels novel functions for these proteins in oncogenic pathways. We discovered that the knockdown of ERK2 caused a reduction in STAT3 protein levels in the osteosarcoma cell line U2OS. Consequently, we investigated if the knockdown of ERK2 by siRNA had the same effect on STAT3 in another cell type (i.e. NSCLC cells). Here, we used two NSCLC cell lines (HCC193 and A549) that were different from the ones used in the first part of this study because optimisation of siRNA experiments previously performed in the Costa-Pereira laboratory revealed that HCC95 cells were not efficiently transfected and because transfection of HCC78 cells with siRNA led to the upregulation of STAT1. For these reasons, we selected the HCC193 and A549 cells for the experiments involving RNAi.

Similarly to what was observed in U2OS cells, STAT3 protein levels were reduced in HCC193 and A549 cells transfected with siRNA against ERK2, suggesting that ERK2 may play a role in maintaining STAT3 protein levels in different cell types. Treatment with MEK inhibitors suggested that the enzymatic activity of ERK2 was not essential for the maintenance of STAT3 levels. In addition, the knockdown of ERK2 also led to the downregulation of the gp130 receptor in U2OS cells. The knockdown of ERK1 did not affect the protein levels of gp130 but appeared to promote the accumulation of its unglycosylated form (130 kDa) suggesting that it may block the maturation of this receptor. The results obtained here thus demonstrate a role for ERK2 in the regulation of STAT3 and gp130 levels, and also suggest that ERK1 might be required for the maturation of gp130. These observations corroborate the idea that although ERK1 and ERK2 are very similar proteins with analogous activities, kinetics and substrates (Chen *et al.*, 2001; Roux and Blenis, 2004), they can also perform distinct functions (Aouadi *et al.*, 2006; Vantaggiato *et al.*, 2006; Bessard *et al.*, 2008; Fremin *et al.*, 2009). Furthermore, these findings strengthen the relevance of ERK1/2 in cancer, as we provided evidence that they regulate the expression of STAT3 and gp130, two important players in pathways relevant to proliferation, survival and drug resistance in cancer cells.

MEK/ERK signalling plays a pivotal role in cancer progression and, accordingly, this pathway has been targeted by several researchers aiming to develop more efficient anti-cancer therapies (Roberts and Der, 2007; Friday and Adjei, 2008). New small-molecule inhibitors of MEK recently developed are currently being evaluated in the clinic, where they are showing encouraging results (Adjei *et al.*, 2008; Bennouna *et al.*, 2010). Our work reinforces the idea that the inhibition of MEK in conjunction with traditional chemotherapy may prevent the occurrence of chemoresistance and contribute to a more successful cancer treatment.

In this study, novel players in FGF-2-induced resistance have been revealed. Similarly to what was previously observed in SCLC cells (Pardo *et al.*, 2002; Pardo *et al.*, 2006), FGF-2 protected U2OS osteosarcoma cells from cisplatin-mediated apoptosis through a mechanism that required the activation of MEK/ERK and involved the physical interaction of B-RAF, PKC ϵ and S6K2. Here we demonstrated that FGF-2-mediated chemoresistance also required the JAK family members JAK1, JAK2 and TYK2. We observed that the knockdown of JAK1, JAK2 or TYK2 by siRNA, as well as chemical inhibition of JAK2 impaired the ability of FGF-2 to rescue U2OS cells from cisplatin-induced cell death. We did not explore the requirement for JAK3 in the FGF-2-mediated chemoresistance because JAK3 is preferentially expressed in leukocytes unlike the other three JAKs that are ubiquitously expressed (reviewed by Schindler *et al.*, 2008). In this

study, we showed that FGF-2 induced the phosphorylation of TYK2 in U2OS cells and preliminary data suggested that FGF-2 may also phosphorylate JAK1 and JAK2. These data indicate that JAK proteins are likely activated downstream of FGF-2. This is also supported by results from other authors, who observed phosphorylation of JAKs downstream of this growth factor in BMVECs, contributing to their migration, invasion, and angiogenesis (Yang *et al.*, 2009). In our study we demonstrated that JAK1, JAK2 and TYK2 interacted with B-RAF and PKC ϵ upon FGF-2 stimulation in U2OS cells. In particular, TYK2 was required for the full activation of ERK1/2 and for the upregulation of the anti-apoptotic protein MCL-1 in response to FGF-2. Although the participation of JAKs in this mechanism is still not fully elucidated, the data obtained here strongly supports our initial working hypothesis that JAKs play a role in FGF-2-mediated chemoresistance.

Interestingly, FGF-2-mediated chemoresistance did not require the STAT family members STAT1, STAT3 and STAT5, which are commonly deregulated in many cancers (Yu and Jove, 2004). Furthermore, under the conditions tested, FGF-2 did not induce STAT1, STAT3 or STAT5A/B phosphorylation/activation in these cells. This suggests that these STATs are unlikely to play a role in the pathway downstream of FGF-2 studied here. We still do not know if other STAT family members (STAT2, STAT4 and STAT6) play a role in FGF-2-mediated chemoresistance. Although JAKs commonly exert their effects through STATs, it was shown by other authors that some responses are mediated by JAKs independently of STATs (Packham *et al.*, 1998; Wen *et al.*, 2001; Dawson *et al.*, 2009). Taken at face value, our data is consistent with the notion that the regulation of cell death in U2OS cells upon cisplatin treatment involves JAKs but not STAT1, STAT3 or STAT5. At this stage, we cannot, however, rule out the possibility that there may be compensatory roles amongst these STATs in this particular context.

Our findings strongly suggest that JAKs are potential targets for treatment of chemoresistant cancers, as their inhibition blocked FGF-2-mediated survival in osteosarcoma cells. Targeting these molecules may be particularly relevant in cancer treatments also because JAK family members have been shown to modulate activation of several other pathways (such as MEK/ERK or p53 pathways) that are implicated in malignant transformation, cell growth and apoptosis (Kumar *et al.*, 1994; Quelle *et al.*, 1998; Mizuguchi *et al.*, 2000). Furthermore, JAK mutations were found to contribute to tumourigenesis in MPDs (Constantinescu *et al.*, 2008). At present, while a number of JAK inhibitors have been developed, just a few have been used in the clinic due to problems related to low potency, off-target effects and drug toxicity (reviewed by Wilks, 2008). But some JAK inhibitors that are currently being tested in clinic are showing promising results

mainly in the treatment of MPDs (e.g. Pardanani, 2008). Our findings support the inhibition of JAKs in additional tumour types. Indeed, this may be a potentially good strategy to overcome chemoresistance and improve the survival of cancer patients.

In summary, the results presented here assign to FGF-2 an important role in the survival of osteosarcoma cells and pave the way to further studies for the elucidation of relevant mechanisms of chemoresistance. Our study has provided compelling evidence for a key role played by JAKs in the anti-apoptotic pathways triggered by FGF-2 in addition to previously identified molecules (i.e. ERK1/2, B-RAF, PKC ϵ and S6K2). In addition, these results suggest that targeting JAKs may provide a novel therapeutic strategy for cancer treatment, particularly in osteosarcoma. Our findings are also consistent with the notion that the signalling downstream of FGF-2 is complex and that the main oncogenic signalling pathways cross-talk and synergise to promote cell survival and resistance to apoptosis. Moreover, our observations further highlight the relevance of ERK1/2 in cancer, as they regulate the expression of many important players in pathways relevant to cancer cells, in addition to their well-described and recognised mitogenic roles.

5.2. FUTURE DIRECTIONS

The present work brings new perspectives on the context of chemoresistance, one of the major obstacles in the successful treatment of cancer. Although a more thorough investigation will be needed to better understand and clarify the mechanisms involved, our findings clearly establish the previously unrecognised importance of JAKs in FGF-2-mediated chemoresistance signal transduction pathways.

The results presented here show that FGF-2 can drive chemoresistance in a bone cancer cell line in a similar manner to that previously observed in lung cancer cells (Pardo *et al.*, 2002; Pardo *et al.*, 2006). In fact, in the osteosarcoma U2OS cell line, FGF-2 conferred protection from cisplatin-induced cell death *via* a MEK-ERK-dependent mechanism, which involved interactions between B-RAF, PKC ϵ and S6K2, and induced the expression of anti-apoptotic proteins, such as XIAP and several BCL-2 family members. This emphasises the generality of this phenomenon and it highlights the importance of this pathway in chemoresistance and, by inference, in tumour progression.

In U2OS cells, FGF-2 likely signals through FGFR1 and FGFR2IIIc. We would like to confirm by Western blotting analysis that these receptors are expressed in U2OS using specific antibodies but the antibodies commercially available are not ideally suited. The use of siRNA against the different FGFRs will confirm the requirement for these receptors in FGF-2 signalling in this cell line. Because we observed that HCC78 cells responded to FGF-2, it would also be interesting to know if, in these cells, FGF-2 signal through FGFR1 only or can use other receptors (i.e. FGFR3 and FGFR4).

Based on what was observed in the SCLC cells (Pardo *et al.*, 2002; Pardo *et al.*, 2006) we hypothesise that the proteins B-RAF, PKC ϵ and S6K2 and their interaction are required for the upregulation of anti-apoptotic proteins, and are thus also relevant for chemoresistance in the U2OS cells line. Importantly, we will need to clarify if B-RAF, PKC ϵ and S6K2 are in the same complex formed upon FGF-2 stimulation in U2OS cells as was observed in SCLC (Pardo *et al.*, 2006). The use of siRNA against these proteins and the expression of mutant proteins will be used in the future to confirm these assumptions.

The activation of B-RAF, PKC ϵ and S6K2 in response to FGF-2 will also be investigated in U2OS cells. Western blot analysis using antibodies that recognise specific phosphorylated sites will be used to determine the activation of these proteins in response

to FGF-2. Since these proteins are kinases it is likely that one, or more, may phosphorylate other components of the complex and that their interaction is a requirement for their activation. We will investigate this using specific inhibitors, *in vitro* kinase assays, immunoprecipitations and co-immunoprecipitations. These experiments will likely help to understand the complex's dynamics, and to establish and identify crucial steps in the pathway.

We observed an increase of S6K2 protein upon stimulation with FGF-2 for a short period of time (ten minutes). Accordingly, it is unlikely that this increase is due to *de novo* protein synthesis. Because S6K protein levels can be regulated by ubiquitination (Wang *et al.*, 2008), we will investigate if the S6K2 degradation rate is decreased in response to FGF-2, for example, by Western blot analysis using antibodies that recognise ubiquitin residues. This very interesting, and potentially important, observation has not been reported yet and will be further pursued. We could also perform pulse chase experiments to assess how the absence or presence of FGF-2 treatment alters the protein half-life of S6K2.

Here, we observed that in U2OS cells FGF-2 induced the upregulation of anti-apoptotic proteins (MCL-1, BCL-2, BCL-X_L and XIAP). Using Western blotting, we detected approximately a 1.5-fold increase in these proteins in response to FGF-2. We will repeat these experiments and extract proteins with a more stringent buffer that may allow a better extraction of BCL-2 family members, which are predominantly associated with the mitochondria membrane (e.g. Breckenridge and Xue, 2004). We will also confirm that these proteins are involved in the chemoresistance provided by FGF-2 using siRNA against these molecules. We will try as well to inhibit their upregulation by inhibiting protein synthesis (using cycloheximide) and observing if this impairs FGF-2-mediated rescue from cisplatin-induced cell death.

The upregulation of anti-apoptotic proteins in response to FGF-2 reported in SCLC H-510 cells was shown to be *via* a translation mechanism (Pardo *et al.*, 2002; Pardo *et al.*, 2003). We will investigate how FGF-2 induces the upregulation of these anti-apoptotic proteins in U2OS. Using real-time PCR we will assess if treatment with FGF-2 induces an increase in mRNA transcripts coding for MCL-1, BCL-2, BCL-X_L and XIAP. Pulse-chase experiments will be conducted to ascertain if the increase in protein levels results from *de novo* protein synthesis or as a consequence of an increase in the proteins' half-lives.

Under the conditions used here, FGF-2 did not rescue the NSCLC cell lines HCC95 and HCC78 from cisplatin-mediated cell death. Rescue experiments will be performed on additional NSCLC cell lines. These experiments will be crucial to establish if FGF-2 is able to also mediate chemoprotection in this type of lung cancer cells.

This work provides the first direct evidence for the involvement and requirement of JAKs in FGF-2-mediated chemoresistance. These proteins were previously associated with tumourigenesis in MPDs by virtue of mutations which affect JAK kinase activity (reviewed by Verma *et al.*, 2003). Here, we have established that they also regulate drug resistance in tumour cells. We have access to a number of cell lines deficient in JAK1, JAK2 or TYK2 (both human and murine) and to matched cell lines complemented with wild type JAKs, or kinase-dead JAKs. These will be utilised in the future to confirm the results obtained using siRNA and further delineate the requirement of JAKs.

Inhibition of JAK2 activity with an inhibitor of its autophosphorylation (Sandberg *et al.*, 2005) also blocked FGF-2-mediated chemoresistance. Studies with additional JAK inhibitors will be useful to confirm the results obtained with the JAK2 inhibitor II and to establish whether the activity of other JAK family members is also required for this process. The use of these inhibitors will be important to understand the requirement of JAK1 and TYK2 activity for FGF-2-mediated chemoresistance mechanism(s).

A more thorough investigation will be needed to understand the precise molecular mechanism of action of JAKs downstream of FGF-2. In response to FGF-2, TYK2 and possibly JAK1 and JAK2 become phosphorylated (albeit more weakly than what is typically observed for the IFNs and other cytokines), a fact which is entirely consistent with a role in FGF-2 signalling. We will confirm the preliminary results regarding JAK1 and JAK2 in U2OS by immunoprecipitation using larger amounts of protein extracts. The possible interaction of JAKs with FGF-2 canonical receptors should also be investigated. In particular, it should be determined if JAKs are recruited to FGF receptors and/or are required for the stability, or expression of these molecules [as seen, for example for TYK2 and IFNAR1 (Ragimbeau *et al.*, 2003)]. Co-immunoprecipitation assays will be conducted to investigate if JAKs bind to FGFRs. The use of *in vitro* kinase assays would be useful to firmly establish that FGF-2 not only leads to JAK phosphorylation/activation.

Preliminary data obtained here suggested that JAK1 and TYK2 were required for the interaction between B-RAF and PKC ϵ (but not the interaction of S6K2 with B-RAF or PKC ϵ) in response to FGF-2 in U2OS cells. These experiments need to be repeated so that the effects can be quantified and statistical significance established. In addition, the requirement for JAK enzymatic activity in the formation of B-RAF/ PKC ϵ / S6K2 complex(es) will be investigated using specific JAK inhibitors.

We showed that JAK1, JAK2 and TYK2 interacted with B-RAF and PKC ϵ upon FGF-2 stimulation. It remains to be established if they also interact with S6K2. Co-immunoprecipitation using a higher concentration of cell lysate may allow the detection of co-immunoprecipitated proteins. Because JAKs are tyrosine kinases it is unlikely that

they catalyse the phosphorylation of B-RAF, PKC ϵ or S6K2 (Levy and Darnell, 2004; Yamaoka *et al.*, 2004) as these three proteins become activated upon phosphorylation of threonine and serine residues (Parekh *et al.*, 1999; Zhang and Guan, 2000; Minami *et al.*, 2001; Cenni *et al.*, 2002; Phin *et al.*, 2003). Many scenarios can be proposed to interpret the interaction between these proteins. It is possible that these interactions are a requirement for the activation and/or function of these molecules, their intracellular localisation or interaction with the translation machinery. It is also plausible that JAKs may function as chaperone proteins required for bringing at least B-RAF and PKC ϵ together. It will be crucial to determine if B-RAF, PKC ϵ and S6K2 become phosphorylated upon FGF-2 stimulation and if the knockdown of JAKs impairs these molecular events. Clearly, it remains central to determine if JAKs bind to S6K2, if they have compensatory roles, or if they have specific functions. The fact that the kinetics of recruitment were not identical for all the JAKs suggests a complex interplay between these molecules. This will be addressed in greater detail in future studies.

We established that MCL-1 upregulation in response to FGF-2 required TYK2 and it would, therefore, be extremely interesting to establish if the upregulation of other anti-apoptotic proteins by FGF-2 are also affected by the absence of JAK1, JAK2 or TYK2. It remains a possibility that JAKs are modulators that are only required for the expression of a subset of target molecules.

The role for JAK3, another JAK family member, in FGF-2-mediated chemoresistance has not been studied yet. This JAK family member was found to be deregulated in several lymphoproliferative disorders (Gee *et al.*, 2001; Yared *et al.*, 2005; Lai *et al.*, 2005; Tomita *et al.*, 2006; Krejsgaard *et al.*, 2006; Walters *et al.*, 2006; Marzec *et al.*, 2008) and to contribute to the oncogenesis of other types of cells, such as colon cancer (Lin *et al.*, 2005) and breast cancer (Kim *et al.*, 2009). Therefore, we will investigate if the osteosarcoma cell line U2OS expresses JAK3 and if so, if this kinase also participates in FGF-2-mediated chemoresistance.

We observed that STAT1, STAT3 and STAT5 were not required for FGF-2-mediated chemoresistance in U2OS cells. We choose to investigate these particular STAT family members because they are the most commonly deregulated STATs in many cancer types (Yu and Jove, 2004), making them prime candidates for playing a role in chemoresistance. Although STAT2, STAT4 and STAT6 do not seem to have such a prominent role in cancer biology, one cannot exclude their involvement in FGF-2-induced chemoresistance. Accordingly, the requirement of STAT2, STAT4 and STAT6 for the FGF-2-mediated chemoresistance will be investigated in the future.

The involvement of other pathways in FGF-2-mediated chemoresistance in U2OS cells will be determined. Accordingly, specific chemical inhibitors will be used in this context. Since JAKs and SRC family kinases (SFK) can work together to activate many signalling molecules (Ingle and Klinken, 2006), the study of SRC within this context remains an obvious possibility and it may contribute to further understanding of the mechanism by which JAKs participate in FGF-2-induced chemoresistance.

It would be interesting to determine if JAKs are required for FGF-2-mediated chemoresistance in SCLC cell lines, where this phenomenon was initially described. This will be carried out using JAK inhibitors prior to the “rescue experiment” as these cells are particularly difficult to transfect with siRNA (Michael Seckl and Olivier Pardo, personal communication). In addition, understanding why FGF-2 was not able to protect the NSCLC HCC95 and HCC78 cell lines from cisplatin-mediated killing will likely provide information regarding functional molecules and/or signalling pathways that are required for FGF-2-induced chemoresistance. Based on our data, we hypothesise that the JAKs may be the ‘missing link’ in these cells and this is something that should be investigated in the future.

Importantly, our work has also unravelled novel roles for ERK2 and ERK1, which appear non-redundant. These proteins have been extensively associated with several oncogenic pathways and are almost universally recognised as potent mitogens. During the course of this work we have found that ERK2 regulates the expression of STAT3 and gp130. These molecules are important players not only in cancer-related pathways, but are also vital players in cytokine responses. Although these results were made possible by the advent of RNAi we are confident that they reflect *bona fide* molecular requirements, as we have used several different siRNA molecules and have also observed these phenomena in other cell lines.

Currently, there is another PhD student in the Costa-Pereira group already investigating how ERK2 regulates STAT3 and gp130 protein levels. ERK2 can be i) a regulator of the their transcription, ii) a modulator of mRNA translation of these proteins, or iii) a molecule required for the stabilisation and protection from protein degradation and/or cleavage. Real-time PCR will be used to verify if *STAT3* and *gp130* transcripts are also reduced in cells transfected with siRNA against ERK2. If they are, then it will be investigated if ERK2 binds to the promoters of these proteins using chromatin IP (ChIP). If ERK2 does not regulate the expression of *STAT3* and/or *gp130* genes, it will be investigated if ERK2 is required for the translation of these proteins. Pulse-chase experiments will be conducted to ascertain if the translation of these proteins is affected by the siRNA. Additionally, polysome analysis can be conducted to ascertain if decreased

levels of ERK2 lead to a decrease in ribosomal binding to mRNA molecules. If none of these processes is being controlled by ERK2, it will be assessed if the decrease in ERK2 is causing a decrease in the proteins' half-lives. Proteasomal degradation will be blocked using proteasomal inhibitors such as MG132 and protein ubiquitination using antibodies that recognise ubiquitin modifications. In addition, also it will be interesting to investigate if STAT3 is cleaved by caspases and if so, if ERK2 is involved in the inhibition of caspases, such as caspase-9 and -3 as previously reported (Allan *et al.*, 2003). For this purpose, caspase inhibitors will be used.

Since inhibition of ERK kinase activity did not impact on STAT3 levels, it seems likely that ERK2 may have a non-enzymatic role as yet unidentified. These observations could be further investigated by transfecting cells with plasmids containing inactive ERK2, as well as plasmids containing wild type ERK2. Inactive and wild type ERK1-plasmids could be used as controls. The prediction is that the transfection of cells with plasmids containing dominant forms of inactive ERK2 will not affect STAT3 protein levels. A connection between the decrease of STAT3 and gp130 will also be investigated in the future. Indeed, it remains to be established if ERK2 modulates STAT3 and gp130 levels by the same mechanism. siRNA could be used to determine if the knockdown of one protein affects the expression of the other.

In contrast, we have observed that inhibition of ERK1 led to modification of gp130 (as assessed by Western blotting) but did not affect STAT3 levels. The data are consistent with ERK1 being required for the processing of gp130 and, presumably, for its transport from the Golgi to the plasma membrane. If our hypothesis is correct, then experiments using endoglycosidase H will confirm that the 150 kDa band detected in untransfected cells corresponded to the glycosylated form of gp130 and the 130 kDa band detected in siERK1-transfected cells corresponded to the unprocessed form. It will be critical to investigate if, in the absence of ERK1, the gp130 receptor is functional. For that purpose, cells could be transfected with siRNA against ERK1, treated with IL-6 or OSM, and phosphorylation of STAT3 and STAT1 detected by Western blotting. In addition, detection of gp130 by immunofluorescence would help clarify if the knockdown of ERK1 affected the cellular localisation of the receptor.

Taken together, these data not only demonstrate that ERK1 and ERK2 have cellular non-redundant roles but also that, as amply recognised, they cross-talk with many other important signalling pathways. Our results emphasise the importance of thoroughly understanding differential functions of ERK1 and ERK2, which are likely to impact on the design of targeted therapies for cancer.

In conclusion, our findings highlight the importance of JAKs in chemoresistance and contribute to the further understanding of the regulation of STAT3 and gp130. These molecules are all essential mediators of cytokine responses and are thus vital for the appropriate maintenance of fundamental biological processes that include immune and acute phase responses. The 'JAK/STAT field' was born just over 10 years ago. Since then, many more functions have been attributed to JAKs and STATs and it is now clear that they also play key roles in cancer biology. The proposed future directions aim to expand our understanding of such molecular processes: much remains to be done, many questions remain to be answered but the proposed work will likely provide new promising perspectives for the development of more effective cancer therapies.

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ABBREVIATIONS

'	Minutes
µg	Microgram
µl	Microliter
µM	Micromole
AIDS	Acquired immune deficiency syndrome
ALL	Acute lymphoblastic leukemia
AMKL	Acute megakaryoblastic leukemia
AML	Acute myeloid leukemia
AP2	Adaptor protein-2
APAF-1	Apoptotic protease activating factor-1
APS	Ammonium persulphate
ASK1	Apoptosis signal-regulating kinase 1
ATM	Ataxia Talangiectasia-Mutated
ATP	Adenosine tri-phosphate
BAD	BCL2-associated agonist of cell death
BAK	BCL2-antagonist/killer
BAX	BCL2-associated X protein
BCL-2	B-cell leukaemia/lymphoma-2
BCL-X _L	BCL-2-like 1 protein
BCR	Breakpoint cluster region
BH	BCL-2 homology domains
BIK	BCL2-interacting killer
BMVECs	Brain microvascular endothelial cells
BrdU	5-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
CamKII	Calcium/calmodulin kinase II
Caspases	CysteinyI-aspartic acid proteases
CBP	CREB-binding protein
ChIP	Chromatin IP
CI	Clathrin-independent
cIAP-1	Cellular inhibitor of apoptosis 1
CK2	Casein kinase 2
CME	Clathrin-mediated endocytosis
Co-IP	Co-immunoprecipitation
COX-2	Cyclooxygenase-2

cm	Centimeters
CML	Chronic myeloid leukemia
CR UK	Cancer Research UK
CRD	Cysteine-rich domain
C-term	C-terminal
DAG	Diacylglycerol
DBD	DNA binding domain
ddH ₂ O	Deionised water
DIABLO	Direct inhibitor of apoptosis-binding protein with low pI
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DRM	Detergent-resistant membrane
DTT	Dithiothreitol
DUSP	Dual specific phosphatases
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
Epo	Erythropoietin
ER	Endoplasmic reticulum
ERK	Extracellular regulated kinase
ERM	ETS-related molecule
ETS	E26 transformation-specific
FADD	Fas-associated death domain
FAK	Focal adhesion kinase
FCS	Foetal calf serum
FERM	Four-point-one, ezrin, radixin and moesin
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FRS2	Fibroblast growth factor receptor substrate 2
FSC	Forward scatter
g	Gravity
G ₁	Gap 1 phase
G ₂ /M	Gap 2 / mitosis phase
GAS	Gamma interferon activated site
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-CSF	Granulocyte colony-stimulating factor
GDP	Guanosine diphosphate
GEFs	Guanine nucleotide-exchange factors
GPCR	G-protein-coupled receptor

GRB2	Growth factor receptor-bound 2
GTP	Guanosine triphosphate
h	Hours
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hESCs	Human embryonic stem cells
HGF	Hepatocyte growth factor
HLGAGs	Heparan-like glycosaminoglycans
HP1	Heterochromatin protein 1
HSGAG	Heparan sulphate glycosaminoglycan
HSP90	Heat shock protein 90kDa
HSPG	Heparan sulfate proteoglycans
HUVECs	Human umbilical vein endothelial cells
IAP	Inhibitor of apoptosis proteins
IFNAR1	Interferon-alpha receptor 1
IFN	Interferon
IL	Interleukin
IP	Immunoprecipitation
IP ₃	Inositol triphosphate
IRF	Interferon-regulatory factor
ISG γ	Interferon- γ -stimulatable genes
ISGF3	Interferon-stimulated gene factor 3
JAK	Janus Kinase
JH	JAK homology domains
kDa	Kilo Dalton
KSHV	Kaposi's sarcoma-associated herpesvirus
KSR	Kinase suppressor of RAS
Kss1	kinase suppressor of Sst2
LD ₅₀	Lethal dose, 50%
LMO2	LIM domain only 2
LMP2	Low molecular mass polypeptide 2
LRI	London Research Institute
M	Molar
MAPKs	Mitogen-activated protein kinases
MCL-1	Myeloid cell leukemia sequence 1
MCM	Minichromosome maintenance
MEFs	Mouse embryonic fibroblasts
MEK	Mitogen-activated protein kinase kinase
mg	Milligram
MHC	Major histocompatibility complex
min	Minutes
MKP	MAP kinase phosphatase

MKs	MAPK-activated protein kinases
ml	Millilitre
MPDs	Myeloproliferative disorders
MRAS	Muscle RAS oncogene homolog
MSKs	Mitogen- and stress-activated kinases
mTOR	Mammalian target of rapamycin
mTORC	mTOR complex
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
n.	Number
NF-κB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells
ng	Nanogram
NH ₂	Amino terminal domain
NK	Natural killer
NLS	Nuclear localization sequence
NP-40	Nonidet-P40
N-region	Negative-charge regulatory region
NRTK	Non-receptor tyrosine kinases
NS	Non-specific
NSCLC	Non-small cell lung cancer
OD	Optical density
OSM	Oncostatin M
OSMR	Oncostatin M receptor
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate buffered saline
PCD	Programmed cell death
PCM1	Human autoantigen pericentriolar material
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PDK1	Phosphoinositide-dependent kinase-1
PE	Phycoerythrin
PEA3	Polyomavirus enhancer activator 3
PEST	Proline (P), glutamic acid (E), serine (S) and threonine (T)
PET	Positron emission tomography
<i>p</i> -FA	Paraformaldehyde
PH	Pleckstrin-homology
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PIAS	Protein inhibitors of activated STATs
PIP ₂	Phosphatidylinositol bisphosphate
PIP ₃	Phosphatidylinositol triphosphate
PKB	Protein kinase B

PKC	Protein kinase C
PLC- γ	Phospholipase C- γ
PMSF	Phenylmethanesulphonyl fluoride
PP5	Protein phosphatase 5
PRAS40	Proline-rich AKT substrate 40 kDa
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
PTMs	Post-translational modifications
PVDF	Polyvinylidene difluoride
RAF	raf murine leukaemia viral oncogene homolog
Raptor	Regulatory-associated protein of mTOR
RAS	ras viral oncogene homolog
RBD	RAS binding domain
Rictor	Rapamycin-insensitive companion of mTOR
RIPA	Radio immunoprecipitation assay buffer
RNA	Ribonucleic acid
RNAi	RNA Interference
RNAse	Ribonuclease
RPMI	Roswell Park Memorial Institute
rRNA	Ribosomal RNA
RSKs	Ribosomal S6 kinases
RT	Room temperature
RTKs	Receptor tyrosine kinases
RT-PCR	Reverse transcription polymerase chain reaction
S	Synthesis phase
S6K	p70 S6 kinase
S6K1/2	p70 S6 kinase α and β
SCID	Severe Combined Immunodeficiency
SCLC	Small cell lung cancer
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEF	Similar expression to fgf
SEM	Standard error of the mean
Ser	Serine
SFK	SRC family kinases
SH	Src homology
SIE	v-Sis inducible elements
sIL-6R	Soluble interleukin-6 receptor
siRNA	Small interfering RNA
SMAC	Second mitochondria-derived activator of caspase
SOCS	Suppressor of cytokine signalling proteins
SOS	Son of sevenless

SPRY	Sprouty
SRC	v-src sarcoma viral oncogene homolog
SSC	Side scatter
STAT	Signal transducer and activator of transcription
Sub-G ₁	Sub Gap 1 phase
T _a	Annealing temperature
TAD	Transcriptional activation domain
TBST	Tris-buffered saline/Tween
TGF- β	Transforming growth factor- β
TH	T helper
Thr	Threonine
TEL	ETS translocation variant 6
TEMED	Tetramethylethylenediamine
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis inducing ligand
TSC	Tuberous sclerosis complex
TYK2	Tyrosine kinase 2
Tyr	Tyrosine
Tris	Tris(hydroxymethyl)aminomethane
UK	United Kingdom
USA	United States of America
v	Volume
VEGF	Vascular endothelial growth factor
WB	Western blot
WCL	Whole cell lysate
w	Weight
XFLRT3	<i>Xenopus</i> homolog of the fibronectin-leucin-rich transmembrane protein 3
XIAP	X-linked IAP

Figure 1. The twenty most common causes of death from cancer in the United Kingdom in 2006.	11
Figure 2. Relapse following successful chemotherapy in a patient with SCLC.	15
Figure 3. Simplified diagram of the main events in the intrinsic and extrinsic apoptotic pathways.	20
Figure 4. Structural organisation of RAF proteins.	24
Figure 5. Structural organisation of S6K proteins.	29
Figure 6. Structural organisation of PKC proteins.	32
Figure 7. Structural organisation of JAK and STAT proteins.	36
Figure 8. Mechanisms of STAT signalling.	42
Figure 9. Structural organisation of FGFRs.	51
Figure 10. Signalling in response to FGF.	55
Figure 11. FGF-2-mediated chemoresistance pathways in SCLC cells.	63
Figure 12. Example of a population cell cycle profile obtained by flow cytometry.	80
Figure 13. ERK1/2 phosphorylation in response to FGF-2 in U2OS cells.	90
Figure 14. ERK1/2 phosphorylation in response to FGF-2 in HCC95 cells.	92
Figure 15. ERK1/2 phosphorylation in response to FGF-2 in HCC78 cells.	94
Figure 16. Expression of FGF-2 receptors FGFR1 and FGFR2IIIc in NSCLC HCC95 and HCC78 and U2OS cell lines determined by RT-PCR.	96
Figure 17. FGF-2 induced proliferation of U2OS but not of NSCLC cells.	98
Figure 18. Effect of serum starvation on cell morphology and density of U2OS.	100
Figure 19. Cisplatin-induced cell death in U2OS, HCC95 and HCC78 cells.	101
Figure 20. FGF-2 protected U2OS but not NSCLC HCC95 and HCC78 cells from cisplatin-mediated cell killing.	103
Figure 21. FGF-2 rescued U2OS cells from cisplatin-induced cell death.	107
Figure 22. FGF-2 failed to reduce cisplatin-induced cell death in HCC95 cells.	109
Figure 23. FGF-2 failed to reduce cisplatin-induced cell death in HCC78 cells.	110
Figure 24. Inhibition of MEK/ERK signalling blocked FGF-2-mediated rescue in U2OS cells.	113
Figure 25. Controls for PKC ϵ , B-RAF and S6K2 immunoprecipitations.	116
Figure 26. FGF-2 induced interactions between PKC ϵ , B-RAF and S6K2 in U2OS cells.	119
Figure 27. Interaction between B-RAF, PKC ϵ and S6K2 in HCC95 cells.	121
	268

Figure 28. FGF-2 induced the up-regulation of anti-apoptotic proteins in U2OS cells.	124
Figure 29. siRNA oligonucleotides can specifically knockdown JAK1, JAK2, TYK2, STAT1, STAT3 and STAT5A/B in U2OS cells.	137
Figure 30. Knockdown of JAK1, JAK2 or TYK2 affected the ability of FGF-2 to protect U2OS cells from cisplatin-induced cell death.	141
Figure 31. Knockdown of JAK1, JAK2 or TYK2 affected the ability of FGF-2 to block cisplatin-induced PARP cleavage in the U2OS cells.	144
Figure 32. Inhibition of JAK2 activity impaired FGF-2-mediated rescue in U2OS cells.	147
Figure 33. FGF-2 was able to rescue U2OS cells from cisplatin-induced cell death in the absence of STAT1, STAT3 or STAT5.	151
Figure 34. FGF-2 was able to reduce cisplatin-induced PARP cleavage in U2OS cells with reduced levels of STAT1, STAT3 or STAT5.	154
Figure 35. FGF-2 did not activate STAT1, STAT3 or STAT5A/B in U2OS cells.	157
Figure 36. FGF-2 potentially activated JAK1 in U2OS cells.	160
Figure 37. FGF-2 potentially activated JAK2 in γ 2A.JAK2 cells.	162
Figure 38. FGF-2 induced the phosphorylation of TYK2 in U2OS cells.	164
Figure 39. TYK2 knockdown in U2OS led to decreased ERK1/2 phosphorylation in response to FGF-2.	166
Figure 40. JAK1 and TYK2 may be required for the interaction between B-RAF and PKC ϵ induced by FGF-2 in U2OS cells.	169
Figure 41. FGF-2 induced interaction of JAK1, JAK2 and TYK2 with PKC ϵ and B-RAF in U2OS cells.	173
Figure 42. TYK2 is required for the up-regulation of the anti-apoptotic protein MCL-1 induced by FGF-2 in U2OS cells.	176
Figure 43. siRNA against ERK2 (but not ERK1) reduced STAT3 protein levels in U2OS cells.	188
Figure 44. Decrease of STAT3 protein levels in cells transfected with individual siRNA oligonucleotides against ERK2 correlated with the degree of ERK2 knockdown.	190
Figure 45. siRNA against ERK2 reduced STAT3 protein levels in lung cancer A549 and HCC193 cells.	192
Figure 46. Inhibition of MEK/ERK did not affect STAT3 protein levels in U2OS cells.	194
Figure 47. IL-6-induced activation of STAT1 and STAT3 was impaired in U2OS cells with reduced levels of ERK2.	197
Figure 48. siRNA against ERK2 reduced the levels of the gp130 receptor on the surface of U2OS cells.	200
Figure 49. siRNA against ERK2 and ERK1 altered gp130 protein expression pattern in U2OS cells.	202

Table I. Cell lines.	73
Table II. Sequences and annealing temperatures for each set of PCR primers.	82
Table III. siRNA transfection conditions.	83
Table IV. Sequences of each set of siRNA oligonucleotides (Dharmacon).	84

APPENDIX I – COMPARISON BETWEEN THE WST-1 ASSAY AND CELL COUNTING

The results obtained with the cell viability assay WST-1 used in this work in some experiments were compared with the ones obtained using cell counting (cf. Material and methods). The results obtained with these two methods were identical (Fig. 50).

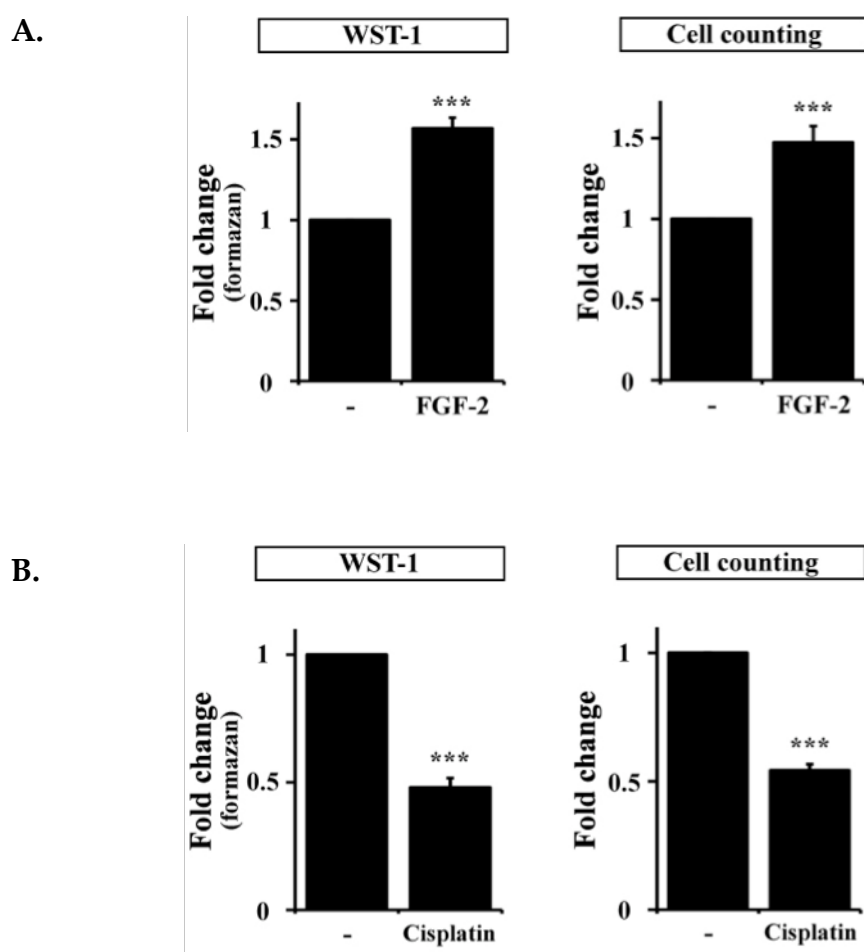


Figure 50. Results obtained with the cell viability assay WST-1 are identical to the results obtained with cell counting. Cells were plated in 96-well plates (3,000 cells per well) or in 6-well plates (2×10^5 cells per well), incubated overnight in serum-free media and then treated with FGF-2 (10 ng/ml) (**A.**) or with the LD₅₀ of cisplatin (**B.**) for 24 hours. Cell viability was measured using the WST-1 cell viability assay (graphs on the left panel). Values correspond to the OD of coloured formazan compounds derived from cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases. Cell number was determined by cell counting using trypan blue exclusion (graphs on the right panel). Mean $\pm$ SEM from three independent experiments are shown in graphs. Values are expressed as fold change over untreated controls. Statistical analysis was performed using Student's t-test (***) – $p < 0.005$ versus untreated control).

APPENDIX II – CISPLATIN BATCH-TO-BATCH VARIATION

The titration of the batch of cisplatin used in the first part of this work is shown in **Figure 19** (Section 4.1.5). The second part of this study was done in U2OS cells with a new batch of cisplatin. The LD₅₀ in U2OS using the new batch was approximately 60 μ M for this cell line ($p < 0.005$) (**Fig. 51**). Denis Akan performed these experiments. 60 μ M of cisplatin was therefore used routinely to perform the rescue experiment in U2OS cells.

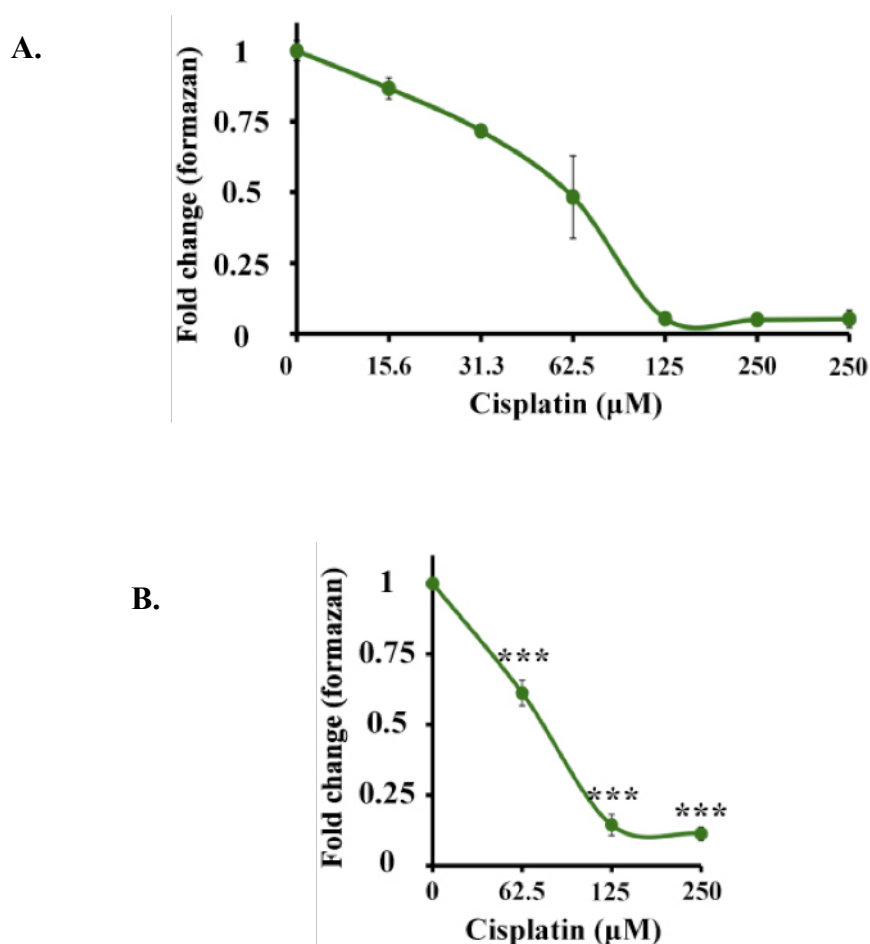


Figure 51. Cisplatin-induced cell death in U2OS cells. Cells were plated in 96-well plates (3,000 cells/well) and incubated in serum-free media overnight. Cells were treated with increasing doses of cisplatin for 24 hours. Cell viability was measured using the WST-1 cell viability assay. **A.** Results from one experiment are presented as fold induction relative to untreated controls. Each condition was set up in triplicate, and the results are shown as mean $\pm$ standard deviation. **B.** Mean $\pm$ SEM of three independent experiments performed in triplicate are shown in a graph. Values correspond to the OD of coloured formazan compounds derived from cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases. Values are expressed as fold change relative to untransfected controls. Statistical analysis was performed with Student's t-test (***) - $p < 0.005$ *versus* untreated control). (I am indebted to Denis Akan for these data).

The DNA content of cells was measured by flow cytometry after staining cells with PI, a DNA-binding dye. Data mining and analysis was done using FlowJo (Tree Star Inc., Oregon, USA). Gates were used to exclude cell doublets and cell fragments from the analysis. The gating used was hierarchical (gate 3 is contained within gate 2 and gate 2 is contained within gate 1) (**Fig. 52**).

The values of PE (phycoerythrin) are the values measured by the red channel and correspond to the amount of PI (proportional to the DNA content) inside the cells. By plotting a graph with PE-A (pulse area) *versus* PE-H (pulse height) and drawing a gate (gate 1) that included the cells with PE-A proportional to PE-H, we excluded the doublet cells (**Fig. 52 A**). The number of events recorded during the acquirement was 10000 within gate 1. To further exclude potential doublet cells we drew a second gate within gate 1. For that purpose we plotted a graph with forward scatter-area (FSC-A), a measurement related to cell size *versus* side scatter-width (SSC-W), a measurement related to the granularity of the cells (**Fig. 52 B**). A third gate within gate 2 was drawn to exclude cell fragments. This was achieved by plotting a graph with FSC-A *versus* SSC-A (**Fig. 52 C**). Gate 3 comprises all cells contained within gate 1 and 2 to include all sizing variables except small fragments. The histogram was obtained with cells within gate 3 and shows a typical cell cycle profile (**Fig. 52 D**). **Figure 52 E** shows the single cells that were used in the analysis (blue dots) and the ungated population that was excluded (black dots).

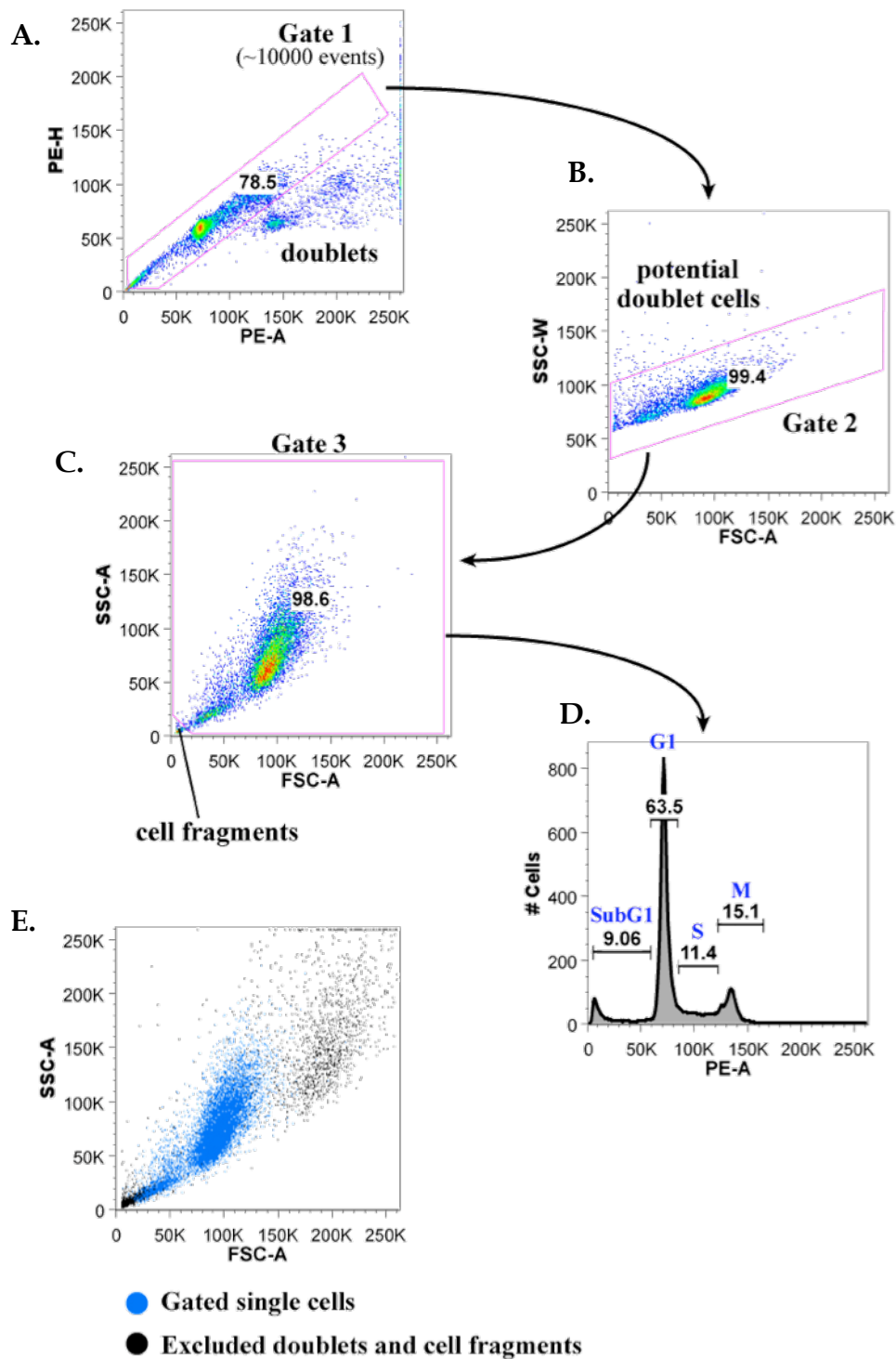


Figure 52. Flow cytometric cell cycle analysis. Cells were harvested, fixed and permeabilised with 70% ethanol and stained with propidium iodide (PI), a DNA-binding dye. DNA content was measured by flow cytometric analysis using a FACSCanto and results were analysed using FlowJo software. Hierarchical gating was done to exclude doublets and cell fragments from the analysis. **A.** Gate 1 selects single cells. The number of events recorded was 10000 within this gate. **B.** Gate 2, contained within gate 1, was drawn to exclude any potential doublet cells not excluded from the analysis using gate 1. **C.** Gate 3, contained within gate 2, was drawn to exclude cell fragments (debris). **D.** Cell cycle profile of the cell population within gate 3. G₁ corresponds to the first phase after mitosis when cells have 2n DNA content. In S phase, cells synthesise DNA. In G₂/M phase cells are ready to complete division and have the double of DNA content of a cell in G₁. The Sub-G₁ population corresponds to the percentage of cells with a DNA content of less than 2n and represents cells with fragmented DNA. **E.** Graph showing gated (blue dots) and ungated (black dots) events. PE – phycoerythrin, A – area, H – height, FSC – forward scatter, SSC – side scatter W – width. Numbers correspond to the percentage of cells within each gate.

In this work, the FGF-2-mediated rescue experiment was also done in SCLC cells, where the phenomenon was initially described (Pardo *et al.*, 2002). The purpose of these experiments was to reproduce the rescue previously observed ensuring that the experimental settings were properly conducted and to ascertain that the results obtained with additional cell lines were *bona fide*.

H-510 cells were treated using the conditions described by Pardo and colleagues (2002). Cells were starved in RPMI-1640 medium supplemented with insulin, transferrin, sodium selenite, BSA, penicillin and streptomycin for 3 days. Cells were then aliquoted at a density of 5×10^4 cell/ml (1 ml per aliquot) in serum-free RPMI. After 1 hour of incubation in serum-free media, cells were pre-treated with 0.1 ng/ml of FGF-2 for four hours and treated with etoposide for 96 hours. Cells were harvested, fixed and permeabilised with 70% ethanol and stained with PI. DNA content was measured by flow cytometry and cell cycle analysis. We observed an increase of the sub-G₁ peak after treatment with etoposide, which was substantially reduced following pre-incubation with FGF-2. This reduction of the sub-G₁ population indicates a reduction in cell death induced by etoposide (**Fig. 53 A**). After 96 hours of drug treatment proteins were extracted from cells, run on a SDS-PAGE gel and analysed by Western blot. Lamin B cleavage was assessed. In H-510 cells, etoposide induced the cleavage of lamin B, which can be seen by the increase on the lower band (45 kDa) and preincubation with FGF-2 reduced this effect (**Fig. 53 B**). These results indicate that, as previously shown (Pardo *et al.*, 2002), FGF-2 protected H-510 cells from etoposide-mediated cell death.

In parallel with the rescue experiments performed, phosphorylation of ERK1/2 in response to FGF-2 was monitored in order to confirm the functionality of FGF-2 (**Fig. 53 C**). Proteins extracted from cells treated with 0.1 ng/ml of FGF-2 for 5 minutes (as indicated in Pardo *et al.*, 2002) were run on a 7.5% SDS-PAGE gel extracted and analysed by Western blot. Membranes were probed with an antibody against phosphorylated ERK1/2 and upon stripping of the antibody the membrane was re-probed with an ERK1/2 antibody which detects total levels of these proteins. β -actin was also probed and used as a loading control. We observed that FGF-2 induced the activation of ERK1/2 in H-510 cells, clearly demonstrating that the FGF-2 used in the rescue experiment was active.

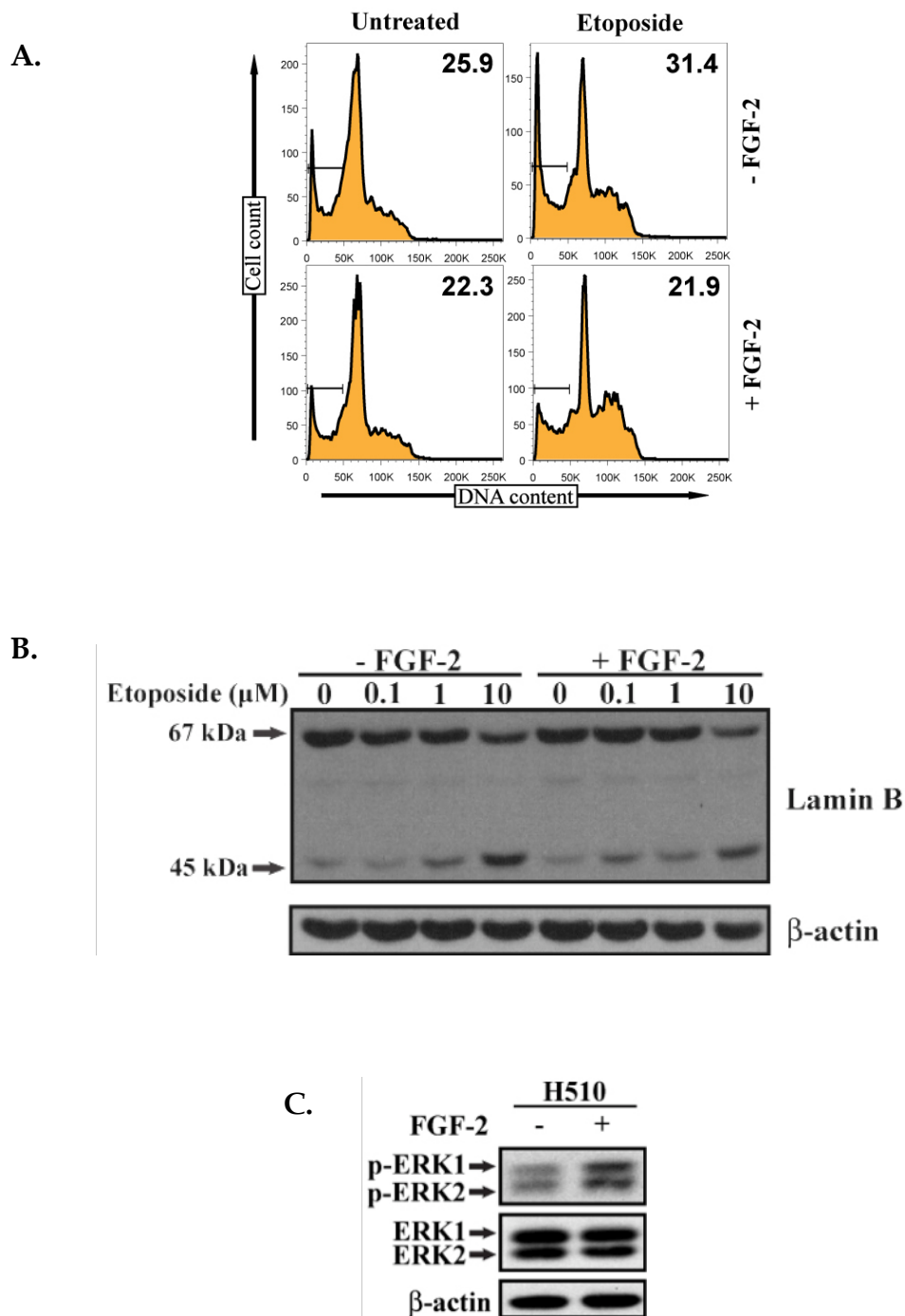


Figure 53. FGF-2 reduced etoposide-induced cell death in H-510 cells. H-510 cells were starved for three days and incubated in serum-free media for one hour. **A.** After 4 hours pre-treatment with 0.1 ng/ml of FGF-2, cells were treated with 1 μ M of etoposide for 96 hours. Subsequently, cells were harvested, fixed, permeabilised and stained with PI (50 μ g/ml). Cell death was assessed by flow cytometric analysis using a FACSCanto and the results were analysed using FlowJo software. Cell cycle profiles from one experiment are shown. Numbers refer to the percentage of dead cells represented within the sub-G₁ population. These results are representative of two independent experiments. **B.** After 4 hours pre-treatment with 0.1 ng/ml of FGF-2, cells were treated with increasing doses of etoposide for 96 hours. Subsequently, cells were harvested; proteins were extracted and separated on a 7.5% SDS-PAGE gel. Western blotting analysis was performed using total cell lysates and antibodies against lamin B. β -actin was used as a loading control. This experiment was performed once. **C.** Cells were stimulated with 0.1 ng/ml of FGF-2 for 5 minutes and proteins were extracted and separated on a 10% SDS-PAGE gel. Western blotting analysis was performed on total cell lysates using antibodies against pERK1/2-Thr²⁰²/Tyr²⁰⁴ and total ERK1/2. β -actin was used as a loading control. These results are representative of two independent experiments.