PhD program in MOLECULAR MEDICINE

PhD Thesis

siRNAs targeted against cell cycle related genes as tools to down regulate cell-proliferation in hepatocellular carcinoma and vascular smooth muscle cells

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Anno Accademico 2005-2007 (XX ciclo)
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Exuberant and non-controlled cellular proliferation underlines the ethio-pathogenesis of many human pathological conditions, including tumour and non-tumour diseases. Thus, the possibility to control this complex process can be extremely useful in terms of prevention/control of disease progression, especially in the light of the limited efficacy of current therapeutic approaches. In this project we draw our attention on the down regulation of cell proliferation in the context of two human diseases, namely hepatocellular carcinoma (HCC), as an example of tumour pathology and in stent- restenosis, example of non-tumour pathology. To explore the possibility to down regulate cell growth, we targeted the transcription factor E2F1 and the serum response factor (SRF) and cyclins E1/E2, all genes implicated in cell cycle progression. As tools to down regulate the expression of the target genes, we used small interfering RNAs, short double stranded RNA molecules able to induce the specific degradation of a homologue mRNA.

The presented results indicate that SRF depletion impairs cell proliferation, in primary VSMC and in the most differentiated HCC cell line HepG2, but not in the less differentiated HuH7 and JHH6. Additionally, the depletion of CyE1, CyE2 and E2F1 is effective in preventing all HCC cell expansion, regardless of the differentiation status. However, whereas in HepG2 the major mechanism is the induction of apoptosis, in HuH7 and JHH6 it is the down modulation of cell growth.

In conclusion, our project based on the inhibition of cell growth in tumour and non tumour cells by means of siRNAs, can contribute to better understand the complex mechanisms regulating cell proliferation in HCC and in vascular smooth muscle cells. Moreover, these data support the rationale to continue the studies for the development of future novel anti HCC and in-stent restenosis approaches based on the use of siRNAs.
LIST OF PAPERS INCLUDED IN THE THESIS


LIST OF PAPERS NOT DIRECTLY RELEVANT TO THE THESIS


LIST OF ABBREVIATIONS

HCC: Hepatocellular carcinoma
VSMC: Vascular Smooth Muscle Cells
siRNAs: small interfering RNA
SRF: Serum Response Factor
CyE1: Cyclin E1
CyE2: Cyclin E2
E2F1: transcription factor E2F1
GL2: Luciferase gene
CyA: Cyclin A
CyD1: Cyclin D1
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
pRb: phosphorylated form of the Retinoblastoma protein
P-pRb: hyperphosphorylated form of the Retinoblastoma protein
INF: Interferon
PDGF: Platelet-Derived Growth Factor
NG: Normal glucose
HG: High glucose
INTRODUCTION

1. Hepatocellular carcinoma.

Hepatocellular carcinoma (HCC), the major type of primary liver cancer, is one of the commonest cancers worldwide and a leading cause of death in many countries (Murray and Lopez, 1997; Bosch et al., 1999), with an estimated 564,000 new cases and almost as many deaths in 2000 (Thorgeirsson and Grisham, 2002). There is no clinically tested useful therapy for the advanced stage of the disease and any effective treatment has not been assessed in large controlled randomized trials for this kind of hyper-vascular and chemotherapy-resistant tumour. Chronic hepatitis B and C and associated liver cirrhosis represent major risk factors for HCC development, being implicated in more than 70% of HCC cases worldwide. Additional etiological factors, which often represent co-factors of an underlying HBV- or HCV-related chronic liver disease, include toxins and drugs (e.g., alcohol, aflatoxins, microcystin, anabolic steroids), metabolic liver diseases (e.g., hereditary hemochromatosis, α-1-antitrypsin deficiency), steatosis, non-alcoholic fatty liver diseases and diabetes.

Hepatocarcinogenesis is a multistep process that can last for decades and involves the progressive accumulation of different genetic alterations ultimately leading to malignant transformation. Regardless of the etiological agent, malignant transformation of hepatocytes is believed to occur through an increased liver cell turnover, induced by chronic liver injury and regeneration, in a context of inflammation and oxidative DNA damage. The detailed analysis of HCC development in experimental animals and the comparison of the results with HCC in humans has identified a variety of genomic and molecular alterations in fully developed HCC and, to a lesser extent, in morphologically defined pre-neoplastic precursor lesions. At least five pathways that regulate either cell proliferation or cell death (i.e., the phospho-retinoblastoma protein (pRb), cyclin E, p53, transforming growth factor-β (TGF-β) and β-catenin pathways) can be affected in HCCs. These changes drastically alter the matrix and microenvironment of the liver (Grisham J.W., 2001; Buendia, 2000).

1.1 Sequential morphological changes in the liver leading to HCC.

Hepatocarcinogenesis in humans unfolds during a process that may take more than 30 years after chronic infection with HBV or HCV is first diagnosed (Buendia, 2000; Tong et al., 1995) (Fig.1). Cirrhosis and HCC occur routinely in a fraction of patients who develop chronic
infection with HBV or HCV. HCC arises with increasing frequency in livers that are the site of chronic hepatitis and cirrhosis, particularly from dysplastic hepatocytes. The tissue lesions that commonly precede HCC (chronic hepatitis and cirrhosis containing foci of phenotypically altered and dysplastic hepatocytes) provide a framework for identifying the temporal order of genomic alterations development during hepatocarcinogenesis (Thorgeirsson and Grisham, 2002).

Fig.1 Chronologic sequence of cellular lesions culminating in the development of hepatocellular carcinoma in human.

1.2 Genomic alterations during the preneoplastic phase.

Although HCC development is a multistep process and its appearance in children suggest also the contribution of a genetic predisposition (Chang et al., 1989), it is difficult to identify most of the predisposing genes for hepatocarcinogenesis. During most of the long preneoplastic stage leading to HCC, alterations in gene expression are almost entirely quantitative, occurring by epigenetic mechanisms in the absence of detected changes in the structures of genes and chromosomes. Elevated expression of transforming growth factor-α (TGF-α) (Grisham J.W., 2001; Kawakita et al., 1992) and insulin-like growth factor-2 (IGF-2) (Grisham J.W., 2001) is responsible for accelerated hepatocyte proliferation. Upregulation of these two genes results
from the combined actions of cytokines produced by chronic inflammatory cells that infiltrate the carcinogen-damaged liver (Grisham J.W., 2001), viral transactivation (Grisham J.W., 2001; Brechot, 1998; Diao et al., 2001; Smela et al., 2001) and the regenerative response of the liver to cell loss (Grisham J.W., 2001).

It should be emphasized that in addition to TGF-α and IGF-2 upregulation, tumor and non-tumor liver contain multiple other changes. Moreover, there is variability in the profile of genomic alterations among different patients even within single studies (Feitelson et al., 2002). Variability in the number and types of genetic changes has also been observed geographically and depends upon the etiology of the tumor (viral, chemical or both) (Wong et al., 2000).

1.3 Genomic changes in HCC.

The early epigenetic changes and some of the early structural alterations of genes or loci are not sufficient to induce malignant phenotypes in hepatocytes, as HCCs ultimately develop in only a fraction of the livers with chronic hepatitis or cirrhosis. Epigenetic changes in gene expression that occur in HCCs appear to act indirectly by creating conditions that increase the chance of generating hepatocyte populations containing critical combinations of structurally and functionally aberrant genes.

Central to the concept of molecular carcinogenesis are mutations of oncogenes and tumor suppressor genes as well as genetic instability of cellular DNA, including mismatch repair efficiency and impaired chromosomal segregation. Overall, there are a variety of molecular and immunological mechanisms by which endogenous, environmental and viral factors may play an interactive role in HCC development (Bergsland, 2001; Blum, 2002; blum HE and Moradpour D, 2002; Hassan et al., 2002; Chen and Chen, 2002).

New technologies should allow to identify genes critical in early stages of tumorigenesis (Chen and Chen, 2002)
1.3.1 Promoters of cell proliferation.

Genetic analyses have revealed that one of the most commonly altered gene in HCC is the cell-cycle regulator cyclin E (Li et al., 2003). Cyclin E is believed to control G1-S phase progression by associating with cyclin-dependent kinase Cdk2 and activating its kinase activity shortly before entry of cells into the S phase (see also paragraphs 3.1.1) (Sauer and Lehner, 1995; Reed, 1997). Its expression near the G1-S phase transition is thought to be critical for the initiation of DNA replication and duplication of the centrosomes. The timely appearance of cyclin E is crucial as excessive activity of the cyclin E-Cdk2 complex drives cells to duplicate the DNA prematurely, resulting in genome instability (Spruck et al., 1999) and carcinogenesis (Bortner and Rosenberg, 1997). Notably, overexpression of cyclin E was found in 70% of HCC patients and was correlated with poor prognosis (Jung et al., 2001).

Other studies reveal that over-expression of the transcription factor E2F1 promotes hepatocarcinogenesis and that accelerates liver cancer development (Conner et al., 2000). E2F1 is involved in regulating key cellular activities including growth and death (DeGregori et al., 1997; Nevins et al., 1997). When over-expressed, E2F1 is capable of driving quiescent cells into S phase in the absence of other mitogenic stimuli (Johnson et al., 1993). In addition to providing
a continuous proliferative signal, E2F1 is also a potent inducers of apoptosis and operate at least through one common pathway involving p53 (Kowalik et al., 1998). Thus, oncogenic activity of E2F1 is associated with a persistent increase in hepatocyte proliferation, above all in the pre-neoplastic phases of liver growth (Conner et al., 2003).

1.3.2 Tumor suppressor genes.

HCCs have recurrent allelic alterations (Grisham J.W., 2001; Buendia, 2000; Kondo et al., 2000; Kawai et al., 2000; Chen et al., 2000a; Okabe et al., 2000) and regional losses and gains (Grisham J.W., 2001; Buendia, 2000; Chen et al., 2000b; Guan et al., 2000; Marchio et al., 2000; Tornillo et al., 2000; Wong et al., 2000; Zondervan et al., 2000; Balsara et al., 2001; Rao et al., 2001; Wilkens et al., 2001) on several chromosomes. In general, these genetic alterations appear to occur at later stages of HCC development.

p53 gene is probably the most common molecular target involved in human carcinogenesis (Levine et al., 1991). A G to T mutation at the third base position of codon 249 of the p53 gene, leading to a substitution of arginine to serine, was found in a significant number of HCC patients (Blum, 2003). Moreover, frequent allelic deletions at chromosome 13q have been associated in human cancer with the inactivation of the tumor suppressor Rb, which is located on 13q14 (Friend et al., 1986) and which represents a pivotal cell-cycle control gene (see also paragraph 3.1.1). Notably, the disruption of the pRb pathway renders cells insensitive to antiproliferative signals that induce growth arrest at the G1 phase. In HCC, pRb chromosomal allelic losses have been found in 25-48% of cases (Murakami et al., 1991; Fujimoto et al., 1994; Zhang et al., 1994; Kuroki et al., 1995), and pRb expression down regulation was found in 30-50% of HCC tumors (Zhang et al., 1994; Hsia et al., 1994).

While mutations of the pRb gene itself have not been documented so far in HCC, there are many different ways to inactivate pRb, including the inactivation of p16$^{INK4A}$. The protein product of p16$^{INK4A}$ tumor suppressor gene binds to cyclin-dependent kinase 4 and 6 and inhibits their interaction with cyclin D1 (Serrano et al., 1993). As the progression through the G1 phase of cell cycle is also mediated by cyclin D1 phosphorylation of pRb, defects of p16$^{INK4A}$ can result in an increased proliferation reate for the cell. The genetic changes of the p16$^{INK4A}$ gene include homozygous deletion and intragenic mutation (Gonzalgo and Jones, 1997). Moreover, recently, epigenetic changes in p16$^{INK4A}$ gene have been recognized as importans events leading to the occurrence of HCC. In particular, p16$^{INK4A}$ promoter methylation has been associated with reduced expression (Grisham J.W., 2001; Buendia, 2000; Kondo et al., 2000; Kawai et al., 2000; Chen et al., 2000a; Okabe et al., 2000).
Finally, a novel pathway leading to pRb inactivation has recently been discovered by the finding of a new oncogene termed gankirin (Higashitsuji et al., 2000), an ankrin-repeat protein homologous to the p28 subunit of the 26S proteasome. Gankyrin, over expressed in all HCCs, binds to pRb and promotes its degradation by the ubiquitin-proteasome pathway (Buendia, 2000).

1.3.3 DNA mismatch repair genes.
In addition to oncogenes and tumor suppressor genes, DNA mismatch repair genes have recently been identified as a class of susceptibility genes involved in the pathogenesis of human tumors. Defective DNA mismatch repair can lead to the accumulation of mutations and microsatellite instability in the cellular genome and thus increase the chance of malignant transformation (Blum, 2003). The role of DNA mismatch repair defects in HCC development is currently unknown.

1.3.4 Telomerase activation.
Telomeres correspond to the ends of eukaryotic chromosomes and are specialized structures containing unique (TTAGGG)n repeats (Blackburn, 1991). Telomeres protect the chromosomes from DNA degradation, end to end fusions, rearrangements, and chromosome loss (de Lange, 1994). Because cellular DNA polymerase cannot replicate the 5’ end of the linear DNA molecule, the number of telomere repeats decreases during aging of normal somatic cells. The progressive shortening of telomeres may control the proliferative capacity of normal cells (Harley et al., 1990) and serves as control mechanism against unregulated cellular proliferation. Telomerase, a ribonucleic acid-protein complex, adds hexameric repeats of 5’-TTAGGG-3’ to the end of telomeres to compensate for the progressive loss (Greider and Blackburn, 1985). Although normal somatic cells do not express telomerase, its expression has been found in most tumor cells. The activation of telomerase activity may play a significant role in hepatocarcinogenesis (Liu et al., 2003).

1.3.5 Growth factors.
As in most other forms of cancer, the downregulated expression of growth factors and of components of their signalling pathways may play an important role in hepatic oncogenesis. Indeed, overexpression of certain growth factors was found in HCC, including insulin-like growth factor II (IGF-II), transforming growth factor (TGF), and hepatovyte growth factor (HGF) (Blum, 2003). The role of growth factors in promoting HCC is still incomplete and
Introduction

Additional factors are likely to emerge as potentially important candidates involved in hepatocarcinogenesis. Among these, it is worthy of mentioning Serum response factor (SRF). SRF is a transcription factor, which binds to a serum response element (SRE) associated with a variety of genes including immediate early genes such as c-fos, fosB, junB, egr-1 and -2, neuronal genes such as nur1 and nur77 and muscle genes such as actins and myosins. By regulating the expression of these genes, SRF controls cell growth and differentiation. Recently, it has been proposed that SRF is implicated in HCC cell growth (Shao et al., 2005).

1.3.6 Other factors.

Other host factors beyond those addressed above may be genetic polymorphisms of enzymes metabolising environmental xenobiotics, such as alcohol and the hydrocarbons in tobacco smoke (Hassan et al., 2002).

Fig.3 Model of stepwise hepatocarcinogenesis (LOH: loss of heterozygosity).
2. **In-stent Restenosis.**

In-stent restenosis (ISR) is a relevant clinical problem which often follows therapeutic interventions aimed at the revascularization of coronary arteries partially/totally obstructed by atherosclerotic plaques.

2.1 **Arterial anatomy.**

The normal artery wall consists of three layers which, from the inner to the outmost part of the artery are represented by: the intima, the media, and the adventitia (Fig. 4).

The intima is constituted by a continuous layer of endothelial cells (ECs) connected each other by a series of junctional complexes (Miyoshi and Takai, 2005) and adherent to a layer of loose connective tissue named “basal lamina”. Endothelial cells form a barrier that controls the entry of substances from the blood into the arterial wall. Under physiological conditions, they control the passage of circulating molecules by active transport (endocytosis and exocytosis) and elaborate the connective tissue components which form their own substrate. Additionally, ECs prevent clotting partly by elaboration of prostaglandin (such as PGI$_2$) that inhibit platelet function. In case of EC layer damage, platelets adhere to the inner vessel surface and form a clot. Damaged ECs favour clot formation by synthesising a variety of molecules among which factor VIII represents a key player. In addition to produce molecules which can affect clot formation, ECs can secrete substances that influence the contraction-relaxation of the subjacent vascular smooth muscle cells (VSMCs).

The intima is delimited from the outer layer called “media” by the internal elastic lamina, an anatomical structure particular prominent in large and medium calibre arteries and absent in capillaries. The media consists of only one cell type, the VSMCs organised in either a single
layer (as in small muscular arteries) or in multiple lamellae (as in elastic arteries). The cells of the inner part of the media receive the nutrients from the vessel lumen while the cells of the outer part are nourished by small vessel termed “vasa-vasorum” which originate in the adventitia, the outer layer of arteries.

In the media, VSMC are surrounded by small amounts of collagen and elastic fibers which they produce together with other extra-cellular molecules such as proteoglycans. VSMCs are highly specialized cells whose principal function consists of the contraction and regulation of blood vessel tone-diameter, blood pressure, and blood flow distribution (reviewed in (Owens et al., 2004)). VSMCs within adult blood vessels proliferate at an extremely low rate, exhibit very low synthetic activity, and express a unique repertoire of contractile proteins, ion channels, and signalling molecules required for the cell’s contractile function (Owens, 1995). Thus, under physiological conditions they display a so called “contractile phenotype”, indicating that their activities are mainly devoted to regulated vessel tone (Adachi et al., 1998). However, unlike skeletal and cardiac muscle that are terminally differentiated, VSMCs retain remarkable plasticity and can undergo rather profound and reversible changes in phenotype in response to changes in local environment (Owens, 1995). In this regard, a classical example is represented by vascular injury, where the VSMCs dramatically increases their rate of proliferation, migration, and the capacity to produce different extra-cellular matrix molecules (synthetic phenotype), in order to repair the vessel damage. The possibility to switch from the contractile to the synthetic or proliferating phenotype is defined as “phenotypic modulation”. This ability, however, predisposes the cell to abnormal environmental signals that can lead to adverse phenotypic switching and acquisition of characteristics that can contribute to the development and/or progression of vascular disease.

The outermost layer of the artery, delimited from the media by a non continuous sheet of elastic tissue (external elastic lamina), is named adventitia. This external coat consists of a loose mixture of collagen, elastic fibres, VSMCs, fibroblasts and contains vasa vasorum and nerves.

2.2 Atherosclerosis.

Arteriosclerosis represents a generic term indicating the thickening and hardening of the arterial wall, which is responsible for the majority of deaths in most westernised societies (Bierman, 1995). One type of arteriosclerosis, defined atherosclerosis, is a disorder which involves larger arteries and which underlies most coronary artery disease, aortic aneurism, arterial diseases of the lower extremities and cerebrovascular diseases.
Atherosclerosis can be considered a form of chronic inflammation resulting from the interaction between modified lipoproteins, monocyte-derived macrophages, T cells and the normal cellular elements of the arterial wall, i.e. ECs and VSMCs (Ross, 1999). This inflammatory process can ultimately lead to the development of complex lesions, also defined plaques, that protrude into the arterial lumen.

Atherosclerotic lesions begin as “fatty streaks” underlying the endothelium of large arteries (Navab et al., 1996). The transition from the relatively simple fatty streak to the more complex lesion is characterized by the immigration of VSMCs from the medial layer of the artery wall through the internal elastic lamina to reach the intimal, or subendothelial, space. Intimal VSMCs take up modified lipoproteins, contributing to foam cell formation. Additionally, they show increased growth rate and are subjected to a number of other variations collectively referred to as “phenotypic modulation” (Owens et al., 2004).

Although advanced atherosclerotic lesions can lead to ischemic symptoms as a result of progressive narrowing of the vessel lumen, acute cardiovascular events can also result from plaque rupture (Lee and Libby, 1997). Plaque rupture exposes plaque lipids and tissue factor to blood components, initiating the coagulation cascade, platelet adherence thus leading to thrombosis and to a sudden vascular occlusion with the related ischemic symptoms (Fiotti et al., 2006).

2.3 Artery reocclusion.

In order to restore blood flow in artery totally/partially occluded by atherosclerotic plaques, different approaches have been developed. The first to be established was the coronary artery bypass grafting (CABG) followed by percutaneous transluminal coronary angioplasty (PTA) (late 1970s). Subsequently, PTA has been combined with the implantation of either intravascular bare metal stents (late 1980s) or drug eluting stents (beginning 2000s).

2.3.1 Percutaneous transluminal angioplasty (PTA).

In order to revascularize stenotic coronary arteries, since 1979 (Grunzig et al., 1979) it has been introduced the so called percutaneous transluminal coronary angioplasty (PTCA). This is a non-surgical method that has been shown to be safe and effective in comparisons to CABG (Pocock et al., 1995), a surgical approach used to circumvent artery stenosis.

PTCA (Fig 5) involves:

- advancing a balloon catheter to an area of coronary narrowing
- inflating the balloon
retrieving the catheter following balloon deflation.

Angioplasty can be applied to broad groups of patients, reducing the severity of coronary stenosis and diminishing or eliminating ischemia. More than 500,000 percutaneous coronary intervention procedures are performed yearly in the USA, and about 1 million procedures worldwide (American Heart Association, 2001). However, PTCA has been shown to induce (Fig. 6) the development of symptomatic re-occlusion (restenosis) caused by early elastic recoil, intimal thickening, late constricting remodelling of the vessel (Ruygrok et al., 2003) and formation of mural thrombus in about 30-50% of treated patients (Califf, 1995).
2.3.2 **Bare metal stents.**

To try to overcome the PTCA related problems, the expansion of the balloon during angioplasty has been associated with the deployment of a stent. This is an expandable metal tubular mesh usually made of stainless steel or titanium/nickel (nitinol) alloys (Fig. 7), firstly developed in 1987 (Sigwart et al., 1987). The deployment of the stents has significantly reduced the rates of early elastic recoil and late constructive remodelling of the vessel reducing restenosis rate down to 20-30% (Serruys et al., 1994; Fischman et al., 1994). The partial success of the stents is due to the induction of the intimal thickening (in-stent restenosis, ISR), a phenomenon particularly evident in small calibre vessels (Ruygrok et al., 2003; Moreno et al., 2004) and charaterized by an exuberant proliferation of VSMCs. Clinical ISR produces recurrent angina or evidence of myocardial ischemia. By angiography, a vessel is considered affected by ISR when its lumen at the dilated segment is occluded for more than 50%.

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**Fig.6** Possible complications of PTCA (from (Bult, 2000)).
2.3.3 Drug eluting stents.

With the aim to overcome the excessive VSMC proliferation observed after bare metal stent implantation, devices able to locally deliver anti-proliferative drugs (drug-eluting stent (DES)) have been developed (Fig.8). Currently, more than 85% of all coronary interventions in the United States are performed with drug-eluting stents (Kandzari et al., 2005). Two pivotal trials, Sirolimus-Eluting Balloon Expandable Stent in the Treatment of Patients With De Novo Native Coronary Artery Lesions (SIRIUS) (Moses et al., 2003) and Treatment of De Novo Coronary Disease Using a Single Paclitaxel Eluting Stent (TAXUS)-IV (Stone et al., 2004), demonstrated striking reductions in angiographic restenosis and revascularization rates with sirolimus- and paclitaxel-eluting stents, respectively, compared to bare metal stents (9% ISR rate in sirolimus-stent compared to 21% in bare metal stents; 8% ISR rate in paclitaxel-stent compared to 26% in bare metal stents). Sirolimus (Rapamycin®) is an immunosuppressor of T-lymphocytes also capable of reducing the inflammatory response and the proliferation of VSMCs. Moreover, sirolimus promotes apoptosis in VSMC (Giordano et al., 2006). Paclitaxel (Taxol®) is a microtubule stabilizing agent which reduces the concentration of free tubulin required for new tubulin formation. This in turn down regulates biological processes such as proliferation and
migrates VSMC (reviewed in (Liuzzo et al., 2005)). In addition, Paclitaxel induces apoptosis and cell death.

![Diagram of drug eluting stent](image)

**Fig. 8** Drug eluting stent

The current U.S. Food and Drug Administration approved indication for drug-eluting stents is for low risk patients with discrete, de novo lesions in native vessels with reference vessel diameters of 2.5 mm to 3.5 mm (Rockville, 2007). Nevertheless, the unrestricted implementation of drug-eluting stents seems to be widely accepted in contemporary interventional clinical practice. This widespread adoption has largely been driven by non-randomized registry experience and has been fuelled by enthusiastic dissemination and acceptance of data and aggressive marketing. However, some concerns are now emerging (reviewed in (Tung et al., 2006; Serruys and Daemen, 2007)). First of all, the impressive results on ISR in the pivotal drug-eluting stent trials may have been overestimated. Careful examination reveals inordinately high ISR rates in the bare metal stent control group, nearly 50% higher than that observed in contemporary randomized trials and in real-world clinical experience. The inferior performance of bare metal stents may be explained by the use, in the above cited trials, of thick-strut stents which are known to have higher ISR compared to thinner-strut stents (Pache et al., 2003). Thus, the impressive ISR reduction rate observed using DES may be exaggerated because of the use of sub-optimal control bare metal stents. Not only over-estimation of the beneficial effects of DES may have occurred, also under-estimation of adverse events, such as thrombosis, may have occurred. Stent thrombosis usually occurs before re-endothelialization of the treated artery (healing) has been completed. Stent thrombosis rarely occurs beyond 2 to 4 weeks for bare metal stents. In contrast, in a large observational study of DES use in real-world settings, the incidence of stent thrombosis was 1.3% (29 of 2229 cases) at 9 months (Iakovou et al., 2005). This implies
a prolonged antiplatelet therapy with aspirin and clopidogrel for at least 6 months or, in some cases, for longer or perhaps indefinitely.

Finally, it should be also pointed out that the re-vascularization benefit with DES is attenuated in high-risk patients (diabetes; the acute coronary syndromes, including ST-segment elevation myocardial infarction; smaller-diameter lesions and longer lesions; several stents or overlapping stents), compared to low risk patients displaying, for example, 26% ISR rate in coronary bifurcation lesions (Colombo et al., 2004).

2.4 ISR patho-physiology

According to Mehran et al. (Mehran et al., 1999) four classes of ISR patients can be distinguished:

- **Class I Focal in-stent restenosis group**: lesions are ≤10 mm in length and are positioned at the unscaffolded segment, the body of the stent, the proximal or distal margin (but not both), or a combination of these sites (multifocal in-stent restenosis).

- **Class II “Diffuse intrastent” in-stent restenosis**: lesions are >10 mm in length and are confined to the stent(s), without extending outside the margins of the stent(s).

- **Class III “Diffuse proliferative” in-stent restenosis**: lesions are >10 mm in length and extend beyond the margin(s) of the stent(s).

- **Class IV In-stent restenosis with “total occlusion”**.

Many predictors of ISR have been identified so far. Several studies (reviewed in (Scott, 2006)) have demonstrated an association between ISR and stent length, multiple stents, smaller final minimal lumen diameter, lack of use of intravascular ultrasound, the female gender, the presence of diabetes, and polymorphism in the angiotensin converting enzyme gene. Beside these factors, it is now accepted that stent implantation following PTCA represents a traumatic event which is the primary cause of ISR, both in the presence of bare metal stent and DES (Karthikeyan and Bhargava, 2004).

The first traumatic event takes place during the balloon-driven expansion of the stent (Fig. 4). The trauma of this procedure is often exacerbated by the inability of the balloon to expand the stent fully to its nominal size. This requires a second dilation of the balloon called post-dilation, which leads to the over-expansion of the blood vessel wall (Brodie et al., 2003; Vernhet et al., 2002). Second, inadequate stent deployment is likely to alter the blood flow conditions increasing the risk of reduced laminar flow and increased pressure gradients. These flow disturbances and pressure gradients have been advocated as possible factors predisposing to the
formation of a neointimal thickening (Muller-Hulsbeck et al., 2001). Third, an over-expansion of the vessel wall is induced by the presence of the device. Indeed, some types of stents seems to enlarge progressively after implantation (Yu et al., 2002). Additionally, endo-vascular stenting produces a significant decrease in arterial wall compliance and extendibility (Vernhet et al., 2003). In physiologic conditions the arterial wall constantly undergoes physical deformation due to the pulsatile change in aortic pressure and consequent coronary perfusion pressure. These mechanical stimuli cause changes in the coronary vessel size, generating cyclic mechanical strain and cyclic flexion. Conversely, in patients with implanted stents, the vessel wall compliance to these stimuli is decreased as the local artery extendibility is impaired and regional stresses are augmented by the stent. These non-physiologic cyclical strains have been shown to induce the proliferation of the VSMCs, heavily contributing to thickening of the intima (Sudhir et al., 2001).

Above all, the damage caused by the expansion of the stent to the vessel wall is considered the most important event leading to ISR. The destruction of the tissue integrity, paralleled by the presence of a foreign body (i.e., the stent), is likely to impair the normal wound healing, which instead develops into hyperplasia (Virmani and Farb, 1999), mainly characterized by an exuberant proliferation of VSMCs which leads to the thickening of the intima. Although reduced in the frequency, these problem also plagues DES, especially in high risk patients.

2.4.1 Intimal thickening.

The neointima thickening, directly related to the degree of penetration of the stent within the vessel wall media (Farb et al., 2002), follows a temporal evolution (Fig.9). Within ten days from stent implantation the constant presence of platelet-rich thrombi surrounding the stent struts is evidenced. This fibrin clot is almost completely resorbed after 12 days. A gradual formation of VSMC-rich tissue, is observed between 12 and 30 days after implantation. These cells are typically associated to a proteoglycan-rich extracellular matrix (ECM). Inflammatory cells are always present. Among them, neutrophils are prevalent during the early phases of implantation, but their number tend to decrease between 12 and 30 days of implantation. Conversely, macrophages are constantly present. Notably, the score of the inflammatory cells contacting the strut is higher in those cases where the damage of the media is more pronounced. From 3–18-months after implantation (Farb et al., 2004; Skowasch et al., 2004), the restenotic tissue is characterized by a proteoglycan-rich (versican and hyaluronan) ECM. This ECM is constantly infiltrated by $\alpha$-actin-positive VSMCs, thus pointing towards the relevance of VSMC hypercellularity which is considered to be relevant in the pathogenesis of ISR. The
hypercellularity mainly results from the previously occurred VSMC switch from the contractile to the synthetic phenotype. Hypercellularity also depends on the mobilization of progenitor cells from the bone marrow to the injured vessel area (Shoji et al., 2004). Additionally, macrophages, which can differentiate into myofibroblasts, also contribute to the hypercellularity (Bayes-Genis et al., 2002). Notably, VSMC proliferation, although reduced in extent, persists for months after stent implantation.

![Events leading to instent restenosis](image)

**Fig. 9** Events leading to instent restenosis  (Modified from:(Edelman and Rogers, 1998))

The hypercellular ECM mostly contains collagen Type III during the first 9 months, and is progressively substituted by collagen Type I at later stages. After 18 months of implantation, decorin-positive staining and collagen Type I prevail. VSMCs and inflammatory cells density are significantly reduced after 18 months (Skowasch et al., 2004; Sakai et al., 2004). Such an ECM is typical of a tissue not fully healed (Farb et al., 2004). Notably it has also been observed that the neointimal tissue can undergo a “contraction” comparable to that occurring in a wound healing process. The contraction seems to be induced by the colonization of the ECM by myofibroblasts which interact with the ECM components (Turley, 2001). Finally, it has to be pointed out that in ISR the endothelium in the stented region is dysfunctional. This has been proved observing the altered endothelium reactivity to acetylcholine (Caramori et al., 1999). Moreover, in the presence of DES, re-endothelization is considerably delayed due to the anti-
proliferative/apoptotic effects exerted by the released drugs (Tung et al., 2006). This phenomenon is probably responsible for the increased thrombosis rate observed for DES compared to bare metal stents (Tung et al., 2006).

Taken together, these data indicate that the process of ISR can be divided in two main phases:

- The wound healing process, where the activation of the clotting system is followed by an inflammatory response, and by the proliferation and migration of VSMCs;

- The neointima organization, where VSMCs reduce their proliferation rate to enter a synthetic phenotype and secrete a hyaluronan-rich ECM.

Neointimal thickening can be observed within any discreet location of the stented segment or can appear in a diffuse pattern. Although certain systemic characteristics (e.g., the presence of diabetes) or anatomical variables (small vessel diameter, long lesion length) increase the probability of ISR, the presence or absence of these factors does not completely explain the specific location of neointimal formation. In this regard, it has been shown that alterations in shear stress could cause important compensatory changes in both luminal and vessel diameter (Glagov et al., 1987).
3. **Cell cycle and proliferation.**

The relevance of VSMC and HCC cell proliferation in the pathogenesis of ISR and HCC, requires a description of the major events ruling this process. Moreover, these informations are useful to properly understand the strategy here adopted to explore possible novel therapeutic approaches.

Somatic cells proliferate to support tissue and organism growth and to replace damaged cells. Proliferation is also a fundamental response that underlies cellular mechanisms involved in immunity, inflammation, hematopoiesis, neoplasia, and other biological responses. Cell growth, replication and division in eukaryotic cells occur according to a highly controlled series of events called the cell cycle.

The cell cycle can be subdivided into two major stages: interphase (a phase between mitotic events) and mitosis (Fig.12).

![Cell cycle phases](image)

Fig.12 Cell cycle phases

There are three distinct, successive stages within interphase, called G1, S and G2 phases. During G1, cells “monitor” their environment and upon receipt of specific signals, induce the synthesis of protein required in the next phases. If proper conditions occur, cells “commit” to DNA synthesis (S phase) and replicate their chromosomal DNA. A G2 phase follows in which cells continue to prepare themselves for mitosis. In mitosis (M), there are four successive phases called prophase, metaphase, anaphase and telophase that are accompanied by cytoplasmic division (cytokinesis) giving rise to two daughter cells. For the most part, upon completion of the process, each daughter cell contains the same genetic material as the original parent cell.

In addition to these specific stages, the G0 phase has been described for cells that exit from the cell cycle and enter a quiescent, non-dividing state. In response to external stimuli, some quiescent cells may undergo reactivation and express early response genes. The G0-G1 transition
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is marked by measurable increases in newly-synthesized RNAs and proteins and by the expression of the Ki67 antigen (Gerdes et al., 1983).

3.1 Cell cycle regulators.

Many regulators of the different cell cycle phases have been described so far (Donjerkovic and Scott, 2000). Among them, cyclin E, the transcription factor E2F1 and the transcription factor SRF represent relevant mediators.

3.1.1 Cyclin E family.

Cyclin E1 (formerly called cyclin E) and the recently described cyclin E2 belong to the family of E-type cyclins that operate during the G1/S phase progression in mammalian cells. The two E-cyclins share a catalytic partner, cyclin-dependent kinase 2 (CDK2), which they activate at similar times during cell cycle progression. Despite these similarities, it is unknown whether the two proteins perform distinct functions, or, alternatively, they control S-phase entry of different cell types in a tissue-specific fashion.

Cyclin E1 is a nuclear protein (Hwang and Clurman, 2005; Moroy and Geisen, 2004) first identified through its ability to complement the proliferative defects in cyclin-deficient yeast cells (Koff et al., 1991). The gene for the human cyclin E protein encodes several polypeptides with molecular weights ranging from 39 to 52 kDa. The “regular” cyclin E1 protein contains a domain spanning from amino acid position 129–215, which is called “cyclin box”. Its sequence is to a certain degree conserved among cyclins (Fig. 13).

![Fig.13 Cyclin E1 protein structure](image)

A conserved stretch of hydrophobic amino acids within the cyclin box is responsible for the recognition of the RXL-motif substrates or inhibitors (Adams et al., 1996). Further towards the C-terminal end, cyclin E1 protein bears another “substrate recognition motif” which binds the retinoblastoma protein, pRb (Kelly et al., 1998). Finally, a PEST box sequence also known as “destruction box” present at the very C-terminal end of cyclin E1 at a position 370–385, is at least partially responsible for targeting the protein to degradation (Lew et al., 1991). In addition to the regular form of cyclin E1, different splice variants have been described (Ohtsubo et al.,
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1995) (Keyomarsi et al., 1995). It is unclear to date, whether the presence of a particular splice variant of cyclin E1 correlates with a specific cell cycle phenotype or a particular cellular state.

Expression of cyclin E1 varies during the cell cycle, peaking at the G1–S-phase boundary. Expression is regulated by proteins of the E2F-transcription factor family (Duronio and O'Farrell, 1995). More in detail, the transcriptional activation of the cyclin E1 gene depends on the activity of D-type cyclins which, coniugated with the respective cyclin dependent kinase – cdk2/4-, phosphorylate the retinoblastoma protein (pRb) family leading to the release of E2F/DP heterodimer from pRB complexes thus allowing E2F transactivation function. Since cyclin E1, coniugated with its cyclin dependent kinase- cdk2-, also phosphorylate pRb family protein, the concentration of free E2F progressively rises and leads to a further amplification of cyclin E transcription (Geng et al., 1996) (Fig. 14). This constitutes a classical feedback mechanism, conferring cyclin E1 the ability to stimulate its own transcription.

Repression of the cyclin E1 gene during G2-M and the early G1 phases of the cell cycle is mediated through the assembly of a multiprotein complex containing hypophosphorylated pRb, histone deacetylase (HDAC), and SWI/SNF which binds the E2F transcription factors bound to the cyclin E1 promoter, thus silencing cyclin E1 transcription. Another important factor in regulating cyclin E1 levels is represented by its degradation at the protein level which occurs via two distinct pathways involving the ubiquitin-proteasome system (Hwang and Clurman, 2005) and is regulated, at least in part, by autophosphorylation on Thr-380.

Fig. 14 Cyclin E/D regulation of the pRB/E2F1 complex.
A second member of the cyclin E family, emerging as a novel potential cell cycle regulator, is called cyclin E2. This protein shares at the protein level 47% overall similarity to cyclin E1 and contains a cyclin box motif that is characteristic of all cyclins (Lauper et al., 1998). The human cyclin E2 gene encodes a 404-amino-acid protein that is most closely related to cyclin E (Payton and Coats, 2002). Cyclin E2 mRNA levels peak at the G1/S transition. As cyclin E1, cyclin E2 controls the initiation of DNA synthesis by activating CDK2. Cyclin E2 associates with CDK2 in a functional, catalytically active kinase complex, which phosphorilates pRb leading to the release of E2F. Abnormally high levels of cyclin E expression have frequently been observed in human cancers; unlike cyclin E1, which is expressed in great majority of proliferating normal and neoplastically transformed cells, cyclin E2 levels are low in non-transformed cells and increase significantly in neoplasm-derived cells (Gudas et al., 1999).

In addition to the E2F1-mediated regulation of cyclin E1 expression, recently, the liver receptor homolog 1 (LRH-1, NR5A2) has been shown to be able to regulate cyclin E1 expression. Indeed in cyclin E1 promoter, one perfect consensus LRH-1-RE was identified (Botrugno et al., 2004). LRH-1 is a nuclear orphan receptor that binds as a monomer to the DNA sequence 5’-YCAAGGYCR-3’, the recognition motif for the fushi tarazu factor 1 (Ftz-F1) subfamily of nuclear receptors. It is predominantly expressed in tissues of endodermal origin including liver, exocrine pancreas, and intestinal (Fayard et al., 2004). The ligand binding domain of this receptor has been shown to adopt an active conformation in the absence of a bound ligand, providing an explanation for its constitutive transcriptional activity (Sabin et al., 2003). It has been shown recently that LRH-1 induces cell proliferation through the concomitant induction of cyclin D1 and E1, an effect that is potentiated by its interaction with β-catenin. Whereas β-catenin coactivates LRH-1 on the cyclin E1 promoter, LRH-1 acts as a potent tissue-restricted coactivator of β-catenin on the cyclin D1 promoter (Wang et al., 2005). This observation highlights on an additional function of this orphan nuclear receptor, which controls cell proliferation via crosstalk with the β-catenin signaling pathway.

3.1.2 The transcription factor E2F1.

E2F1 belongs to the E2Fs family (reviewed in (Bracken et al., 2004; DeGregori and Johnson, 2006) which all contain a DNA-binding domain (DB) (Fig. 15).
 Except E2F7 and 8, they also contain a dimerization domain (DIM) to form heterodimer with DP proteins. Sequences for transcriptional activation (TA) and pRB family protein binding (PB) are present in E2F1-5 only. Moreover, E2F1-3 share a cyclin-A-binding domain (cA), absent in the other members. DPs protein are distantly related to the E2F family, sharing homology in the DB and DIM domains.

The E2F transcription factors can be divided into four subgroups based on their main function. E2Fs 1–3a, the ‘activating’ E2Fs, are required for the transactivation of target genes involved in the G1/S transition and, hence, for correct progression through the cell cycle. E2Fs 3b, 4 and 5 are considered to possess predominantly repressive activity, because they are mainly nuclear in G0/G1 cells where they are bound to members of the retinoblastoma protein (pRB) family (Muller et al., 1997). E2Fs 6, 7 and 8 are the founding members of the last two subgroups and are also considered to be transcriptional repressors (Morkel et al., 1997). Six of the E2Fs protein, including E2F1, require, for proper activity, to form heterodimers with the DP proteins (Dp1 2/3 and 4), also considered to be part of E2Fs family (Bandara et al., 1993) (Fig.15).

The transcriptional activity of E2F1 through E2F5 is regulated through their association with pRB or the related pocket proteins, p107 and p130. Pocket protein binding blocks the transcriptional activity of E2F-DP heterodimers by masking the transcriptional activation domains located in the carboxy terminus of E2F1-5. This association prevents the E2F-DP heterodimer from recruiting the basal transcription factor TFIID, as well as transcriptional co-activators, such as p300/CBP, GCN5, TRAPP, Tip60 and ACTR/AIB1 (Louie et al., 2004). The expression pattern for E2F1-3 members is cell cycle dependent and mainly dependent of E2F1-3 itself as, for example, E2F1-3 contain E2F binding sites on their promoter (Bracken et al., 2004). Upon growth factor stimulation, E2F1-3 and DP1 mRNA levels progressively increase with a pick at the G1/S boundary, approximately reaching a 4 folds increase, compared to the basal expression (reviewed in (Slansky and Farnham, 1996). With the progression of the S phase and till the next G1 phase, their mRNA levels drops considerably. The same patter is followed by their protein levels which are mainly regulated, as describe for cyclin E, by ubiquitin-directed degradation (reviewed in Dyson (Dyson, 1998). In contrast to E2F1-3, E2F4 and 5 are constitutively expressed (DeGregori and Johnson, 2006).

3.1.3 The Serum Response Factor.

Serum Response Factor (SRF) is a phylogenetically conserved protein of 67Kda that belongs to MADS box family of transcription factors (Norman et al., 1988). SRF protein contains 508 amino acids; it contains three major domains: a Serum Response Element (SRE) DNA binding domain, a transactivation domain and several phosphorylation sites.
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SRF is a widely expressed transcription factor essential for murine embryogenesis (Arsenian et al., 1998). SRF not only controls the expression of immediate early genes (IEGs), such as c-fos or egr1, which are involved in proliferation of many cell types, but it also regulates selectively the expression of smooth muscle cells marker genes, such as smooth muscle α-actin, calponin-1, or transgelin (SM22α) (Dalton and Treisman, 1992; Hipskind et al., 1991; Manabe and Owens, 2001). Thereby, both the type of protein interaction partner and the kind of signal transduction determine SRF activity, including its cell-type- or target gene-specificity.

In contrast to the well-established function of SRF in cellular development, a possible role of SRF in cellular proliferation has not yet directly been addressed. Suggestive evidence exists, however, implicating SRF in cell proliferation. For instance, sphingosine 1-phosphate mediated VSMC proliferation has been shown to correlate with Erk-mediated phosphorylation of the SRF cofactor Elk-1 (Lockman et al., 2004). Also, ectopic overexpression of SRF in the A7R5 cell line established from rat thoracic aorta SMCs was associated with a twofold increased proliferation (Chai et al., 2004). Moreover, it seems possible that SRF plays an important role also in the HCC cell growth (Shao et al., 2005).

SRF is a stable protein with an half life of about 12 hours, that is not actively synthetized in serum-starved cells, but only upon serum stimulation, with kinetics that are delayed relative to Fos induction (Misra et al., 1991). Mature SRF protein results from a series of successive posttranslational modifications that retard its migration on SDS-polyacrylamide gels compared to the newly synthetized form. Newly synthetized SRF migrates as a 63- to 65-Kda species, which upon modifications is converted to a 67-Kda mature form (Misra et al., 1991). Recent studies have shown that the newly synthetized SRF is converted to mature SRF by phosphorylation and glycosylation events (Schroter et al., 1990).
4. RNA interference.

RNA interference (RNAi), firstly discovered in Nematodes (Fire et al., 1998) derives from an evolutionarily conserved mechanism, involved in cellular defense against viral invasion, transposon invasion and post-transcriptional regulation (Zamore, 2001; Tuschl, 2001; Sharp, 2001; McManus and Sharp, 2002; Hannon, 2002; Hutvagner and Zamore, 2002).

As a novel biological pathway, RNAi has quickly distinguished itself as a valuable reverse genetic tool. The functional intermediates in the RNAi pathway, small interfering RNAs (siRNAs), may be synthesized by a variety of means and also introduced intracellularly for research purposes to induce changes in gene expression. The potency and specificity of siRNA-mediated gene silencing exploits a naturally occurring pathway, thus distinguishing it from other nucleic acid-based silencing technologies such as antisense oligonucleotides (Scanlon et al., 1995) and ribozymes (Grassi et al., 2004) (Fig.10).

![Fig.10 Silencing pathways. (A: Protein activity may be silenced by an inhibitor that binds the protein directly; B: An antisense oligonucleotides may bind complementary target mRNA and thereby block translation; C: A ribozymes may promote degradation of target MRNA molecules; D:siRNAs may incorporate into the RISC and catalyze cleavage of complementary mRNAs.](image-url)
4.1 RNA interference mechanism.

The original phenotypic observations of RNAi were made over a decade ago by researchers working in plant and fungal genetics (Jorgensen, 1990). In the late 1990’s, major strides in understanding the mechanics underlying RNAi were made, most notably by Andrew Fire, Craig Mello and their colleagues. Working with *C. Elegans*, the founders of this emerging field in RNA biology observed that injection of double-stranded RNA (dsRNA) induced sequence-specific reduction of the target mRNA. Moreover, the authors documented that only a few molecules were required to reduce the population of the target mRNA, thus suggesting an extremely potent mechanism of action (Fire, 1999).

RNAi pathways are guided by small RNAs that include small interfering RNA (siRNA) and microRNAs (miRNAs). The siRNA pathway begins with the cleavage of long double-stranded RNA (dsRNA) by the Dicer enzyme complex into siRNA. These siRNAs are incorporated into Argonaute 2 (AGO2) and the RNAi-induced silencing complex (RISC). The sense strand of the duplex siRNA is then cleaved by AGO2 so that the antisense strand can direct the cleavage of the target RNA by RISC.

The microRNA pathway begins with endogenously encoded primary microRNA transcripts (pri-miRNAs) that are transcribed by RNA polymerase II (Pol II) and are processed by the Drosha enzyme complex to yield precursor miRNAs (pre-miRNAs). These precursors are then exported to the cytoplasm by exportin 5 and subsequently bound to the Dicer enzyme complex, which processes the pre-miRNA for loading onto the AGO2–RISC complex. When the RNA duplex loaded onto RISC has imperfect sequence complementarity, the passenger (sense) strand is unwound leaving a mature miRNA bound to active RISC. The mature miRNA recognizes target sites (typically in the 3’-UTR) in the mRNA, leading to direct translational inhibition (Fig.11).
4.2 SiRNA delivery in vitro.

Due to their ability to specifically induce the destruction of a given target RNA, siRNAs have been used to down modulate the expression of deleterious genes in cellular and animal models. However, due to their chemical nature, siRNAs cannot be delivered to the target cells as naked molecules. Two main categories of siRNAs delivery systems exist: viral and non-viral. Viral constructs have the advantage of being able to guarantee a long lasting expression of the siRNA of interest. However, they can induce serious deleterious effects (Chen et al., 2003). Non-viral approaches are considered to have far less draw backs, although multiple administrations are required for a long lasting presence of the siRNA within the cells (Luo and Saltzman, 2000). To
overcome this limitation, a number of innovative approaches are intensively pursued (Agostini et al., 2006).

Among the non-viral approaches, those most commonly used include lipid-mediated transfection, electroporation, calcium phosphate-mediated delivery and microinjection. Successful introduction of siRNA into cells using any of these methods depends on a number of factors including delivery reagent chemical composition and charge, siRNA amounts and cell membrane characteristics. For this reason, delivery methods demand comprehensive optimization to achieve effective silencing while minimizing toxicity.

4.2.1 Lipid-mediated transfection.

Lipid-mediated transfection is currently the most popular method for introducing siRNA into cell cultures (Billy et al., 2001; Caplen et al., 2000; Elbashir et al., 2001; Elbashir et al., 2002; Brummelkamp et al., 2002; Weil et al., 2002; Hutvagner et al., 2001). For lipid-mediated delivery to be successful, multiple dynamic categories must be taken into consideration, including lipid concentrations, total siRNA concentrations and cell type characteristics (i.e., adherent or non-adherent, differentiated or undifferentiated, primary cell or transformed cell line). Transfection protocols are often unique for each cell type. In most cases, cells should be in an exponential growth phase and actively dividing at the time of transfection. The growth rate will determine optimal cell densities for plating and transfection. Cell densities that are too high should be avoided as overgrown cell populations limit access to siRNA:lipid complexes and have a greater sensitivity to the toxic effects associated with transfection. Similarly, cell densities that are too low may also lead to toxicity due to excessive exposure and uptake of siRNA:lipid complexes. Moreover, the composition and concentration of lipid plays a significant role in lipid-mediated transfection. Different cell types may exhibit greater or lesser levels of viability and transfection efficiencies with different transfection reagents. Lastly, the presence or absence of serum should be rigidly controlled during transfection procedures. Some reagents are incompatible with the presence of serum and require serum-free conditions to prevent potential binding to serum proteins and to ensure efficient transfer of siRNA:lipid complexes. Finally, total siRNA amount, the siRNA:lipid ratio and the duration of the transfection are important parameters to be considered when optimizing transfection procedures. Optimizing the interplay between the important transfection factors is critical for each cell line.

In contrast to in vitro studies, siRNAs for in vivo use must also have additional attributes that permit appropriate biodistribution and that result in the desired pharmacokinetic properties (absorption, distribution, metabolism, excretion) to achieve silencing. Rapid infusion or
hydrodynamic injection of siRNA achieves the greatest overall delivery efficiency to a limited set of vascularized tissues, with the primary recipient tissue being the liver. Recently, macromolecules have been proposed to ferry the siRNAs through the bloodstream into target tissues. These include conjugation of a cholesterol moiety to one of the strands of the siRNA duplex (Soutschek et al., 2004) (Wolfrum et al., 2007). It has been demonstrated that cholesterol-conjugated siRNAs can silence gene expression in vivo. Efficient uptake of these siRNA conjugates depends on interactions with lipoprotein particles, lipoprotein receptors and transmembrane proteins. But not only cholesterol is able to bind siRNAs to lipoprotein particles. There are other possible lipophilic conjugates, such as long-chain fatty acids and bile acids, that are also effective in binding to lipoproteins like LDL or HDL and which lead to siRNA uptake into cells. The role of LDL and HDL binding of lipophilic siRNA conjugates is very important, because the cellular uptake is mediated by their corresponding uptake receptors. HDL directs siRNA delivery into liver, gut, kidney and steroidogenic organs, whereas low-density lipoprotein (LDL) targets siRNA primarily to the liver (Wolfrum et al., 2007).
AIMS OF THE STUDY

The aim of this thesis is to provide the rational for the development of novel therapeutic approaches for the treatment of Hepatocellular carcinoma (HCC) and In-stent Restenosis (ISR). HCC is the third leading cause of cancer death worldwide, with an estimated 564,000 new cases and almost as many deaths in 2000. Despite current therapies, local recurrences are the rule and life expectancy is short. ISR still represents a major non completely solved problem which often follows the revascularization of coronary arteries partially/totally obstructed by atherosclerotic plaques. To restore blood flow, plaques are first mechanically destroyed by percutaneous transluminal angioplasty (PTA) and subsequently a stent is placed at the site of intervention. Although stent can prevent early artery elastic recoil and late remodeling, it induces a particularly pronounced intimal hyperplasia characterized by exuberant proliferation of vascular smooth muscle cells (VSMC). The use of stents releasing anti-proliferative drugs substantially contributed to reduce ISR rate in arteries bearing single primary lesions by down regulating VSMC growth. However, the limited efficacy of conventional therapies for complex coronary lesions suggests the development of alternative approaches.

Here we plan to inhibit HCC derived cells and VSMC growth by targeting the transcription factor SRF. SRF has been implicated in the control of the expression of genes involved in the proliferation of many primary cell types, but direct evidence of its involvement in VSMC and HCC derived cells are lacking. Furthermore, SRF has been recently shown to be implicated in the proliferation of HCC cells in vivo. As an additional strategy to target HCC proliferation we plan to knock down two pivotal cell cycle promoting genes, cyclin E1 and the transcription factor E2F1. The choice of these genes as targets is particularly attractive because of the existence of a feed-forward loop between them which amplifies cell proliferation promoting signals. Moreover, they both have been implicated in the development of HCC. Finally, as it is becoming evident the role of the second member of the cyclin E family, i.e. cyclin E2, in the E2F1-cyclin E1 pathway, also cyclin E2 was considered as target in our research.

As tools to inhibit target gene expressions, we plan to use small interfering RNAs (siRNAs) whose potential application in many different human disease has been recently reported.
MATERIALS AND METHODS

1. Cell cultures.
In the case of HCC, three different cell lines were evaluated: HepG2, HuH7 and JHH6. These three cell lines have been chosen as they reflect different phenotypes: HepG2, HuH7 and JHH6 were assigned to high, medium and low hepatocytic differentiation grade on the basis of the capacity to synthesize albumin, a known marker of hepatic differentiation (Rothschild et al., 1972). HepG2 exhibit a relatively normal hepatocyte phenotype, including many hepatic inducible enzymes and an aromatic hydrocarbon receptor (Darlington et al., 1987; Ellsworth et al., 1986). HuH7 and JHH6 cell lines derives from a differentiated and undifferentiated hepatoma respectively (Fujise et al., 1990). Despite the almost undetectable albumin production, JHH6 were able to synthesize ferritin, thus showing a residual hepatic phenotype (Grassi et al., 2007).

HepG2 and HuH7 cell lines were cultured in DMEM High Glucose medium (Celbio); JHH6 cell line was cultured in William’s medium (Sigma). All media contained 10% of heat inactivated foetal bovine serum (Euroclone, Celbio), 100 U/mL penicillin, 100 μg/mL streptomycin and 2mM L-glutamine (Euroclone, Celbio). HCC cell lines were grown at 37°C in a humidified atmosphere at 5% CO2.

With regard to in-stent restenosis, primary cultures of human vascular smooth muscle cells (VSMC) (Promocell), isolated from three different donors, were purchased from Promocell. For all experiments, cells in the third passage were used. VSMC were cultured in a medium containing one third Smooth Muscle Cell Basal Medium (Promocell), one third Nutrient Mixture F-12 (Euroclone, Celbio) and one third Waymouth Medium (Invitrogen) supplemented with 15% of heat inactivated foetal bovine serum and 100 U/mL penicillin and 100 μg/mL streptomycin (Euroclone, Celbio) at 37°C in a humidified atmosphere containing 5% CO2.

2. Selection of siRNAs targeted agains SRF, Cyclin E1, Cyclin E2 and E2F1.
A preliminary selection of siRNAs was conducted according to the previously reported guide lines based on a mathematical algorithm (Poliseno et al., 2004a; Poliseno et al., 2004b). In the case of siRNAs targeted against SRF, a previously selected molecule was used (werth et al., 2007).
3. **Uptake studies.**

For each cell type considered, uptake studies were performed in the presence of variable amounts of transfection reagent and siRNAs conjugated with a fluorescein-isothiocyanate (FITC) molecule in order to optimize the weight ratio siRNAs/transfection reagent. Transfection time was also optimised for each cell type considered (1-6 hours); uptake efficiencies were studied soon after transfection by evaluating the amount of FITC positive cells using a fluorescence microscope (Leica Mycrosystem); a more quantitative evaluation was carried out counting FITC positive cells by flow citometry (FACScanto, Becton Dickinson, DIVA software) after cell trypsinization.

**Optimized HCC cell transfection conditions:** 7.65, 2.55 or 2.55 μg of liposome (Lipofectamin2000 -1mg/ml, Invitrogen) for HepG2, HuH7 and JHH6, respectively, were mixed in 125 μl of appropriate medium (Optimem, Invitrogen) for 15 minutes at room temperature. 2.55 μg of the selected siRNAs were dissolved in 125 μl of Optimem for 15 minutes at room temperature and subsequently mixed with the liposome solution prepared as above. After 20 minutes, necessary to allow complexes formation, the mixture was added to 800 μl of decomplemented DMEM High Glucose medium to reach a final siRNA concentration of 220 nM and administered to the cells seeded in 6-well plate at density of 3.8x10^3 cells/cm^2. After three hours, the supernatant was replaced by 3 ml of complete medium (Fig.1).

![Fig.1: Transfection protocol.](image)
Optimized VSMC transfection conditions: 12.0 μg of liposome (CellFectin -1mg/ml, Invitrogen), were mixed in 125 μl of appropriate medium (Smooth Muscle Cell Basal Medium, Promocell) for 15 minutes at room temperature. 4.8 μg of the selected siRNAs were dissolved in 125 μl of Smooth Muscle Cell Basal Medium for 10 minutes at room temperature and subsequently mixed with the liposome solution prepared as above. After 20 minutes, the mixture was added in 1500 μl decomplemented Smooth Muscle Cell Basal Medium to reach a final concentration of 220 nM and administered to the cells cultured in 6 cm plastic dishes at density of 3.3x10³ cells/cm². After two hours, the supernatant was replaced by 4 ml of VSMC complete medium (Fig.1).

3.1 Experimental set up.
HCC cell lines were seeded in complete medium at a density of 3.8x10³ cells/cm² in 6-well plate and allowed to attach overnight. The experiments were performed with actively growing cells (proliferating cells) and transfections were conducted the day after seeding (see protocol 1) (Fig.2). In a variation of protocol 1, cells were re-transfected at day three and then collected at day six.

As regard with VSMC, 3.3x10³ cells/cm² (Grassi et al., 2005b) were seeded into 6 cm plastic dishes in the presence of complete medium. In the experiments performed with actively growing cells (proliferating cells), transfections were conducted the day after seeding (Protocol 1). In the case of Protocol 2, one day after seeding, cells were kept under starving conditions for 48 hours and then transfected (re-proliferating cells) (Fig.2).

![PROTOCOL 1](imageurl)

Fig. 2: Transfection scheme.
In a variation of Protocol 2, after transfection, VSMC were cultured in the presence of platelet-derived growth factor-ββ (PDGF-ββ) (Sigma) in place of serum. In another variation of Protocol 2, cells were cultured in the presence of 22mmol/l glucose (high glucose HG) to simulate the high glucose concentration found in diabetic patients.

Different commercially available transfection reagents, i.e. Oligofectamin, Cellfectin, Lipofectin and Lipofectamin2000 (Invitrogen) were tested.

Under final optimized conditions, non treated cells, cells treated with selected siRNAs, cells treated with the transfection system only and cells treated by a control siRNA directed against the luciferase gene (siGL2), were analysed in parallel.


The evaluation of cell morphology and number were performed by direct microscopic observation (Nikon Eclipse TS100) and by Thoma’s haemocytometer chamber. Cell viability was evaluated by 0.05% Trypan Blue dye staining (w/v in PBS) (1:1,v/v). Total viable cell number was calculated by the formula:

\[ X_n = N^o \times 2 \times 10^4 \]

where \( X_n \) is the number of cells in 1 ml of sample, \( N^o \) is the number of viable cells, 2 is the dilution’s factor with Trypan Blue dye and \( 10^4 \) is the conversion’s factor of Thoma’s haemocytometer.
5. Evaluation of cell colony number and area.
After siRNA treatment (Protocol 1), cells were gently washed by PBS and then added with fresh medium. Analysis were performed with living cells in order not to alterate the morphological characteristics of cells and evaluated by an inverted microscope (Leica Mycrosystem). Colony morphometrical analysis and amount were calculated using the Sigma Scan Pro program. Five fields for each treatment, each containing at least 20 events (magnification = 5x), were considered.

6. Quantitative real time RT-PCR.
Total RNAs were extracted from siRNA treated and control cells (siGL2 and non treated cells) by using the RNeasy Mini kit (Qiagen). The quantification of total RNA was evaluated by spectrophotometric determination using NanoDrop ND-1000 (CelBio). The RNA quality was controlled by a Lab-on-Chip-System Bioanalyser 2100 (Agilent).
Reverse transcription reactions were performed using 500ng of each RNA sample; the master mix for reverse transcription was prepared by adding the reagents in the order and in the proportions shown in table 1:

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>25mM MgCl$_2$ Solution</td>
<td>5mM</td>
</tr>
<tr>
<td>10x PCR Buffer II</td>
<td>1x</td>
</tr>
<tr>
<td>dGTP</td>
<td>1mM</td>
</tr>
<tr>
<td>dATP</td>
<td>1mM</td>
</tr>
<tr>
<td>dCTP</td>
<td>1mM</td>
</tr>
<tr>
<td>dTTP</td>
<td>1mM</td>
</tr>
<tr>
<td>Rnase Inhibitor</td>
<td>1U/μl</td>
</tr>
<tr>
<td>MuLV Reverse Transcriptase</td>
<td>2.5U/μl</td>
</tr>
<tr>
<td>Random Hexamers</td>
<td>2.5μM</td>
</tr>
</tbody>
</table>

*Table 1: Reverse Transcription Master Mix*

Using Random Hexamers as reverse transcriptase primer, allow samples to incubate at room temperature for 10 minutes: the room temperature incubation allows for the extension of the
exameric primers by reverse transcriptase. The extended primers will then remain annealed to
the RNA template upon raising the reaction temperature of 42°C.

Real-Time polymerase chain reaction was performed using the SYBRGreen Master Mix
(Applied Biosystems) on a 7900/HT Sequence Detection System (Applied Biosystems). The
primers (MWG Biotech, GA) and the T_m used are reported in Table 1.

Amplifications, conducted in triplicate, were performed in a final volume of 35μl of SYBRGreen
Master Mix buffer, containing primers (300nM each) and 1μl cDNA. 28s rRNA and all the other
target genes were submitted to 40 cycles of amplification, preceded by pre-denaturation at 95°C
for 10 minutes, denaturation at 95°C for 15 seconds, annealing at proper temperature for 60
seconds and extension at 72°C for 30 seconds. A final extension at 72°C for 10 minutes and a
dissociation stage (95°C/60°C/95°C for 15 seconds each) was then added. The relative amounts
of the mRNA of target genes were normalized by 28s rRNA content according to Pfaffl (Michael
W.Pfaffl, 2004).
Table 1. Primers and annealing temperature.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer pair</th>
<th>Tm</th>
<th>Length (bp)</th>
<th>Amplification Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>28S (M11167)</td>
<td>Fw 5’–TGG GAA TGC AGC CCA AAG- 3’</td>
<td>60°C</td>
<td>84</td>
<td>282-365</td>
</tr>
<tr>
<td></td>
<td>Rev 5’–CCT TAC GGT ACT TGT TGA CTA TGC–3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRF (J03161)</td>
<td>Fw 5’- AGT GCA GGC CAT TCA AGT G-3’</td>
<td>60°C</td>
<td>128</td>
<td>1426-1554</td>
</tr>
<tr>
<td></td>
<td>Rev 5’-ACG GAT GAC GTC ATG ATG GTG-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin E1 (NM 001238)</td>
<td>Fw 5’-TGC CTG TAC TGA GCT GGG CA-3’</td>
<td>60°C</td>
<td>112</td>
<td>497-608</td>
</tr>
<tr>
<td></td>
<td>Rev 5’-GGC TGC AGA AGA GGG TGT TG-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin E2 (NM 057749)</td>
<td>Fw 5’-CTT CCA AAC TTG AGG AAA TC-3’</td>
<td>62°C</td>
<td>94</td>
<td>696-789</td>
</tr>
<tr>
<td></td>
<td>Rev 5’-TCC ATC CTT AAG ATA TCC TC-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2F1 (NM 005225)</td>
<td>Fw 5’-CCA GGA AAA GGT GTG AAA TC-3’</td>
<td>62°C</td>
<td>74</td>
<td>466-539</td>
</tr>
<tr>
<td></td>
<td>Rev 5’-AAG CGC TTG GTG TGC AGA TT-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin D1 (NM 053056)</td>
<td>Fw 5’-CCG TCC ATG CGG AAG ATC-3’</td>
<td>62°C</td>
<td>70</td>
<td>369-438</td>
</tr>
<tr>
<td></td>
<td>Rev 5’-CCT CCT CCT CGC ACT TCT GT-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin A1 (NM 003914)</td>
<td>Fw 5’-GTC AGA GAG GGG ATG GCA T- 3’</td>
<td>58°C</td>
<td>90</td>
<td>804-894</td>
</tr>
<tr>
<td></td>
<td>Revf 5’- CCA GTC CAC CAG AAT CGT G -3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LRH-1 (NM 205860)</td>
<td>Fw 5’-CCG ACA AGT GGT ACA TGG AA-3’</td>
<td>60°C</td>
<td>112</td>
<td>1382-1493</td>
</tr>
<tr>
<td></td>
<td>Rev 5’-ACT CAT GAG GTT GTG GAG G-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p21waf1/cip1 (NM 000389)</td>
<td>Fw 5’-AGT GGA CAG CGA GCA GCT GA-3’</td>
<td>62°C</td>
<td>83</td>
<td>166-248</td>
</tr>
<tr>
<td></td>
<td>Rev 5’-CGA AGT TCC ATC GCT CAC GG-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p27kip1 (NM 004064)</td>
<td>Fw 5’-CGG TGG ACC ACG AAG AGT TAA-3’</td>
<td>60°C</td>
<td>66</td>
<td>569-634</td>
</tr>
<tr>
<td></td>
<td>Rev 5’-GGC TCG CCT CTT CCA TGT C-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OAS1 (NM016816)</td>
<td>Fw 5’-TCC AAG GTG GTA AAG GGT GG-3’</td>
<td>60°C</td>
<td>68</td>
<td>272-339</td>
</tr>
<tr>
<td></td>
<td>Rev 5’-AGG TCA GCG TCA GAT CGG C-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
7. Protein extraction.

After siRNA treatment (Protocol 1 or Protocol 2), cells were collected by centrifugation at 1000 rpm for 5 min and rinsed with PBS. The cells were lysed with an extraction buffer containing 45mM Tris-HCl (pH 6.8) (Sigma), 0.2% N-Lauroylsarcosine (Fluka), 0.2 mM of phenylmethanesulfonyl fluoride (PMSF) (Sigma), proteases and phosphatases inhibitors. Because of the instability of PMSF in aqueous solution, this component was added to the buffer shortly before use. Twenty μl of buffer/10⁵ cells were added and incubation performed at room temperature for 5 minutes. Protein extracts were then stored at –80°C (Wu W. et al., 2004).

The protein content was determined by the bicinchoninic (BCA) protein assay. The BCA method is based on the reduction of Cu⁺² to Cu⁺¹ by protein in alkaline medium (the biuret reaction) and their subsequent reaction with BCA to form a water-soluble complex that exhibits a strong absorbance at 570nm, proportional to the protein concentration.

Standards solutions of BSA were prepared to have a calibration curve in order to determine the concentration of protein extracts. 20 μl of each standards and of protein sample were pipetted into a 96 well flat-bottomed microplate in duplicate. 200 μl of the freshly prepared working reagent (mixture of BCA and CuSO₄ at a ratio of 50:1) were added and put in the microplate at 37°C for 30 minutes. The samples are then cooled at room temperature and the absorbance measured at 570 nm using a spectrophotometer (Spectra Max plus 384, Molecular Devices). The concentration of each protein sample was determined from a plot of concentration against the absorbance obtained for the standard solutions (calibration curve).
8. Immunoblotting.

30 μg of protein extract were loaded on 12% SDS-PAGE (29:1, acrylamide:bis-acrylamide) gel according to Laemmli’s procedure (Laemmli, 1970) and then transferred onto a 0.22 μm nitrocellulose membrane (Schleicher & Schuell) using a transblot semi-dry apparatus system (Biorad) with 60 mM Tris-HCl, 40 mM glycine containing 0.05 % SDS and 10% methanol. The membranes were then stained by Ponceau S (Sigma Chemicals) to verify the efficiency of the transfer. The membrane was blocked with non-fat dried milk. The IgG and the hybridisation conditions used are reported in the table 2:

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Dilution</th>
<th>Non-fat Milk</th>
<th>Incubation time</th>
<th>Secondary antibody</th>
<th>Incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E2F1</strong> (Santa Cruz)</td>
<td>1:100</td>
<td>5%</td>
<td>o/n a 4°C</td>
<td>Anti-Mouse HRP</td>
<td>1 h</td>
</tr>
<tr>
<td><strong>Cyclin E1</strong> (BD Pharmigen)</td>
<td>1:1000</td>
<td>3% + Tween</td>
<td>1 h a RT</td>
<td>Anti-Mouse HRP</td>
<td>1 h</td>
</tr>
<tr>
<td><strong>Cyclin A1</strong> (Santa Cruz)</td>
<td>1: 500</td>
<td>5% + Tween</td>
<td>o/n a 4°C</td>
<td>Anti-Rabbit HRP</td>
<td>1 h</td>
</tr>
<tr>
<td><strong>pRb</strong> (BD Pharmigen)</td>
<td>1:400</td>
<td>5% + Tween</td>
<td>1 h a RT</td>
<td>Anti-Mouse HRP</td>
<td>1 h</td>
</tr>
<tr>
<td><strong>PARP</strong> (BD Pharmigen)</td>
<td>1 :1000</td>
<td>5% + Tween</td>
<td>o/n a 4°C</td>
<td>Anti-Mouse HRP</td>
<td>1 h</td>
</tr>
<tr>
<td><strong>Cyclin E2</strong> (Santa Cruz)</td>
<td>1 :100</td>
<td>5%</td>
<td>o/n a 4°C</td>
<td>Anti-rabbit HRP</td>
<td>1 h</td>
</tr>
<tr>
<td><strong>Cyclin D1</strong> (Santa Cruz)</td>
<td>1:500</td>
<td>5% + Tween</td>
<td>o/n a 4°C</td>
<td>Anti-Mouse HRP</td>
<td>1 h</td>
</tr>
<tr>
<td><strong>P27</strong> (Santa Cruz)</td>
<td>1 :100</td>
<td>3%</td>
<td>o/n a 4°C</td>
<td>Anti-Mouse HRP</td>
<td>1 h</td>
</tr>
<tr>
<td><strong>p-Cyclin E1</strong> (Santa Cruz)</td>
<td>1 :100</td>
<td>5% + Tween</td>
<td>1 h a RT</td>
<td>Anti-goat HRP</td>
<td>1 h</td>
</tr>
<tr>
<td><strong>GAPDH</strong> (Santa Cruz)</td>
<td>1:800</td>
<td>5% + Tween</td>
<td>1 h a RT</td>
<td>Anti-Rabbit HRP</td>
<td>1 h</td>
</tr>
</tbody>
</table>

**Table 2:** IgG and hybridisation conditions.
The membranes were washed twice by PBS containing 0.05% Tween 20 and then develop by enhanced chemiluminescence’s detection kit (Pierce). Band intensities were quantified by densitometry (Model GS-700 Imaging Densitometer, Biorad and Molecular Analyst software, Biorad). GAPDH was used as internal loading control.


Cytotoxicity was evaluated by lactate dehydrogenase (LDH) assay kit (BioVision Prod., Mountain View CA). LDH activity is determined by a coupled enzymatic reaction: LDH oxidizes lactate to pyruvate which then reacts with tetrazolium salt to form formazan. The formazan dye is water soluble and can be detected by spectrophotometer at 500nm. 100 μl of supernatant were transferred to corresponding wells in an optically clear 96-well plate. 100 μl of Reaction Mixture (containing Catalyst Solution: Dye Solution = 1 : 45) were then added to each well and incubated for 30 minutes at room temperature protecting the plate from light. Absorbance of sample was performed at 500nm using a spectrophotometer (Spectra Max plus 384, Molecular Devices). As positive control, triton X-100 (1% final concentration) treated cells were considered; as negative control, free medium was used. The percentage of cytotoxicity was determined according to the formula:

\[
\text{Cytotoxicity (\%)} = \frac{(\text{Test sample} - \text{Negative control})}{(\text{Positive control} - \text{Negative control})} \times 100
\]

10. Apoptosis evaluation.

Apoptosis was evaluated by the Annexin V test (Bender – Med System, Burlingame, CA) and Propidium Iodide (PI) staining (BD Pharmigen™). Annexin V binds to phosphatidylserine when it is translocated from the inner to the outer part of the membrane, an early event in apoptosis. As annexin V is conjugated to a fluorochrome (FITC) it is possible to identify annexin V positive cells (apoptotic) by flow-cytometric identification. PI, which stains the DNA of cells with damaged cytoplasmic membrane, allow to identify apoptotic and necrotic cells. HepG2 were collected two days after siRNA transfection, HuH7 and JHH6 three days after siRNA transfection; then cells were resuspended in binding buffer at the concentration of $2\times 5 \times 10^5$ cell/ml and incubated with annexin V Ig for 15 min. Cells were then sedimented,
resuspended in the binding buffer and incubated with propidium iodide PI for 20 min at room
temperature. After a final washing step, cells were re-suspended in 0.5 ml of 1x PBS/0.5% BSA
(bovine serum albumin) and analyzed by flow cytometry (FACS Canto, BD) using the DIVA
software. As positive control a sample of cells kept in complete medium was incubated with
1 μM staurosporin (Sigma Chem. Co, St. Louis) 3 h prior harvesting.

11. Proliferation assays.
The distribution of cell cycle phases can be performed by the measurement of bromodeoxyuridine (BrdU) incorporation into newly synthesized cellular DNA and DNA staining by Propidium Iodide. Moreover, Ki67 staining can be used to discriminate the G0-phase of cell cycle. Ki67 is a nuclear cell proliferation-associated antigen expressed in all stages of the cell cycle, except of G0.

BrdU pulse and DNA staining: twelve hours before harvesting, cells were pulsed with bromodeoxyuridine (BrdU) at a concentration of 10 μM. Then cells were trypsinized and resuspend in ice cold 70% EtOH over night. These fixed samples can be storeded in EtOH at 4°C up to 3 days. Afterwards, a washing step with PBS containing 0.5% BSA was performed; then cells were treated with 1 M HCl/0.5% BSA for 1 hour (only 20 minutes in the case of VSMC). After a further washing step, cells were resuspended in 0.1 M sodium borate, pH 8.5, for 2 minutes and washed again. Each sample was incubated with fluorescein-isothiocyanate (FITC) – conjugated mouse monoclonal antibody (BD PharMigen™) anti BrdU or with FITC-conjugated mouse IgG1 Isotype Control (as background control) for 60 minutes (only 20 minutes in the case
Materials and Methods

of VSMC). Another washing step preceded incubation with 4 μl PI Staining Solution (BD Pharmigen™) and 100 μl Ribonuclease A 100 μg/ml (Sigma) for 1-2 hours. After a final washing step, cells were resuspended in PBS containing 0.5% BSA and analyzed by flow cytometry (FACScanto, Becton Dickinson) using the DIVA software.

Detection of Ki67: cells were harvested, counted and pelleted as above. Then cells were resuspended in 200 μl ice cold 70% EtOH overnight. Fixed cells can be stored at -20°C for up to 60 days prior to staining. After washing with PBS containing 1% BSA and 0.09% NaN₃, cells were incubated with 7 μl of FITC-conjugated Anti-Human Ki67 (BD Pharmigen™) or with 7 μl of FITC-conjugated mouse IgG₁ Isotype Control (as background control) for 60 minutes (only 20 minutes in the case of VSMC). After a further washing step, 4 μl PI Staining Solution (BD Pharmigen™) and 100 μl Ribonuclease A 100 μg/ml (Sigma) were added and the incubation allowed for 30 minutes. After a final washing step, cells were resuspended in PBS containing 0.5% BSA and analyzed by flow cytometry.

12. Statistical analysis.

If not otherwise indicated, data are expressed as the mean ± SEM, and statistical significance (P value) was calculated using the ANOVA one way test led by MS Excel. P values ≤ 0.05 were considered to be statistically significant.
RESULTS

Selection of siRNAs and uptake studies

1. Selection of siRNAs targeted against SRF, Cyclin E1, Cyclin E2 and E2F1.

Guide lines for the identification of active siRNAs against the mRNA targets chosen (SRF, CyE1, CyE2 and E2F1), were as described (Poliseno et al., 2004a; Poliseno et al., 2004b).

To interfere with SRF expression, we selected two different SRF-specific siRNAs, siSRF820 and siSRF797, thereby specifically targeting sequences encoding the SRF MADS-box. Targeted region showed no significant homology to any other transcript including MEF2 (myocyte enhancer factor-2) transcripts, which possess a very homologous MADS-box to SRF, nor did they significantly affect transcript levels of MEF2 genes as judged by gene expression profiling (werth et al., 2007). Also in the case of siRNAs targeted against Cyclin E1, Cyclin E2 and E2F1, two different specific siRNAs were selected. After some preliminary investigations, only the more effective siRNAs, in terms of the effects on the specific protein levels (data not shown), were chosen for our experiments and are reported in the table 1. A luciferase siRNA (siGL2), as sequence unrelated control, was used.

<table>
<thead>
<tr>
<th>siRNAs</th>
<th>sense</th>
<th>antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>SiSRF797</td>
<td>GAUGGAGUUCAUCGACAACAA</td>
<td>GUUGUCGAUGAACUCCAUCCUU</td>
</tr>
<tr>
<td>siCyclin E1-1415</td>
<td>GAGCGGAAGAAGCAGAGC</td>
<td>GCUCUGCUUCUUACCUCUC</td>
</tr>
<tr>
<td>siCyclin E2-647</td>
<td>GCUGCAGUGAAGAGGAAU</td>
<td>UCUCUCUCUCACUGAAGC</td>
</tr>
<tr>
<td>SiE2F1-1324</td>
<td>GAGGAGUCAUCAGCCUUU</td>
<td>AAAGGCUGAUGACUCCUC</td>
</tr>
<tr>
<td>siGL2</td>
<td>CGUACGCGGAUUACUUCGA</td>
<td>UCGAAGUAAUUCGCGUACG</td>
</tr>
</tbody>
</table>

Table 1: siRNAs sequences.
2. Uptake studies.
To evaluate uptake efficiencies, a siRNA, siGL2, conjugated with a fluorescein-isothiocyanate (FITC) molecule, was transfected to the target cells. The following transfection parameters were considered:

a. percent of cell transfected;
b. cellular distribution of the molecules;
c. persistence of the molecules in the cell.

The percentage of cell transfected (a) and the persistence of the molecules in the cell (c) were evaluated by flow cytometry. Cellular distribution (b), which was evaluated by fluorescence microscopy, is an important parameter for our studies. Our experience in the field of HHRzs (Grassi et al., 2005a; Grassi et al., 2001) and data showed by other groups (Bramlage et al., 1999) indicate that different cellular localization of RNA molecules dramatically affect their activities. This variable was investigated by fluorescence microscopy.

a. percent of cell transfected: as it is not possible to predict a priori the optimal transfection conditions for each cell type considered, transfections were performed in the presence of different commercially available transfectant, variable ratios of transfection reagent/FITC-labeled siRNAs and variable transfection times (see Materials and Methods).

Lipofectamin 2000, at a siGL2-FITC/Lipofectamin 2000 weight ratio of 1:3 and after a transfection time of three hours gave the highest transfection rates in HepG2 cell line (Fig. 1).

![Transfection efficiencies in HepG2 cell line](image.png)

**Fig.1:** transfection efficiency in HepG2 cell line evaluated by flowcitometry (siRNAs conc=220nM, density of 1*10^4 cells/cm^2 in 6well plate).
Similar results were obtained for HuH7 and JHH6 (data not shown) but using a siGL2-FITC/Lipofectamin2000 weight ratio of 1:1. Vascular smooth muscle cells (VSMC) were better transfected by cellfectin at a weight ratio siGL2-FITC/Cellfectin of 1:2.5 with a transfection time of two hours (Fig. 2). Similar experiments as described above were conducted prolonging cell-liposomes interaction to six hours, with no significant increase of transfection rate (data not shown).

![Transfection efficiency in VSMC](image)

**Fig. 2:** transfection efficiency in VSMC evaluated by flowcitometry (siRNAs conc=220nM, density of $3.3 \times 10^3$ cells/cm$^2$ in 6 cm dishes).

In all these experiments, the concentration of siRNAs was of 220nM for all cell types used. At higher siRNAs concentrations, the pronounced increase in cell death, as visualized microscopically (data not shown), suggested us not to overcame in our experiments this concentration.

In VSMC an excellent transfection rate was achieved under the conditions reported in Fig.2 and using a cell seeding density of $3.3 \times 10^3$ cells/cm$^2$. In all HCC cell lines seeded at $1 \times 10^4$ cells/cm$^2$, the amount of positive cells never exceed 60%, suggesting that further optimisation was required. For this purpose the best conditions selected in Fig.1 were tested in the presence of different cell seeding density (Fig.3):
Results

Fig. 3: transfection efficiency in HepG2 cell line evaluated by flowcitometry
(siRNAs conc=220nM in 6well plate)

Reducing cell density, transfection efficiency increased from about 60% to more than 80% in HepG2 cell line (Fig.3) and in JHH6 and HuH7 cell lines (siGL2-FITC/Lipofectamin2000 weight ratio of 1:1) (data not shown).

In conclusion, the final optimised transfection conditions for each cell type were as reported in the table 2:

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Seeding density</th>
<th>SiRNAs concentration</th>
<th>weight ratio siRNAs:liposome</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepG2</td>
<td>3.8*10³/cm²</td>
<td>220nM</td>
<td>1:3</td>
</tr>
<tr>
<td>HuH7</td>
<td>3.8*10³/cm²</td>
<td>220nM</td>
<td>1:1</td>
</tr>
<tr>
<td>JHH6</td>
<td>3.8*10³/cm²</td>
<td>220nM</td>
<td>1:1</td>
</tr>
<tr>
<td>VSMC</td>
<td>3.3*10³/cm²</td>
<td>220nM</td>
<td>1:2.5</td>
</tr>
</tbody>
</table>

Table 2: Optimised transfection conditions for each cell type.
b. cellular distribution of the FITC-siGL2: the intracellular localization of siGL2-FITC following the above reported transfection conditions was documented with living cells to avoid artifacts introduced by fixation. As reported in Fig.4, in VSMC an even distribution of green fluorescence in the cells was observed. Similar results were observed for the HCC cell lines.

![VSMC transfected cell with siGL2-FITC](image)

**Fig.4:** VSMC transfected cell with siGL2-FITC (siRNAs conc=220nM, density of $3.3 \times 10^3$ cells/cm$^2$, weight ratio siGL2-FITC:Cellfectin = 1:2.5).
3. INF Response.

SiRNAs are believed to be too short to activate the non-specific antiviral response against dsRNA molecules. However, a recent report showed that short hairpin RNAs (shRNAs) can activate an antiviral response (Bridge et al., 2003). Thus, before proceeding with the siRNAs experiments, it was necessary to exclude an INF- triggered response by our molecules. This fact would have profoundly altered the siRNA specificity of action abrogating the relevance of the investigation planned.

By quantitative RT-PCR analysis, we measured the expression of 2’-5’-oligoadenylate synthetase 1 (OAS1) gene, a classical INF target gene that is induced >50-fold upon activation of an INF-response. The mRNA levels of OAS-1 in siRNA-transfected cells varied between 0.5 and 3 fold of controls, thus being far below the reported activation observed in an INF-response (Fig.5).

**Fig. 5:** Quantitative RT-PCR analysis of OAS1.

OAS1 induction by all siRNAs is within 3-fold, which is far less than that reported on activation of an IFN response.
Depletion of SRF in HCC cell lines

For HCC, a leading cause of cancer death world-wide, there is no clinically tested useful therapy. Thus, the development of novel effective therapeutic approaches are of utmost relevance. As a strategy to down modulate HCC cell expansion, we have considered the targeting of the transcription factor SRF by means of siRNAs. SRF has been considered as target in this work because of its implication in the proliferation of the HCC cell line HepG2 (Shao et al., 2005). Moreover, it has been observed that SRF is expressed in HCC tissue but not in normal/cirrhotic liver and that its over-expression is particularly evident in high grade, poorly differentiated, HCC forms (Park et al., 2007). These observation together with the absolute lack of any published data about the investigation of a direct depletion of SRF in HCC cell lines, prompted us to generate a specific siRNA anti SRF and test its effect in three HCC cell lines, i.e. HepG2, HuH7 and JHH6. Suitable transfection systems were undertaken to test siSRF797 effects in HepG2, HuH7 and JHH6 cell lines. Non treated cells, and cells treated by a control siRNA directed against the luciferase gene (siGL2), were analysed in parallel. Optimized analysis times were two and three days after transfection for HepG2 and HuH7-JHH6, respectively. In all reported histogram the data belonging to the three different cell lines and they are normalized to the respective siGL2 control: this implies that it is not possible to compare the reported values among the three different cell lines.
4. siSRF797 effects on SRF mRNA and protein levels.
To investigate the specificity and the efficacy of siSRF797-mediated RNAi, siSRF797 was transfected into all HCC cell lines and the mRNA and protein levels of SRF evaluated. Between two and three days after transfection, siSRF797 induced an evident decrease in the specific target mRNA levels in all HCC cell lines (* p < 0.001) (Fig. 6).

**Fig. 6:** siRNA-mediated reduction of SRF transcript levels evaluated by quantitative RT-PCR (data were normalized to 28S). Data are expressed as means ± SEM, n=6.
* p < 0.001 compared to siGL2 treated cells. NT=not treated cells.

In the reported histogram, as well as in the following, the data belonging to the three different cell lines are normalized to the respective siGL2 control: this implies that it is not possible to compare the reported values among the three different cell lines.

In all HCC cell lines, total SRF protein levels correlated with the mRNA levels: the decrease in SRF mRNA upon transfection with siSRF797 was paralleled by a clear decrease in SRF protein levels as reported in a representative experiment in Fig. 7a and summarized in Fig 7b.

**Fig. 7a:** Reduction of endogenous SRF protein by siSRF797 was detected by immunoblotting. GAPDH protein served as a loading control.
As indicated in Fig. 7a, two bands were detected for SRF. The upper one corresponds to the mature SRF form while the lower one to the immature neo-synthesized form (REF). It should be noted that in general the upper band (mature form) remained visible while the lower one (neo-synthesized) disappeared in the blot in siSRF797 treated cells. This effect was somewhat variable among the three cell lines as in HepG2 the upper band is clearly decreased (similarly to VSMC, as reported in the next chapter) while in HuH7 and in JHH6 it is comparable to the controls. Despite this variability, in all cell lines tested the disappearance of the neo-synthesized SRF form was evident and thus the global protein levels of SRF were reduced.
5. Effects of SRF depletion on cell morphology and cell-counting.

The morphology of HepG2, HUH7 and JHH6 cell lines are reported in figures 8, 9 and 10.

**Fig. 8:** Effect of SRF depletion by siSRF797 on HepG2 morphology, two days after transfection.

Cell morphology was analysed by phase contrast microscopy at 5x and 20x magnification. NT = not-treated cells.

In HepG2 treated cells we observed a clear reduction in cell number compared to controls. Moreover, part of the attached cells started to round up and detach from plates.
**Results**

**Fig. 9 and 10:** Effect of SRF depletion by siSRF797 on HuH7 and JHH6 morphology, three days after transfection.

With regard to HuH7 and JHH6, no evident variations were detected in cell morphology upon siSRF797 treatment compared to controls.
Thus, SRF depletion resulted in a microscopically appreciable reduction in the amount of HepG2 but not of HuH7 and JHH6 cells.

With regard to HepG2, this observation was confirmed by cell counting (41.1% ± 15.0 of reduction compared to siGL2 treated cells) (Fig. 11a). The reduction in cell number compared to controls indicate a specific effect of the siSRF797 (*p<0.05). As HepG2 grow in colonies (Fig 8), amount and areas were also measured. As reported in Fig. 11b and 11c, the reduction in cell number was paralleled by a decrease in colonies area (*p<0.05) but not in number, suggesting a cytostatic effect of SRF depletion.

**Fig. 11:** siSRF797 effects on HepG2 cell line.

a. The amount of cell number was evaluated two days after transfection; (*p<0.05 compared to siGL2 treated cells); data are expressed as means ± SEM, n=4. NT=not treated cells.

b. Colonies area was evaluated two days after transfection (*p<0.05 compared to siGL2 treated cells); data are expressed as means ± SEM, n=100. NT=not treated cells.

c. Colonies number was evaluated two days after transfection; data are expressed as means ± SEM, n=100. NT=not treated cells.
In the case of *HUH7*, no significant reduction in the cell number as well as in colonies area and amount were observed in siSRF797 treated cells compared to siGL2 treated cells; a clear reduction was in contrast observed compared to not-treated cells (Fig.12 a, b, c). This suggest an aspecific reduction in cell number in this cell line upon siRNA treatment.

**a. Hu7 cell counting**

**b. Hu7 colony areas, day3**

**c. Hu7 number of colonies, day3**

**Fig. 12:** siSRF797 effects on HuH7 cell line.

a. The cell number was evaluated three days after transfection; data are expressed as means ± SEM, n=4. NT=not treated cells.

b. Colonies area was evaluated three days after transfection data are expressed as means ± SEM, n=100. NT=not treated cells.

c. Colonies number was evaluated three days after transfection; data are expressed as means ± SEM, n=100. NT=not treated cells.
With regard to the amount of JHH6 cell number upon siSRF797 treatment, this cell line behaved similarly to HuH7 where no clear reduction (15.2% ± 13.7) in cell number was observed compared to siGL2 treated cells (Fig 13). More evident was in contrast the reduction compared to not-treated cells (aspecific reduction). No data in terms of the areas and number of colonies were available as JHH6 do not grow in colonies.

**Fig. 13**: siSRF797 effects on JHH6 cell line: a tendency towards a reduction in cell number compared to siGL2 treated cells was observed three days after transfection; evident is the reduction compared to NT cells; data are expressed as means ± SEM, n=5; NT=not-treated cells.
6. Effects of SRF depletion on cell cycle phase distribution.

Cell cycle progression was evaluated by flow cytometry after bromodeoxyuridine (BrdU) pulse and DNA staining by propidium iodide (PI). This methodology allows the identification of the newly synthesised DNA (BrdU incorporation) and total DNA content (DNA PI staining) permitting to distinguish cells in the different phases of the cell cycle (Dolbeare et al., 1983; Dekens et al., 2003). Thus, this method allowed not only to evaluate the proliferation inhibition potential of siSRF797 but also the consequences of SRF knock down in terms of cell cycle phase distribution in HCC cell lines.

In HepG2, siSRF797 caused a reduction of about 20% of S phase cells compared to control siGL2-treated cells as reported in a representative experiment in Fig. 14 and summarized in Fig. 15 (*p<0.001); a tendency towards an increase of G1/G0 cell was also observed.

**Fig. 14:** A representative dot plot of HepG2 is shown.
A: siGL2 treated cells; B: siSRF797 treated cells.
Q1: S-phase cells
Q2/Q4: G2/M phase cells
Q3: G1/G0 phase cells.
PI-A: Propidium Iodide staining; FITC-H: FITC labelled Ig anti BrdU
Results

Depletion of SRF diminished the S-phase cells by 20% compared to siGL2 treated cells (*p<0.001).

Fig. 15: Cell cycle phase distribution.
Data are expressed as means ± SEM, n=4.
Depletion of SRF diminished the S-phase cells by 20% compared to siGL2 treated cells (*p<0.001).
NT=not treated cells.
This decrease is followed by a tendency of an increase in G0/G1 phase cells.

In HuH7, only a tendency toward the reduction of S-phase cells was observed in siSRF797 treated cells compared to siGL2 treated cells. In contrast, evident was the reduction of S-phase cells in siSRF797 treated cells compared to not treated cells, suggesting an aspecific effect of siRNA transfection in this cell line.

Fig. 16: Cell cycle phase distribution.
Data are expressed as means ± SEM, n=4. NT=not treated cells.
Depletion of SRF resulted in a tendency towards a decrease of S-phase cells compared to siGL2 treated cells.

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In *JHH6*, SRF depletion did not substantially reduced the amount of S-phase cells as reported in Fig.17:

![Cell cycle phase distribution](image)

**Fig. 17:** Cell cycle phase distribution.
Data are expressed as means ± SEM, n=4. NT=not treated cells.
Depletion of SRF did not substantially reduce the amount of S-phase cells compared to siGL2 treated cells.

To discriminate between cells in G1 or G0-phase of cell cycle, Ki67, an antigen expressed in G1/S/G2/M phase cells, but not in G0 phase cells, was quantified by flowcitometry. As no major variations in the amount of G0 phase cells was observed (data not shown), we deduced that the G0/G1 increase was mainly due to a G1 increase.
In *JHH6* and *HuH7* the depletion of the newly synthesized SRF form by siSRF797 was not sufficient to induce a clear reduction in S-phase cells and in the total number of cells. Thus it seems that the presence of the mature SRF form is sufficient to sustain cell proliferation. This hypothesis implies that the levels of the SRF mature form in JHH6 and HuH7 are substantially constant and poorly dependent on the amount of SRF newly synthesized form. This indirectly means that the levels of SRF mature form are mostly independent from the stimulation of physiological inducers, such as serum. In this sense it is interesting to note that in JHH6 and HuH7 the levels of SRF mature form are substantially independent from serum stimulation, as reported in a representative experiment in Fig.18A and fig. 18B and summarized in Fig.19. In contrast, a clear dependence of the amount of SRF mature form to serum stimulation, is evident in *HepG2* and VSMC, undertaken as a normal control (Fig.18C and Fig. 18D). Moreover, whereas the levels of newly synthesized SRF form depends on serum stimulation in HepG2, this is only partially the case in JHH6 and not the case in HuH7.

![Fig.18A: Time course of SRF protein in JHH6 cell line detected by immunoblotting. GAPDH protein served as a loading control.](image1)

![Fig.18B: Time course of SRF protein in HuH7 cell line detected by immunoblotting. GAPDH protein served as a loading control.](image2)
**Fig. 18C:** Time course of SRF protein in HepG2 cell line detected by immunoblotting.
GAPDH protein served as a loading control.

**Fig. 18D:** Time course of SRF protein in VSMC detected by immunoblotting.
GAPDH protein served as a loading control.
These data would suggest an increased stability of the mature form of SRF in JHH6 and HuH7. For this reason, in a variation of protocol 1 (see Materials and Methods) JHH6 were double transfected by siSRF797 at day 0 and at day 3, and the amount of total cells evaluated at day 6. Also under this condition, no evident reduction in cell number was observed. Similar results were obtained with HuH7 (Fig.20)

**Cell counting**

**Fig. 20:** siSRF797 effects on cell counting with modified transfection conditions; data are expressed as means ± SEM, n=3; NT=not-treated cells.
7. Effects of SRF depletion on cell death.

The apoptotic potential of the selected siRNAs in the target cells was investigated by studying the externalisation of phosphatidylserine (PS). In apoptotic cells, PS accumulates on the outer part of the cellular membrane (Geske and Gerschenson, 2001) as a consequence of increased externalisation from the inner part to outer part of the cellular membrane and decreased internalisation. Thus, with a specific antibody direct against PS it is possible to detect the number of apoptotic cells. To distinguish between early and late apoptotic cells, an additional staining with propidium iodide (PI) is required. PI can penetrate the damaged cellular membrane of late apoptotic cells (and stain DNA) but not the normal membrane of early apoptotic cells. Thus, cell positive for both PI and PS staining, represent late apoptotic, cells positive for PS staining only, represent early apoptotic cells, cells positive for PI only represent cell debris and cells negative for both staining represent normal living cells.

As an additional test to confirm apoptosis, Poly-ADPRibose-Polymerase (PARP) cleavage was evaluated. In apoptotic cells, this DNA repair protein of 113kDa is cleaved into two fragments by the caspases cascade. As this event is limited to apoptotic cells, it represents a reliable and specific marker for apoptosis.

With regard to HepG2, SRF depletion resulted in an increase in the amount of apoptotic cells (annexin V positive cells) two days after transfection as shown in a representative experiment in Fig.21 and summarized in Fig.22 (* p<0.001):

![Fig. 21: Effects of SRF depletion on cell apoptosis: the amounts of apoptotic HepG2 cells were determined two days after transfection by annexin V and PI staining, followed by flow cytometry analysis. The percentage of apoptotic cells is indicated in the two right quadrants. FITC-A: annexin V; PI-A: propidium iodide staining.](image-url)
Fig. 22: Annexin V positive cells two days after transfection. Data are expressed as means ± SEM, n=4, (* p<0.001 compared to siGL2 treated cells). NT=not treated cells.

The pro-apoptotic effect was confirmed by the increased cleavage of the Poly-ADPRibose-Polymerase (PARP) in siSRF-treated cells compared to controls (Fig. 23):

Fig. 23: PARP cleavage. GAPDH was used as loading control.

In HuH7 and JHH6, the depletion of the newly synthesized form of SRF did not result in an increase of apoptotic cells compared to controls (Fig 24):

Fig. 24: Annexin V positive cells three days after transfection. Data are expressed as means ± SEM, n=4. NT=not treated cells.
Possible siRNA cytotoxic effects, either depending on the specific depletion of SRF or an aspecific effect of siRNA transfection into the cells, were evaluated by measuring the activity of lactate dehydrogenase (LDH). This enzyme is normally found in the cytosol of all cells but it is rapidly released into the supernatant upon plasma membrane damage. As reported in Fig.25A, B and C, no significant toxicity was observed in all treatments compared to not treated cells. As positive control, triton treated cells were introduced.

**Fig. 25:** LDH test two or three days after transfection.

Data are expressed as means ± SEM, n=4. NT=not treated cells.
8. Effects of SRF depletion on other cell cycle regulators.

Upon treatment with siSRF797 we found some interestingly variations of other relevant cell cycle genes, such as the transcription factor E2F1 and the G1/S cyclin E1.

The reduction of SRF total levels caused an evident decrease in E2F1 protein levels (60%, 70% and 20% of reduction for HepG2, HuH7 and JHH6 respectively) as shown in a representative blot in Fig. 26A and summerised in Fig.26B; the decrease might be attributable to a post-transcriptional effect in HuH7 and JHH6 since E2F1 transcript levels were not affected (Fig.27). In contrast, a transcriptional mechanism can be advocated for HepG2 where E2F1 mRNA level was reduced compared to siGL2 treated cells (Fig.27).

![E2F1 and GapDH protein levels](image)

**Fig. 26A**: Reduction of E2F1 protein by siSRF797 was detected by immunoblotting. GAPDH protein served as a loading control.

![E2F1 protein levels](image)

**Fig. 26B**: E2F1 protein levels.
Data are expressed as means ± SEM, n=3. NT=not treated cells.
Results

Fig. 27: siSrf797-mediated indirect reduction of E2F1 transcript levels evaluated by quantitative RT-PCR (data were normalized to 28S). Data are expressed as means ± SEM, n=6. NT=not treated cells. (*p < 0.001).

In contrast to E2F1, no significant variations in the amount of the phosphorylated form of the retinoblastoma protein pRb were observed (Fig. 28).

Fig. 28: Levels of the iперphosphorylated form of pRb; data are expressed as means ± SEM, n=3. NT=not treated cells.
The depletion of SRF was paralleled by a clear increase in **CyE1** protein levels in HepG2 cell line but not in HuH7 and JHH6 (Fig. 29A and Fig. 29B).

**Fig. 29A**: Increase of cyclin E1 protein by siSRF797 was detected by immunoblotting. GAPDH protein served as a loading control.

**Fig. 29B** CyE1 protein levels.
Data are expressed as means ± SEM, n=3. NT=not treated cells.
The increased CyE1 protein levels in HepG2 prompted us to evaluate whether this was a transcriptionally or post-transcriptionally phenomenon. Compared to controls, a reduction of CyE1 mRNA was observed (Fig.30).

![CyE1 mRNA levels](image)

**Fig.30:** Reduction of CyE1 transcript levels evaluated by quantitative RT-PCR (data were normalized to 28S). Data are expressed as means ± SEM, n=6. NT=not treated cells.

Thus we draw our attention on the stability of CyE1 protein. Notably, the decrease of CyE1 mRNA, was consistent with the reduction of the major transcription factor, i.e. E2F1. Moreover, no increase in the expression level of LRH-1, an hepatic specific transcription factor for CyE1, was observed (data not shown). Therefore, the phosphorylation status of CyE1 Thr395 residue was investigated (phosphorylation at this site is required for proteasome-mediated degradation of CyE1 and the extent of phosphorylation is directly proportional to the degradation rate) as reported in a representative experiment (Fig.31A) and summarized in Fig.31B. A decrease in the amount of phosphorylation on CyE1 Thr 395 residue was observed in Hepg2 compared to controls. No evident variation were in contrast observed in HuH7 and JHH6 cell lines.

![Fig. 31A: Reduction of P-Cyclin E1 by siSRF797 was detected by immunoblotting. GAPDH protein served as a loading control.](image)
Finally no relevant variation in the expression levels of cyclin E2, cyclin D1, cyclin A, p27\textsuperscript{kip1}, p16\textsuperscript{INK4} and p21\textsuperscript{cip1} were observed (data not shown).

In conclusion, our data show the identification of an active siRNA directed against SRF able to specifically and efficiently reduce the SRF mRNA in the hepatic cell line Hepg2, JHH6 and HuH7. SRF mRNA depletion resulted in a significant inhibition of cell proliferation (reduction in S-phase cells) and apoptosis induction in HepG2 but not in JHH6 and HuH7. This is most likely due to the impossibility to deplete the mature SRF form in JHH6 and HUH7. In HepG2, SRF depletion was paralleled by a decrease of E2F1 mRNA and protein levels, a fact which can contribute to explain the down modulation of cell proliferation. Consistently with the E2F1 expression down regulation, cyclin E1 mRNA was also reduced; however, total cyclin E1 protein levels were increased due to reduced phosphorylation at Thr395.
Depletion of CyE1, CyE2 and E2F1 in HCC cell lines

In the previous paragraphs, we have presented data about the possibility to control HCC cell growth by the inhibition of SRF, a widely expressed transcription factor involved in cellular development and proliferation. Only in the case of most differentiated HepG2 we could observe a clear anti-proliferative and pro-apoptotic effect of the selected siSRF797. Thus, in an attempt to find strategies able to down regulate the proliferation also of the less differentiated HCC cell lines, such as HuH7 and JHH6, additional siRNAs targets, i.e. CyE1 and E2F1, were considered. Therefore, siRNAs direct against the position 1415 of cyclin E1 mRNA (siCyE1-1415), the position 647 of cyclin E2 mRNA (siCyE2-647) and the position 1324 of E2F1 mRNA (siE2F1-1324) were used.

Suitable transfection systems were undertaken to test siCyE1-1415, siCyE2-647 and siE2F1-1324 efficiency in HepG2, HuH7 and JHH6 cell lines. Non treated cells, and cells treated by a control siRNA directed against the luciferase gene (siGL2), were analysed in parallel. Optimized analysis times were two and three days after transfection for HepG2 and HuH7-JHH6, respectively according to Protocol 2 (Materials and Methods).

In all reported histogram the data belonging to the three different cell lines and they are normalized to the respective siGL2 control: this implies that it is not possible to compare the reported values among the three different cell lines.
9. siRNAs effects on CyE1, CyE2 and E2F1 mRNA and protein levels.

To investigate the specificity and the effects of siRNAs, siCyE1-1415, siCyE2-647 and siE2F1-1324 were transfected into all HCC cell lines and the mRNA and protein levels of cyclin E1, cyclin E2 and E2F1 evaluated.

Between two and three days after transfection, every specific siRNA was able to strongly reduce the mRNA levels (* p<0.0001 and § p<0.005) of the respective target, compared to siGL2 treated cells (Fig.32).

**Fig. 32:** siRNA-mediated reduction of the respective target transcript levels by quantitative real time PCR (data were normalized to 28S). Data are expressed as means ± SEM, n=6. NT=not treated cells.

(* p < 0.0001; § p < 0.05 compared to siGL2 treated cells).

In the reported histogram, as well as in the following, the data belonging to the three different cell lines are normalized to the respective siGL2 control: this implies that it is not possible to compare the reported values among the three different cell lines.
In all HCC cell lines, the decrease in mRNA upon transfection with the specific siRNA was paralleled by a clear decrease in the respective protein levels as reported in a representative experiment (Fig. 33a-c) and summarized in Fig 33d.

Fig. 33a: Reduction of endogenous cyclin E1 protein by siCyE1-1415 was detected by immunoblotting. GAPDH protein served as a loading control.

Fig. 33b: Reduction of endogenous cyclin E2 protein by siCyE2-647 was detected by immunoblotting. GAPDH protein served as a loading control.

Fig. 33c: Reduction of endogenous E2F1 protein by siE2F1-1324 was detected by immunoblotting. GAPDH protein served as a loading control.
Fig. 33d: CyE1, CyE2 and E2F1 protein levels; data are expressed as means ± SEM, n=3. NT=not treated cells.
10. Effects of CyE1, CyE2 and E2F1 depletion on cell morphology and cell-counting.
The morphology of HepG2, HUH7 and JHH6 cell lines upon siSRNA treatment is reported in Fig.34, Fig.35 and Fig.36, respectively.

**HepG2**

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**Fig. 34:** siRNA effect on HepG2 morphology, two days after transfection.
Cell morphology was analysed by phase contrast microscopy at 5x and 20x magnification. NT = not-treated cells.
withstanding the HepG2 cell line, we observed that upon siRNAs treatment cells rounded up and detached from plates (Fig. 34), no major morphological variations were detected in HuH7 cell line compared to controls (Fig. 35).

**Fig. 35:** siRNA effect on HuH7 morphology, three days after transfection.

Cell morphology was analysed by phase contrast microscopy at 5x and 20x magnification. NT = not-treated cells.
Fig. 36: siRNA effect on JHH6 morphology, three days after transfection.
Cell morphology was analysed by phase contrast microscopy
at 5x and 20x magnification. NT = not-treated cells.

In the case of JHH6 cell line, we observed that transfected cells underwent a change in cell shape: siSRF797 treated cells lost their elongated characteristic shape and became more polygonal (Fig. 36). Finally, for all cell types a clear reduction in cell amount was observed upon specific siRNA treatment.
With regard to *HepG2* and to *HUH7*, upon depletion of cyE1, CyE2 and E2F1, we observed a clear reduction in cell number, paralleled by a reduction in colony areas, while the number of colonies was maintained (Fig.37 and Fig.38).

**Fig. 37**: siRNAs effects on HepG2 cell line.

d. The amount of cell number was evaluated two days after transfection; (*p<0.001 compared to siGL2 treated cells);

data are expressed as means ± SEM, n=4. NT=not treated cells.

e. Colonies area was evaluated two days after transfection

(*p<0.001 compared to siGL2 treated cells);

data are expressed as means ± SEM, n=100. NT=not treated cells.

f. Colonies number was evaluated two days after transfection;
data are expressed as means ± SEM, n=100. NT=not treated cells
Results

**Fig. 38:** siRNAs effects on HuH7 cell line.

a. The cell number was evaluated three days after transfection; (*p<0.05 compared to siGL2 treated cells); data are expressed as means ± SEM, n=4. NT=not treated cells.

d. Colonies area was evaluated three days after transfection (*p<0.001 compared to siGL2 treated cells); data are expressed as means ± SEM, n=100. NT=not treated cells.

e. Colonies number was evaluated three days after transfection; data are expressed as means ± SEM, n=100. NT=not treated cells.
Also in the case of **JHH6** cell number, upon depletion of cyE1, CyE2 and E2F1, we observe a clear reduction in cell number (Fig.39).

No data in terms of the areas and number of colonies were available as JHH6 do not grow in colonies.

**Fig. 39:** siRNAs effects on JHH6 cell line: an evident reduction in cell number compared to siGL2 treated cells was observed three days after transfection; data are expressed as means ± SEM, n=5;

(*p<0.05 compared to siGL2 treated cells).
11. Effects of CyE1, CyE2 and E2F1 depletion on cell cycle phase distribution.

Cell cycle phase distribution was determined by double DNA staining technique as previously described.

SiRNAs treatment affected cell cycle phase distribution of *HepG2* cell line by reducing S-phase cell by about 20% (*p*<0.05) compared to siGL2 treated cells, as shown in a representative experiment in Fig.40 and summarized in Fig.41. This phenomenon was paralleled by a tendency to an increase in G0/G1 cells.

**Fig.40:** A representative dot plot of HepG2 is shown.
A: siGL2 treated cells; B: siSCyE2-647 treated cells.

Q1: S-phase cells  
Q2/Q4: G2/M phase cells  
Q3: G1/G0 phase cells.

PI-A: Propidium Iodide; FITC-H: FITC labelled Ig anti BrdU.
Depletion of CyE1, CyE2 and E2F1 diminished the S-phase cells by about 20% compared to siGL2 treated cells (*p<0.05). NT=not treated cells.

This decrease is followed by a tendency of increase in G0/G1 phase cells.

As regard with HUH7, the data reported in a representative experiment (Fig.42) and below summarized (Fig.43) demonstrated that three days after transfection, CyE1, CyE2 and E2F1 depletion induced an evident decrease of S-phase cells and an accumulation of cells in G0/G1 phases compared to siGL2 treated cells (*p<0.05; §p<0.001).
Depletion of CyE1, CyE2 and E2F1 diminished the S-phase cells from 40% to 80% compared to siGL2 treated cells (*p<0.05; §p<0.001). NT=not treated cells.
This decrease is followed by a tendency of increase in G0/G1 phase cells.

Similar result were obtained also for JHH6: in siRNA treated cells, it was evident the decrease of S-phase cells which is paralleled by a tendency to increase in G0/G1 phases compared to siGL2 treated cells (*p<0.05), as summarized in Fig.44.
Finally, no significant variations in the amount of G0 cells was observed (as evaluated by Ki67 staining –data not shown-). This indicate that the slightly increase in G1/G0-phase cells was due to an increase of G1-phase cells.

12. Effects of CyE1, CyE2 and E2F1 depletion on cell death.
Apoptosis was first investigated by means of annexin V assay. Cells were collected and stained by fluorescein labelled antibody against annexin V and by Propidium Iodide (PI) and then analyzed by flow cytometry.

With regard to HepG2, two days after transfection we observed that siRNAs treated cells shrank, rounded up and detached from plates (as previously shown in Fig.34), suggesting apoptosis had occurred. This increase in the rate of apoptosis is shown in a representative experiment (Fig.45) and summarized in Fig.46 (*p<0.05).

![Fig. 45: Effects of E2F1 depletion on cell apoptosis: the amounts of apoptotic HepG2 cells were determined two days after transfection by annexin V and propidium iodide staining, followed by flow cytometry analysis. The percentage of apoptotic cells is indicated in the two right quadrants. FITC-A: annexin V; PI-A: propidium iodide staining.](image)
Results

Fig. 46: Annexin V positive cells two days after transfection.

Data are expressed as means ± SEM, n=4. (*p<0.05 compared to siGL2 treated cells). NT=not treated cells.

These data were confirmed by PARP cleavage which showed the appearance of the cleaved form of PARP in siRNAs treated cells compared to controls, as summarized in Fig.47.

Fig. 47: Amount of PARP cleavage expressed as ratio between the uncleaved form of PARP and the uncleaved one.
No increased apoptosis was observed for HuH7 and JHH6 cell lines (Fig.48).

**Fig. 48:** Annexin V positive cells three days after transfection.
Data are expressed as means ± SEM, n=4. NT=not treated cells.

Finally, no significant cytotoxicity was observed in siRNAs treated cells compared to controls as evaluated by LDH test (Fig.49).

**Fig. 49:** LDH test two or three days after transfection.
Data are expressed as means ± SEM, n=4. NT=not treated cells.
Triton treated cells were introduced as positive control.
13. Effects of CyE1, CyE2 and E2F1 depletion on cell cycle regulators.

The decrease of S-phase cells and the accumulation of cells in G0/G1 phases induced by siCyE1-1415, syCyE2-647 and siE2F1-1324, prompted us to evaluate the effects of CyE1, CyE2 or E2F1 depletion on some mediators of the G1/S cell cycle phases.

In all HCC cell lines, depletion of CyE1 causes a consistent decrease of E2F1 protein levels as shown in a representative blot in Fig.50A and summarized in Fig.50B. this decrease in protein levels is paralleled by a decrease in mRNA (* p<0.05; § p<0.001) levels as indicated in Fig.51.

![Fig. 50A](image)

**Fig. 50A**: Reduction of E2F1 protein by siCyE1-1415 was detected by immunoblotting.
GAPDH protein served as a loading control.

![E2F1 protein levels](image)

**Fig. 50B**: E2F1 protein levels following siCyE1-1415 transfection; data are expressed as means ± SEM, n=3. NT=not treated cells
Fig. 51: siRNA-mediated reduction of E2F1 transcript levels by quantitative RT-PCR (data were normalized to 28S). Data are expressed as means ± SEM, n=6. NT=not treated cells. (*p <0.05; §p <0.001 compared to siGL2 treated cells).

This data confirm the strong correlation between these two cell cycle promoting proteins also in this model cell lines of HCC (Fig.52).

Fig. 52: E2F1/pRb-Cye1 correlation
In all HCC cell lines, the depletion of CyE2 causes a consistent decrease of E2F1 protein and mRNA levels (*p <0.05) (Fig. 53A and B and Fig.54).

**Fig. 53A**: Reduction of E2F1 protein by siCyE2-647 was detected by immunoblotting. GAPDH protein served as a loading control.

**Fig. 53B**: E2F1 protein levels following siCyE2-647 transfection; data are expressed as means ± SEM, n=3. NT=not treated cells.

**Fig.54**: siRNA-mediated reduction of E2F1 transcript levels by quantitative RT-PCR (data were normalized to 28S). Data are expressed as means ± SEM, n=6.

NT=not treated cells. (*p <0.05 compared to siGL2 treated cells).
These data suggest that CyE2 could have the same role and functions of CyE1 in the HCC cell lines considered (Fig.55).

The results of these experiments, driven us through another question: what's happens to CyE1/CyE2 protein and mRNA levels when E2F1 is knocked down?

In all HCC cell lines, the depletion of E2F1 caused an evident decrease in CyE1 mRNA levels (*p<0.05; §p<0.001), in accordance with the established role of E2F1 in promoting cyclin E1 expression (Fig.56b). Only in HuH7 cell line, the decrease in mRNA levels was paralleled by a decrease in protein levels (Fig.56a).

![Diagram](image_url)

**Fig. 55:** E2F1/pRb-Cye2 hypothesis of correlation.

**a. CyE1 protein levels**

**b. CyE1 mRNA levels**

*Fig. 56:* a. CyE1 protein levels following siE2F1-1324 transfection; data are expressed as means ± SEM, n=3. NT=not treated cells.

b. siRNA-mediated reduction of CyE1 transcript levels by quantitative RT-PCR (data were normalized to 28S). Data are expressed as means ± SEM, n=6. NT=not treated cells. (*p <0.05; §p <0.001 compared to siGL2 treated cells).
This reduction was not observed in HepG2 and JHH6. The increased CyE1 protein levels with a reduced mRNA amount, implies that an increased stability of the proteins may occur. In this regard, in HepG2, it is possible to observe a reduced phosphorylation of CyE1 Thr395 residue, a known target for proteasome-mediated degradation (Fig.57).

![HepG2 cell line](image)

**Fig. 57:** Reduction of p-CyE (Thr395) upon siE2F1-1324 treatment was detected by immunoblotting. GAPDH protein served as a loading control.

In the case of JHH6, no variations in the Thr395 residue were observed (data not shown); this does not exclude that another phosphorylation site involved in protein degradation could have been hypophosphorilated.

Upon E2F1 depletion, we observed a clear reduction of cyclin E2 mRNA levels compared to siGL2 treated cells (*p <0.05) (Fig.58b), indicating the role of E2F1 in promoting cyclin E2 expression in the HCC cell lines considered. In this case, the decrease in mRNA levels was paralleled by a clear and a more moderate decrease in protein levels in JHH6 cell line, and in HepG2-HuH7 cell line, respectively (Fig.58a).

![a. CyE2 protein levels](image)

**Fig. 58a:** CyE2 protein levels following siE2F1-1324 transfection; data are expressed as means ± SEM, n=3. NT=not treated cells.
In addition to induce a decrease of E2F1 expression, depletion of CyE1 caused also a reduction in CyE2 mRNA levels in HuH7 and JHH6 cell lines. Less evident was the effect on the protein levels. No major differences were observed in HepG2 cell line (Fig.59a and b).

**Fig. 58b:** siRNA-mediated reduction of CyE2 transcript levels by quantitative RT-PCR (data were normalized to 28S). Data are expressed as means ± SEM, n=6.

NT=not treated cells. (*p <0.05 compared to siGL2 treated cells).

**Fig. 59:** a. CyE2 protein levels following siCyE1-1415 transfection; data are expressed as means ± SEM, n=3. NT=not treated cells.

b. siRNA-mediated reduction of CyE2 transcript levels by quantitative RT-PCR (data were normalized to 28S). Data are expressed as means ± SEM, n=6.

NT=not treated cells. (*p <0.05 compared to siGL2 treated cells).
Cyclin E2 depletion did not affect the mRNA levels of CyE1 (Fig. 60).

![CyE1 mRNA levels](image)

**Fig. 60:** siRNA-mediated reduction of CyE1 transcript levels by quantitative RT-PCR (data were normalized to 28S).

Data are expressed as means ± SEM, n=6. NT=not treated cells.

This finding was somewhat surprising if we considered the fact that E2F1, the major cyclin E1 transcription factor, was down regulated. In the liver, another cyclin E1 transcription is known, i.e. LRH. We therefore studied the expression level of this gene in the hypothesis it could be upregulated to take over cyclin E1 transcription in place of E2F1. Unexpectedly, no increase in the expression level of LRH-1 was observed (Fig. 61).

![LRH mRNA levels](image)

**Fig. 61:** siRNA-mediated reduction of LRH-1 transcript levels by quantitative RT-PCR (data were normalized to 28S).

Data are expressed as means ± SEM, n=6. NT=not treated cells.
In contrast, an increase of cyclin E1 protein levels were observed (Fig.62).

![CyE1 protein levels](image)

**Fig. 62.** CyE1 protein levels following siCyE2-647 transfection; data are expressed as means ± SEM, n=3. NT=not treated cells.

This result prompted us to investigate a possible increase in stability of cyclin E1 protein, verifying the reduction in the phosphorylation of CyE1 Thr395 residue in HepG2 by Western Blot analysis (Fig.63).

![HepG2 cell line](image)

**Fig. 63:** Reduction of p-CyE (Thr395) by siCyE2-647 was detected by immunoblotting. GAPDH protein served as a loading control.

With regard to HuH7 and JHH6, no variation was detected on Thr395 residue (data not shown).
With regard to other cell cycle related genes, we did not observe any change in the phosphorylation status of pRb and in cyclin A protein levels (data not shown). On the other hand, some variations were observed for cyclin D1 protein and mRNA levels (Fig. 64a and b). The depletion of cyclin E1 caused an increase in cyclin D1 protein levels, paralleled by an increase in mRNA levels in JHH6. In HuH7 this effect was more evident at the mRNA level, while no clear variation were observed for HepG2.

The depletion of cyclin E2 caused a certain decrease in cyclin D1 protein levels, paralleled by a decrease in mRNA levels. No substantially differences in cyclin D1 expression were observed in siE2F1-1324 treated cells compared to controls.

![Graphs showing protein and mRNA levels](image)

**Fig. 64:** a. CyD1 protein levels following siRNAs transfection; data are expressed as means ± SEM, n=3. NT=not treated cells.

b. CyD1 transcript levels by quantitative RT-PCR (data were normalized to 28S).

Data are expressed as means ± SEM, n=6. NT=not treated cells.

(*p <0.05 compared to siGL2 treated cells).
Moreover, whereas no major variations in mRNA levels of the CDK inhibitors p27<sup>kip1</sup> were observed, an increase of p21<sup>cip1</sup> was detected in HuH7 and JHH6 cell line following the depletion of cyclin E1 (Fig 65a and b).

![Fig. 65: a. p27<sup>kip1</sup> mRNA levels](image)

**a. p27<sup>kip1</sup> mRNA levels**

![Fig. 65: b. p21<sup>cip1</sup> mRNA levels](image)

**b. p21<sup>cip1</sup> mRNA levels**

Fig. 65: a. p27<sup>kip1</sup> transcript levels by quantitative RT-PCR (data were normalized to 28S).

Data are expressed as means ± SEM, n=6. NT=not treated cells.

b. p21<sup>cip1</sup> transcript levels by quantitative RT-PCR (data were normalized to 28S).

Data are expressed as means ± SEM, n=6. NT=not treated cells.

In conclusion, we have identified siRNAs targeted against pivotal cell cycle controlling genes, i.e. cyclin E1, cyclin E2 and E2F1, which are able to efficiently down modulate the expansion of the HCC cell lines here considered. Whereas in HepG2 the mechanism is sustained by both apoptosis induction and inhibition of cell proliferation, in HuH7 and JHH6 inhibition of cell proliferation is predominant. The interrelation between cyclin E1, E2 and E2F1 have been also investigated: the data are summarized in tab 3.
To summarize:

<table>
<thead>
<tr>
<th>siRNA treatment</th>
<th>CyE1</th>
<th>CyE2</th>
<th>E2F1</th>
</tr>
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<tbody>
<tr>
<td>siCyE1-1415</td>
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<tr>
<td>siCyE2-647</td>
<td>↑</td>
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<tr>
<td>siE2F1-1324</td>
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Table 3: Protein and mRNA levels of cell cycle related genes upon siRNAs transfection.
**Depletion of SRF in VSMC**

Anti-proliferative drugs released from endo-vascular stents have substantially contributed to reduce in-stent restenosis rates in patients bearing coronary single primary lesions by down regulating VSMC growth. However, the considerably lower drug efficacy shown in the treatment of patients bearing more complex coronary lesions and in diabetic patients, suggests that alternative anti-proliferative approaches can be beneficial. Here, we explored the use of siRNAs as tools to knock down SRF, a transcription factor involved in the proliferation of different cell types and for which only limited data exist about its function in primary human VSMCs.

Suitable transfection systems were undertaken to test siSRF797 effects in vascular smooth muscle cells (VSMC). If not otherwise indicated, protocol 2 has been chosen as transfection protocol (see materials and methods).

Non treated cells and cells treated by a control siRNA directed against the luciferase gene (siGL2), were analysed in parallel. Optimal analysis time was found to be three days after transfection.

**14. siSRF797 effects on SRF mRNA and protein levels.**

To test the activity of siSRF797, it was transfected into VSMC, previously kept under starving conditions for 48 hours. Three days after transfection, a clear reduction (p<0.001) in the amount of SRF mRNA was observed in cells transfected by siSRF797, compared to siGL2 treated cells (Fig.66).

![SRF mRNA levels](image)

**Fig. 66:** siRNA-mediated reduction of SRF transcript levels evaluated by quantitative real time PCR (data were normalized to 28S). Data are expressed as means ± SEM, n=6. NT=not treated cells.

* *p < 0.001 compared to siGL2 treated cells.*
The decrease in SRF mRNA levels was paralleled by a strong decrease in SRF protein as reported in a representative experiment (Fig. 67a) and summarized in Fig 67b. In contrast to what revealed in the HCC cell lines JHH6 and HuH7, a clear reduction of both the mature and the newly synthesized form of SRF was observed.

**Fig. 67a:** Reduction of endogenous SRF protein by siSRF797 was detected by immunoblotting. GAPDH protein served as a loading control.

**Fig. 67b:** SRF protein levels; data are expressed as means ± SEM, n=3. NT=not treated cells.
15. Effects of SRF depletion on cell morphology and cell-counting.

SiSRF797 treatment significantly reduced VSMC number, without affecting significantly cell morphology, as reported in Fig. 68.

![VSMC](image)

**Fig. 68:** Effect of SRF depletion by siSRF797 on VSMC morphology, three days after transfection.

Cell morphology was analysed by phase contrast microscopy at 5x and 20x magnification. NT = not-treated cells.
Compared to control cultures, SRF-depleted VSMC showed a substantially lower confluence three days after transfection (Fig.68), which was accompanied by a significant decrease in cell number according to cell counting (*p<0.05) (Fig.69). Thus, SRF depletion impairs VSMC expansion.

**Fig. 69:** siSRF797 effects on VSMC: a reduction in cell number compared to not-treated cells (NT) and to siGL2 treated cells was observed three days after transfection (*p<0.05 compared to siGL2 treated cells); data are expressed as means ± SEM, n=5. NT=not treated cells.
16. Effects of SRF depletion on cell cycle phase distribution.

For cell cycle analysis, cells were incubated for 12 hours with BrdU and then stained by PI and FITC-conjugated anti-BrdU antibody as described for HCC cell lines. SiSRF797 treatment caused a significant decrease of S-phase cells compared to untreated or control siGL2 treated cells (*p<0.05) as reported in a representative experiment in Fig. 70a and summarized in Fig. 70b; concomitantly, the fraction of G0/G1 cells rose approximately 20% (*p<0.05).

**Fig. 70a:** A representative dot plot of VSMC is shown:

Q1: S-phase cells; Q2/Q4: G2/M phase cells; Q3: G1/G0 phase cells.

PI-A: Propidium Iodide; BrdU FITC-H: FITC labelled Ig anti BrdU.

**Fig. 70b:** Cell cycle phase distribution.

Data are expressed as means ± SEM, n=8. NT=not treated cells.

Three days after transfection, depletion of SRF diminished the S-phase cells by 40% compared to siGL2 treated cells (*p<0.05).

This decrease is followed by a tendency of increase in G0/G1 phase cells (*p<0.05).
To discriminate between cells in G1 or G0-phase of the cell cycle, Ki67 was tested: no significant variations in the amount of G0 cells was observed, indicating that the slightly increase in G1/G0-phase cells was due to an increase of G1-phase cells. Therefore, all these data suggest that SRF impairs VSMC proliferation and that SRF contributes to the G1-S phase transition in VSMC.

In contrast to these results, recently, it has been observed that stable depletion of SRF in rat VSMC resulted in an increase of cell proliferation upon PDGF-ββ or low serum (10%) stimulation. To investigate this point in our experimental set-up, human VSMC were stimulated by PDGF-ββ or low serum (10%) as described (Kaplan-Albuquerque et al., 2005) and cell proliferation evaluated by BrdU incorporation. As a result of this experiment, we could show that also after PDGF-ββ/low serum (10%) stimulation, the depletion of SRF resulted in a decrease of cell proliferation (*p<0.05) (Fig. 71).

![Graph](image)

**Fig. 71:** Data are expressed as means ± SEM, n=4. NT=not treated cells.

Three days after transfection, depletion of SRF inhibited cell proliferation also after PDGF-ββ/low serum stimulation compared to siGL2 treated cells (*p<0.05).
The very early onset of VSMC proliferation in restenotic lesions and the possible presence of pre-existing proliferating CSMCs in atherosclerotic plaques, implicates that, \textit{in vivo}, variable amounts of VSMCs may be proliferating when the therapeutic drug is applied. As it is not possible to precisely reproduce this situation in cultured cells, we decided to evaluate the proliferation inhibitory effects of our siRNA under the two possible extreme conditions. In the first case siRNA were administered to actively growing cells (proliferating cells protocol x) while in the second case cells were starved and then treated with siRNA and foetal calf serum at the same time (re-proliferating cells, protocol x). Proliferation inhibition efficacies were measured evaluating siRNA abilities to alter cell cycle phase distribution. (Fig.72).

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{cell_cycle_phases.png}
\caption{Cell cycle phase distribution.}
\end{figure}

Data are expressed as means ± SEM, n=4. NT=not treated cells.

Three days after transfection, depletion of SRF diminished the S-phase cells by 40% also in proliferating cells (*p<0.05 compared to siGL2 treated cells).

This decrease is followed by a tendency of increase in G0/G1 phase cells (*p<0.05).
The experiments so far presented were performed in condition of normal glucose concentration (5.5mmol/l). However, VSMC hyperproliferation is a patho-fisiological event that frequently occurring in diabetic patients, i.e. in condition of high blood glucose (22mmol/l) (Zanetti et al., 2008). Thus, to understand whether our approach may potentially be effective also in diabetic patients, siSRF797 antiproliferative effect was tested in conditions of high glucose (see Materials and Methods).

**Fig. 73:** Cell cycle phase distribution.

Data are expressed as means ± SEM, n=2. NT=not treated cells.

Three days after transfection, depletion of SRF diminished the S-phase cells by 30% (*p<0.05 compared to siGL2 treated cells) also in conditions of high glucose (HG).

As indicated in Fig.73 a significant reduction of S-phase cell (*p>0.05) was observed also in VSMC cultured in high glucose condition.
17. Effects of SRF depletion on cell death.

The apoptotic potential of the SRF depletion was investigated three days after transfection. Cells were collected and stained by fluorescein labelled antibody against annexin V and by Propidium Iodide (PI) and then analyzed by flow cytometry. Reported is a representative experiment showing similar amounts of apoptotic cells in siSRF797 treated cells compared to control siGL2 treated cells (Fig.74).

![Fig. 74: Effects of SRF depletion on cell apoptosis: the amounts of apoptotic VSMC were determined three days after transfection by annexin V and propidium iodide staining, followed by flow cytometry analysis. The percentage of apoptotic cells is indicated in the two right quadrants.](image)

Citotoxicity was evaluated by LDH test. As reported in Fig.75, no substantial differences were detected among siSRF797 treated cells and the controls. As positive control, triton treated cells were introduced.

![Fig. 75: LDH test three days after transfection. Data are expressed as means ± SEM, n=4. NT=not treated cells.](image)
18. Effects of SRF depletion on cell cycle regulators.

At the molecular level, SRF depletion caused an increase of the CDK2 inhibitor p27<sup>kip1</sup> (25%). Moreover, a decrease in the hyperphosphorylated form of pRb (P-pRb) and in the S/G2/M cyclin A of 45% and 40%, respectively were observed. Representative blots are reported in Fig.76a and summarized in Fig.76b.

Fig. 76a: Increase of p27<sup>kip1</sup>, reduction of P-pRb and cyclin A upon SRF depletion.

GAPDH protein served as a loading control.

Fig. 76b: pRb, CyA and p27<sup>kip1</sup> levels;

data are expressed as means ± SEM, n=3. NT=not treated cells.
The increased of $p27^{kip1}$ might be attributable to a post-transcriptional effect since $p27^{kip1}$ transcript levels were not affected by transfection with siSRF797 (Fig. 76c).

![p27kip1 mRNA](image)

**Fig. 76c:** $p27^{kip1}$ transcript levels evaluated by quantitative real time PCR (data were normalized to 28S). Data are expressed as means ± SEM, n=6. NT=not treated cells.

Additionally, we did not observe any changes in the protein and transcript levels of the CDK4 inhibitor $p16^{INK4}$, the CDK2 inhibitor $p21^{cip1}$, the pRb partner protein E2F1, the G1 cyclin D1 and the G1/S cyclin E1 and E2 (Fig. 77a and b).

![Protein levels](image)

**Fig. 77a:** Protein levels of different cell cycle related genes; data are expressed as means ± SEM, n=3. NT=not treated cells.
**Fig. 77b:** mRNA levels of different cell cycle related genes evaluated by quantitative real time PCR (data were normalized to 28S).

Data are expressed as means ± SEM, n=6. NT=not treated cells.

Since cyclin A, but not cyclin D1, cyclin E1 or cyclin E2 protein levels were affected by siSRF797, a SRF knock-down probably arrest VSMC cell cycle late in G1.

In conclusion, our results show that SRF is crucially involved in the control of human primary VSMC proliferation which complements the well-established role of SRF in VSMC differentiation and demonstrates the important role of SRF in human primary VSMCs. Moreover, the possibility to down modulate VSMC growth in condition of high glucose potentially makes our approach attractive also for the treatment of diabetic patients. At the molecular level, SRF depletion is paralleled by an increase in the protein levels of p27, a decrease of the phosphorylated form of p-RB and of cyclinA1. These observation well correlate with the S-phase reduction and G1-G0 increase induced by SRF depletion in VSMC.
DISCUSSION

Exuberant and non-controlled cellular proliferation underlines the ethio-pathogenesis of many human pathological conditions, including tumour and non-tumour diseases. Thus, the possibility to control this complex process can be extremely useful in terms of prevention/control of disease progression, especially in the light of the limited efficacy of current therapeutic approaches. In this project we draw our attention on the down regulation of cell proliferation in the context of two human diseases, namely hepatocellular carcinoma (HCC), as an example of tumour pathology and in stent- restenosis, example of non-tumour pathology. Beside the obvious advantages deriving from the prevention of cell growth in these two pathological conditions, we thought interesting the comparison between the mechanisms regulating the growth of tumour vs non tumour cells.

1. Hepatocellular carcinoma data

HCC is a leading cause of cancer death world-wide, with an estimated 564,000 new cases and almost as many deaths in 2000 (Thorgeirsson and Grisham, 2002). There is no clinically tested useful therapy for the advanced stage of the disease and any effective treatment has not been assessed in large controlled randomized trials for this kind of hyper-vascular and chemotherapy-resistant tumor. Thus, the development of novel effective therapeutic approaches are of utmost relevance.

Our strategy to prevent HCC development is based on the inhibition of tumour cell proliferation targeting the transcription factors SRF and E2F1 and cyclins E1 and E2 by means of siRNAs, short double stranded RNA molecules able to induce the specific degradation of a homologue mRNA (Scherr et al., 2003).

1.1 Effects of SRF depletion on HCC cell lines.

The transcription factor SRF has been considered as target in this work because of its involvement in the control of the expression of proliferation-related genes in many primary cell types. Furthermore, SRF has been recently shown to be directly implicated in the proliferation of the HCC cell line HepG2 (Shao et al., 2005). Additional evidence of SRF relation to HCC growth has been provided evaluating its expression in human specimens of HCC (Park et al., 2007). As a result of this analysis it was found that SRF is expressed in HCC tissue but not in
normal/cirrhotic liver. Even more interestingly, over-expression of SRF was reported in high grade, poorly differentiated, HCC forms. These observation together with the absolute lack of any published data about the investigation about a direct depletion of SRF in HCC cell lines, prompted us to generate a specific siRNA anti SRF and test its effect in three HCC cell lines, i.e. HepG2, HuH7 and JHH6. The choice of these cell lines was based on the fact that, although they all are HCC tumor-derived cells, they display different phenotypes and thus phenotypic-related effects of SRF depletion can be studied. In particular, HepG2, HuH7 and JHH6 can be assigned to high, medium and low hepatocytic differentiation grade on the base of the capacity to synthesize albumin, a known marker of hepatic differentiation (Grassi et al., 2007). It should be noted that JHH6, the most undifferentiated cells, although not able to synthesize albumin (data not shown) can still produce ferritin, indicating a residual hepatic phenotype (data not shown).

The selected siSRF797 is able to drastically reduce the amount of SRF mRNA in all three cell line tested (Fig.6) compared to the control siGL2 and non treated cells. This observation demonstrates the potent and sequence specific action of siSRF797. The specificity of action is further strengthened by the fact that siSRF797 does not elicit any significant expression of the gene AOS, a known marker of an a-specific IFN response (Fig.5) which double stranded RNAs may trigger (Bridge et al., 2003). Consistently with the reduction of the mRNA levels, also the total SRF protein levels are reduced in all cell lines (Fig.7). However, whereas in HepG2 both the mature (67 kDa) and newly synthesized (63 kDa) SRF form are reduced, in JHH6 and HuH7 only the newly synthesized form is clearly reduced. The reasons for this behavior are unclear. A possibility is that in JHH6-HuH7 the mature form is more stable than in HepG2. Consistent with this anomalous behavior of SRF mature form there is the observation (Fig.18A and B) that in JHH6 and HuH7 SFR protein levels of the mature form are substantially independent from serum stimulation, in contrasts to what occurs in HepG2, and other differentiated cells such as vascular smooth muscle cells (Fig.18C and D) and in fibroblasts (Misra et al., 1991). The fact that in the more differentiated HepG2 cell line but not in the less differentiated JHH6-HuH7, SRF protein forms seem to be regulated in a physiologically fashion, indicates a phenotypic dependent regulation of SRF. It is possible that in more aggressive and less differentiated cells, stable levels of the mature form of SRF confers and advantage in terms of tumor expansion. This hypothesis is in agreement with the very recent observation (Park et al., 2007) that SRF is more abundant in high grade poorly differentiated human HCC tumors. Notably, in this HCC type, SRF increased cell migration and invasion probably providing HCC and advantage in terms of diffusion and expansion.
Despite the possibility to only reduce the protein levels of the newly synthesised form of SRF by siSRF797 in JHH6 and HuH7, a significant reduction of the total SRF protein levels were achieved compared to controls (Fig.7). Therefore, the examination of the effects of SRF down regulation were conducted also in JHH6 and HuH7, in addition to HepG2.

In HepG2 cell line siSRF797 transfection resulted in a clear reduction in cell number (Fig.11) paralleled by a concomitant decrease of colony areas but not colony number. This observation suggest a cyto-static rather than a cyto-toxic effect as also supported by the fact that no significant elevation of LDH enzymes were observed in treated cells compared to controls (Fig.25). Consistently with the above observation, SRF depletion resulted in a cell cycle down modulation (Fig.14 and Fig.15) with a reduction of S-phase cells. It is worth noting that a similar behaviour was observed in VSMC (Fig.70), suggesting a similar mechanism of action for SRF in these two cell type, albeit for what concern the effects on cell cycle.

SRF depletion in HepG2 is not limited to the down modulation of proliferation, it also induces apoptosis (Fig.21-23). This phenomenon, seems to be more contained than cell proliferation inhibition. Whereas the total amount of apoptotic cells rises only up to 4-5%, the decrease in the amount of S-phase cells corresponds to about 20%. Despite this observation, the biological meaning of apoptosis induction should not be underscored. It may represent the response to the confounding effects resulting from outside (serum stimulation) and inside (SRF depletion) the cell. This may indicate a certain capacity of a physiological response of this HCC cell line to confounding signals, a property typically lacking in poorly differentiated tumour cells which in contrast display high resistance towards apoptosis. Notably, apoptosis induction does not seems to be specific for SRF depletion as we (Fig.46 and Fig.47) and other (Li et al., 2003) have observed that also upon cyclin E and E2F1 depletion apoptosis is induced in HepG2.

In contrast to what observed for HepG2, in JHH6 and HuH7 transfection by siSRF797 neither induced a significant reduction in cell number nor significantly affected cell cycle progression and apoptosis rate. These observation may be due to different factors. First, SRF may not be essential to promote proliferation in poorly differentiated HCC cells such as JHH6 and HuH7, in contrast to what occur in the more differentiated HepG2 and the fully differentiated VSMC (see also next paragraph). It is therefore possible that in JHH6 and HuH7 the SRF control on cell proliferation is bypassed by alternative pathways. This hypothesis, however, would contradict the observation of the relevant role exerted by SRF in the development of high grade, poorly differentiated HCC forms (Park et al., 2007). Second, the reduction of total SRF protein levels, although being evident in our experiments, may not be sufficient to abrogate SRF biological functions. Our difficulties to deplete SRF total protein amount may be due to an increased SRF
stability in JHH6 and HuH7. To explore this aspect, siSRF797 was transfected in JHH6-HuH7 twice (protocol 1, transfection at day 0 and 3 and analysis at day 6) instead of only once (protocol 1, transfection at day 0 and analysis at day 3) as done for all the experiments above commented. Disappointingly, also under these conditions of a more prolonged siSRF797 action, no significant decrease in cell number, a parameter taken to indirectly evaluate SRF depletion, was observed. This experiment is in favour of an high stability of SRF mature form in JHH6 and HuH7. Probably, only by means of an expression vector stably producing the siSRF797 within the cells it is possible to properly deplete SRF in these HCC cell lines (work in progress). It should be noted that we were unable to perform more that two consecutive siSRF797 transfection because of the a-specific transfection-induced cell toxicity. A third possible explanation for the lack of any clear biological effect of siSRF797 in JHH6 and HuH7 may reside in the biological role of the mature and newly synthesised SRF forms. It is not possible to exclude that, as in fibroblasts (Misra et al., 1991), the biologically active form is the mature one while the newly synthesised form gradually acquire this ability during the post-trabscriptional modification (mostly phosphorylation). Whether this is the case also in JHH6 and HuH7 remains to be determined.

In HepG2, consistent with the reduction of S-phase cells upon siSRF797 treatment, we observed a clear reduction in the protein level of the transcription factor E2F1 (Fig.26), a relevant promoter of the G1 to S phase transition. This reduction seems to be regulated at the transcription level as the E2F1 mRNA is significantly reduced (Fig.27). Since no SRF binding sites are known on the E2F1 promoter, the down modulation of E2F1 transcription is likely to be an indirect cause of SRF depletion. Furthermore, the fact that in VSMCs the down modulation of SRF does not results in an appreciable reduction of E2F1 levels (see also next paragraph) suggests a cell specific effect. Further analysis (DNA array) will be performed to clarify this aspect. Consistently with the reduction of E2F1 expression, also the mRNA levels of cyclin E1, one of E2F1 transcriptional target gene, are significantly reduced (Fig.30). Notably, however, whereas the mRNA levels decrease, cyclin E1 protein levels are increased (Fig.29) suggesting an increased protein stability. This hypothesis is confirmed by the reduced cyclin E1 phosphorylation at residues Thr 395, a site whose phosphorylation is known to drive cyclin E1 degradation (Hwang and Clurman, 2005). The increased cylin E1 protein levels together with the un-altered protein levels of cyclin D1 and cyclin A, all cyclins concurring to phosphorylate the retinoblastoma protein, are consistent with the presence in SRF depleted cells of an hyper phosphorylation status of p-RB (Fig.28) similar to control. These observation, together with the substantially non modified protein levels of the cell cycle inhibitors p27, p21 and p16, suggest,
in HepG2, a mechanism of reduced G1-S phase transition upon SRF depletion mediated by the
decrease of E2F1. Despite being unbound to pRB, which is in its hyper-phosphorylated form like
control, E2F1 is probably not quantitatively adequate to properly promote the transcription of all
or a part of its target genes. Obviously, other mechanisms cannot be excluded. In this sense,
DNA array will certainly contribute to further understand this aspect.

In contrast to HepG2, in JHH6 no substantial variation in both protein and mRNA levels of E2F1
were observed (Fig.26 and Fig.27), consistently with the lack of any evident reduction of the G1-
S phase transition. As above discussed, it is possible that the permanence of the mature form of
SRF prevents the down regulation of E2F1. In HuH7, a reduction of E2F1 protein levels
comparable to HepG2 is observed (Fig.26). However, in contrast to HepG2, the down regulation
is post-transcriptional as E2F1 mRNA levels are un-affected (Fig.27). Despite this difference, it
remains unclear why the reduction of E2F1 does not result in a reduced G1-S phase transition,
especially in the light of the fact that a direct targeting of E2F1 by an siRNA in HuH7 (see next
paragraph) does result in a delayed G1-S phase transition. The only difference we observe is that
upon SRF depletion in HuH7 a decrease of E2F1 but not of cyclin E1 occurs while the direct
depletion of E2F1 is also accompanied by a marked cyclin E1 decrease (Fig.56). It is therefore
possible that in this last case the reduced cyclin E1 level cannot absolve its role in sustaining
DNA synthesis (Zhang, 2007; Hwang and Clurman, 2005) thus impairing proliferation.

In conclusion, our data show the identification of an active siRNA directed against SRF able to
specifically and efficiently reduce the SRF mRNA in the hepatic cell line HepG2, JHH6 and
HuH7. SRF mRNA depletion resulted in a significant inhibition of cell proliferation and
apoptosis induction in HepG2 but not in JHH6 and HuH7. Thus, the different phenotypes of the
cell line considered, hepatocyte-like for HepG2, intermediately differentiated for HuH7 and
undifferentiated and JHH6, respectively, play a relevant role in the response siSRF797 treatment.
This implies that in vivo not all HCC forms may respond similarly to a siSRF797 treatment.
Despite this fact, we have shown for the first time the involvement of SRF in hepatic cell
proliferation, expanding the cell types in which SRF is implicated in cell proliferation. These
results can pave the way to a better understanding of SRF involvement in cell proliferation and
possibly to identify novel effective anti HCC therapeutic strategies.
**1.2 Effects of E2F1, Cyclin E1/2 depletion on HCC cell lines.**

Whereas in HepG2 siSRF797 was able to reduce cell expansion by decreasing cell proliferation and increasing apoptotic rate, in the less differentiated HCC cell line JHH6 and HuH7 the effect was far less evident if not absent. Thus, with the aim to identify siRNAs also able to down modulate the expansion of less differentiated HCC cells, siRNAs directed against cyclin E1, cyclin E2 and the transcription factor E2F1 were generated and tested in vitro. Cyclin E1 is an ideal candidate to be suppressed for an anti HCC strategy as it is over-expressed in about 70% of HCC patients (Jung et al., 2001) and this over-expression correlates with a poor prognosis. Moreover, cyclin E1 over-expression was found to be stronger in high grade HCC (II and III according to Edmondson classification) compared to low grade (I) (Zhu et al., 2003; Zhou et al., 2003) and it was significantly more elevated that in HCC surrounding cirrhotic tissues (Masaki et al., 2003). Consistent to HCC in humans, also in mice models cyclin E1 has been shown to be over-expressed (Mauriz et al., 2007). Additionally, preliminary observations (Li et al., 2003) indicate that in the HCC cell line HepG2 cell lines over-expressing cyclin E, the inhibition of this gene results in the prevention of cell growth. For the HCC forms with no cyclin E1 over-expression, the inhibition of cyclin E alone does not seem to be sufficient to prevent tumour cell proliferation (Li et al., 2003). To try to down modulate cell cycle progression in these HCC cells and to potentiate it in HCC over-expressing cyclin E, we propose the knock out also of the transcription factor E2F1. The inhibition of E2F1 was chosen because of its strict biological connection to cyclin E1 as evidenced by the existence of a feed-forward loop between them amplifying the G1 to S phase promoting signals (Geng et al., 1996). Additionally, E2F1 has been recently implicated in the development of HCC in vitro (Arakawa et al., 2004) and in animal models (Coulouarn et al., 2006; Pascale et al., 2002; Conner et al., 2000). Finally, as it is becoming evident the role of the second member of the cyclin E family, i.e. cyclin E2, in the E2F1-cyclin E1 pathway, also cyclin E2 was considered as target in our research. The suitability of the specific destruction of E2F1 and cyclin E1/2 mRNAs by means of siRNAs, also depends on the fact that these genes are regulated at the mRNA and protein levels in a cell cycle-dependent manner. This means that they are synthesized de novo at each new G1 phase and then rapidly degraded at the protein and mRNA level with the progression of the cell cycle so that no active protein and mRNA is left for the next cycle.

The selected siRNAs are able to drastically reduce the amount of their target mRNAs (*p<0.05) in all the three cell line tested (Fig.32) compared to the control siGL2 and non-treated cells. This observation demonstrates the potent and sequence specific action of the selected siRNAs. The specificity of action is further strengthened by the fact that they do not elicit any significant
expression of the gene AOS, a known marker of an a-specific IFN response (Fig.5) which double stranded RNAs may trigger (Bridge et al., 2003). Finally, the lack of any detectable citotoxicity as evaluated by LDH test (Fig.49), supports the safeties of the considered siRNAs and of the transfection protocol.

In general, in all the three cell lines, the siRNA against cyclin E1 significantly decreases the amount of cell number by decreasing proliferation via the reduction of S-phase cells and a certain increase of G1 cells (Fig.41, Fig.43 and Fig.44). In HepG2 however, the siRNA directed against cyclin E1 (siCyE1-1415) only modestly reduces S-phase cells (Fig.41). This observation is qualitatively in agreement with a previous observation (Li 2003) but differs from the quantitative point of view as the extent of S phase cell reduction upon cyclin E1 depletion is much less evident. We can exclude that the siRNA we used has lower efficacy in inducing cyclin E1 depletion as comparable degrees of cyclin E1 protein reduction can be observed with Li’s siRNA (compare Fig.33a with Fig.1B Li 2003). Most likely, this discrepancy depends on some biological differences between our and Li’s HepG2 strain. This hypothesis seems to be supported by the fact that our preliminary results (data not shown) indicate a similar behaviour of Li’s siRNA when tested in our HepG2 strain. Despite this partial discrepancy, our and Li’s data support the concept that cyclin E1 targeting by siRNA may have the potential to prevent HCC cell growth. Moreover, our data expand this concept to the far less differentiated HCC cells JHH6. This observation is particularly relevant in the light of the strong cyclin E1 expression in high grade HCC types (Zhu et al., 2003; Zhou et al., 2003). Thus, here we provide evidence for the broad HCC therapeutic potential of anti cyclin E1 siRNAs.

Compared to siCyE1, the E2F1 directed siRNA (siE2F1-1324) resulted to have even broader proliferation inhibition effects as it could significantly reduce the amount of S-phase cells in all three HCC cell lines tested (Fig.41, Fig.43 and Fig.44). In HepG2, our data are in agreement with a previous work (Simile et al., 2004) where the down modulation of E2F1 mRNA expression was achieved by an antisense oligodeoxy nucleotide. However, in this case the extent of HepG2 proliferation inhibition was more evident that in our case. Beside the fact that the partial discrepancy may arise from minor differences between the HepG2 strain used and the fact Simile et al used an ODN (10 μM) and we an siRNA (0.22 μM), it is possible that a technical step accounts for the quantitative difference. Whereas Simile et al used cell synchronized in low serum, we have used a-synchronized growing cells. Compared to synchronized cells, a-synchronized cells have a lower amount of G1 phase cells and an increased S or G2-M cells and are therefore more refractory to siE2F1. In fact, siE2F1 action can only be exerted in G1 phase when the target gene E2F1 is expressed, while in the subsequent cell cycle phases the lack of the
target gene renders siE2F1 action impossible. In support of this hypothesis there is the observation that depletion of E2F1 in synchronized vascular smooth muscle cells by an hammerhead ribozyme (Grassi et al., 2005b) and an siRNA (work in progress) resulted in a more evident reduction in cell proliferation (S-phase cell reduction) compared to a-synchronized vascular smooth muscle cells. In this work we have used a-synchronized cells instead of synchronized to better mimic the in vivo situation where HCC cells have free access to growth factor and are in a-synchronized conditions. Despite this aspect, our and Simile data prove the efficacy of E2F1 depletion in the down regulation of the growth of HepG2, an in vitro model of a differentiated HCC cell type. Moreover, our data extend this concept also to the far less differentiated HCC cells HuH7 and JHH6 thus broadening the attractiveness of an anti E2F1-based strategy. Giving the growing evidence of the E2F1 direct implication in HCC carcinogenesis (Arakawa et al., 2004; Coulouarn et al., 2006; Pascale et al., 2002; Conner et al., 2000) it is evident the potential relevance of our observation.

The depletion of the third siRNA target, i.e. cyclin E2, has never been evaluated in any HCC cell line. Its down regulation gave comparable results as cyclin E1 depletion with regard to cell cycle inhibition in terms of the reduction in S-phase cell amount (Fig.41, Fig.43 and Fig.44). This observation supports the concept that, although sharing only 47% homology with cyclin E1 (Lauper et al., 1998; Mazumder et al., 2004), cyclin E2 has similar functions. As a consequence, also cyclin E2 depletion has the potential to be of interest for a novel HCC therapeutic approach.

Depletion of cyclin E2 like cyclin E1 and E2F1 induces apoptosis in HepG2 but not in JHH6 and HuH7 (Fig.46 and Fig.48). In the case of cyclin E1 depletion, apoptotic induction is qualitatively but not quantitatively in agreement with the work of Li (Li 2003). Also in this case we believe that the difference observed just depends on some biological differences between our and Li’s HepG2 strain. This hypothesis seems to be supported by the fact that our preliminary results (data not shown) indicate a similar behaviour of Li’s and CyE1-1415 siRNA when tested in our HepG2 strain.

In HepG2, apoptosis induction by siE2F1-1324, is more contained than cell proliferation inhibition. Whereas the total amount of apoptotic cells rises only up to 4-5%, the decrease in the amount of S-phase cells corresponds to more then 20% (Fig.41). Despite this observation, the biological meaning of apoptosis induction should not be underscored. It may represent the response to the confounding effects resulting from outside (serum stimulation) and inside (cyclin E1/2 or E2F1 depletion) the cell. This may indicate a certain capacity of a physiological response of this HCC cell line to confounding signals, a property typically lacking in poorly differentiated tumour cells displaying high resistance towards apoptosis. As already observed for the siSRF
treatment, the fact that the depletion of different cell cycle promoting genes induces apoptosis in HepG2 suggest an a-specific response of this type of HCC to contrasting signals. This different behaviour observed between HepG2 and JHH6-HuH7 stress the importance of the cell differentiation grade as a factor influencing the therapeutic response.

At the molecular level, depletion of E2F1 leads to very similar effects with regard to the influence on cyclin E1/2 expression. In all cell lines, E2F1 down regulation results in a reduction of cyclin E1/2 mRNA levels (Fig.56b and Fig.58b). These findings extend the E2F1-mediated cyclin E1-2 expression observed in different other cell types (see (Bracken et al., 2004) for a review) also to the HCC cell line tested. However, at the protein levels some relevant differences can be observed. Whereas upon E2F1 depletion cyclin E2 protein levels are consistently decreased in all cell lines (Fig.58a), cyclin E1 protein levels varies in the different cell lines. No substantial variations are observed in JHH6 cyclin E1 protein levels, while a reduction is observed in HuH7 and a clear increased is observed in HepG2 (Fig.56a). In this last case we could provide evidence of a decreased cyclin E1 phosphorylation at residues Thr395, a fact which can be responsible for reduced protein degradation (Fig.57). Although the molecular reasons for this variable behaviour of the protein levels in the three HCC cell lines are not clear, it is highly probable that the different HCC cell phenotypes play a major role in the reported differences.

Depleting cyclin E1/E2, a reduction in E2F1 protein amounts were observed in all cell lines (Fig.50 and Fig.53). These data are substantially confirmed at the mRNA levels (Fig.51 and Fig.54). This suggests that an E2F1 transcriptional inhibition occurs upon cyclin E1/2 down modulation. As the major E2F1 transcriptional activator is E2F1 itself, one would expect to detect a reduced p-RB phosphorylation status, due to the reduction of cyclin E1/2 levels. Decreased p-RB phosphorylation in turn would reduce the free E2F1 available for its own transcription. However, we could not detect major variation of p-RB phosphorylation status. One possibility is that instead of being involved pRB, other pocket proteins, which also interact with E2F1 (p107 and p130, (Moroy and Geisen, 2004), are involved. In this case the reduction in cyclin E1/2 would reduce p107 and p130 phosphoryation and consequently the amount of free E2F1 (work in progress).

Depletion of cyclin E2 has no substantial effects on the mRNA levels of cyclin E1 in all three HCC cell lines (Fig.60). This finding is somewhat surprising if we consider the fact that E2F1, the major cyclin E1 transcription factor, is down-regulated. In the liver, another cyclin E1 transcription factor is known, i.e. LRH (Fayard et al., 2004). However, we could show that LRH is not up-regulated (Fig.61) and thus it is unlikely that it could have taken over cyclin E1
transcription in place of E2F1. Our findings suggest either an increased cyclin E1 mRNA stability or, most likely, the presence of other unknown cyclin E1 transcription factors. This would not be too surprising considering the described E2F-independent transcription of cyclin E1 (Lukas et al., 1997).

In contrast to the unaffected cyclin E1 mRNA levels upon cyclin E2 depletion, we observed increased cyclin E1 protein levels in all three HCC cell lines (Fig.62). In HepG2 cells, cyclin E1 protein level increase can depend on decreased cyclin E1 phosphorylation at residues Thr395 (Fig.63). In JHH6 and HuH7 no significant variation in the phosphorylation status of cyclin E1 residues Thr395 was observed. In these cases we cannot exclude that other cyclin E1 phosphorylation sites regulating protein degradation are affected.

Whereas cyclin E2 depletion induces in all three HCC cell lines comparable effects on cyclin E1 protein and mRNA levels, depletion of cyclin E1 variably affects cyclin E2 mRNA and protein levels (Fig.59). In HepG2 it does not alter cyclin E1 mRNA/protein levels, in HuH7 and JHH6 it mainly reduces the cyclin E1 mRNA levels, consistently with the reduction of E2F1. These findings suggest that cyclin E1 and E2 may not only complement each other. It is possible that they have unique functions and that these functions depends on the specific cell phenotype.

Finally, it has been proposed a reciprocal expression level between cyclin E1 and cyclin D in HCC tissue samples (Jung et al., 2001). This trend seem to be confirmed in JHH6, the less differentiated HCC cell line considered, where cyclin E1 depletion is paralleled by an increased cyclin D expression (Fig.64). Much less evident is this phenomenon in HuH7 and substantially absent in the most differentiated HepG2. Notably, cyclin E2 depletion does not result in any reciprocal expression effect with cyclin D1, further stressing the concept that cyclin E1 and E2 may have also independent effects on cell cycle.

In conclusion, we have identified siRNAs targeted against pivotal cell cycle controlling genes, i.e. cyclin E1, cyclin E2 and E2F1, which are able to efficiently down modulate the expansion of the HCC cell lines here considered. Whereas in HepG2 the mechanism is sustained by both apoptosis induction and inhibition of cell proliferation, in HuH7 and JHH6 inhibition of cell proliferation is predominant. In contrast to what observed for siSRF studies, the different phenotypes of the cell line considered, hepatocyte-like for HepG2, intermediately differentiated for HuH7 and undifferentiated and JHH6, respectively, have a lower impact on the efficacy of the strategy adopted. These results can contribute to a better understanding of E2F1-cyclin E1-cyclinE2 involvement in cell proliferation and possibly to identify novel effective anti HCC therapeutic strategies.
2. VSMC data

Anti-proliferative drugs (such as rapamycin) released from endo-vascular stents have substantially contributed to reduce in-stent restenosis rates in patients bearing coronary single primary lesions by down regulating VSMC growth. However, the considerably lower drug efficacy shown in treatment of patients bearing more complex coronary lesions and in diabetic patients, suggests that alternative anti-proliferative approaches can be beneficial. In this regard, the use of less aggressive and citotoxic drug has been proposed (Fajadet et al., 2005).

The understanding of the molecular mechanisms controlling VSMCs proliferation is therefore crucial for the development of new therapeutic strategies. Here, we explored the use of siRNAs as tools to knock down SRF, a transcription factor involved in the proliferation of different cell types and for which only limited data exist about its function in primary human VSMCs (Chow et al., 2007).

1.3.1 Effects of SRF depletion on primary VSMCs

SRF has been shown to control cellular processes such as adhesion, migration or cell survival (Schratt et al., 2002) (Schratt et al., 2004), whereas its role in cellular proliferation remains controversial. Additionally, there exist only very few data about the role of SRF in primary human VSMCs (Chow et al., 2007). On the one hand, homozygous inactivation of both SRF alleles does not severely affect the proliferation of murine embryonic stem (ES) cells (Schratt et al., 2001). On the other hand, SRF has been shown to be an essential mediator of PI3-kinase-regulated proliferation of PC12 cells, and ectopic expression of a constitutively active form of SRF, SRF-VP16, was sufficient to induce proliferation of 3T3-L1 fibroblasts (Poser et al., 2000).

We show here that SRF suppression diminishes human primary VSMC proliferation, and inhibits progression from G0/G1 phase to S phase of the cell cycle (Fig. 70). This observation indicates that, at least in more differentiated somatic cells such as VSMCs, fibroblasts, and in the highly differentiated hepatic cell line HepG2 (our results), the SRF seems to be a positive regulator of cell proliferation. Moreover, the discrepancy between the data obtained in differentiated cells and those obtained in ES cells may stem from different experimental approaches. In the case of differentiated cells (Poser et al., 2000) and the present work), SRF was transiently modulated. In contrast, inactivation of SRF was permanent in “knock-out” ES cells (Schratt et al., 2001).

Recently, Kaplan-Albuquerque and collaborators reported that rat aortic vascular SMC clones stably expressing SRF small hairpin RNAs (shRNAs) displayed strongly diminished SRF levels, but, in contrast to our findings, increased rates of proliferation compared to control cells were observed (Kaplan-Albuquerque et al., 2005). The reasons for this discrepancy are unclear.
Potential toxic side effects of the siRNA-lipid complexes we used could not account for the observed anti-proliferative effects of transiently transfected SRF siRNAs, because only the perfectly homologous SRF siRNAs siSRF797 inhibited VSMC proliferation, whereas the sequence-unrelated control siGL2 had no effect (Fig.70). Moreover, Kaplan-Albuquerque et al. studied the effects of SRF depletion in VSMCs stimulated by PDGF-BB or low serum (10%), while we performed most of our investigation in the presence of high serum (15%). However, also under their growth conditions, transient SRF depletion in our human VSMCs resulted in a decreased proliferation rate confirming our data obtained in high serum (Fig.71). Thus, it is possible that the cells stably expressing the shRNA may have adapted physiologically to the reduced SRF levels during the clonal selection process. In contrast to stable shRNA expression, our transient siRNA transfection approach is less likely to be affected by cellular adaptation processes. Additionally, there could be differences between VSMCs from rat and men and between aortic and coronary artery VSMCs. Finally, our results are in agreement with the proliferation-supportive role of SRF in several cell types (Poser et al., 2000; Chai et al., 2004; Lockman et al., 2004; Ding et al., 2001; Soulez et al., 1996; Wheaton and Riabowol, 2004).

Reduced cell proliferation upon SRF suppression is associated with an accumulation of VSMCs in the G0/G1 phase of the cell cycle. This accumulation may be either due to an impaired cell cycle progression through G1 or by a hindered transition from G1 to S phase. In our hands, siRNA-mediated depletion does not substantially affect cyclin D1, cyclin E1 or E2F1 protein levels (Fig.77). Furthermore, expression of the CDK4 inhibitor p16INK4 is not affected by SRF suppression (Fig.77). Taken together, these results suggest that inhibition of SRF expression does not interfere with cell cycle progression during mid G1. However, SRF siRNA induces the CDK2 inhibitor p27kip1 (Fig.76a-b), which might interfere with CDK2 activity, thereby inhibiting pRB phosphorylation and progression into S phase of the cell cycle. In line with this assumption, protein levels of the S/G2/M cyclin A are reduced upon SRF depletion (Fig.76a-b).

SRF-dependent regulation of p27kip1 levels may proceed via transcriptional and posttranscriptional mechanisms, e.g. by the ubiquitin-proteasome pathway. Since p27kip1 mRNA levels were not affected by SRF siRNA transfection (Fig.76c), a transcriptional regulation is unlikely. p27kip1 stability is regulated by at least two different pathways: translocation-coupled cytoplasmic ubiquitination by KPC (KIP1 ubiquitination-promoting complex) at G1 phase, and nuclear ubiquitination by an SKP2 (S-phase kinase-associated protein 2)-containing complex at S and G2 phases. Inhibition of G1/S transition by SRF siRNAs favors a KPC-dependent p27kip1 degradation. In line with this assumption, SKP2 protein levels were not affected by SRF siRNAs (data not shown). Therefore, SRF may influence p27kip1 stability either through affecting its
intracellular localization or through modulation of KPC expression (Boehm et al., 2002; Kamura et al., 2004).

The beneficial effects of an anti-SRF based approach are not limited to the anti-proliferative effects here documented. Unlike commonly used anti-restenotic drugs such as rapamycin, siRNA-mediated SRF suppression does not induce cell death (Roque et al., 2001) (Fig.74). Therefore, it can be speculated that the application of SRF siRNAs may be advantageous over the above-mentioned drug, which in long term may cause cell death associated with arterial wall weakening and retarded re-endothelialization leading to the emergent problem of late stent thrombosis (Serruys and Daemen, 2007). Moreover, SRF depletion is not associated with increased pro-inflammatory cytokine expression (data not shown). This is a desirable feature for a therapeutic approach to pathological conditions such as in-stent restenosis, where pro-inflammatory stimuli contribute to its pathogenesis (Ferns and Avades, 2000). Finally, the SRF siRNAs tested do not compromise CREB, a relevant down-regulator of CASMC proliferation (data not shown) (Reusch and Watson, 2004).

The vast majority of the work (Knoll et al., 2006; Campisi, 2000; Schratt et al., 2004; Schratt et al., 2001) dealing with the development of molecules to be used to prevent VSMC proliferation, was performed using starved cells induced to proliferate after drug administration. This experimental design is based on the simplified assumption that in vivo VSMCs are quiescent before PTA treatment and stent implantation. However, this assumption often does not completely reflect the in vivo situation where the very early onset of VSMC proliferation in restenotic lesions and the possible presence of pre-existing proliferating VSMC in atherosclerotic plaques (Heidenreich et al., 1999) implicates that variable amounts of VSMCs may be proliferating when the therapeutic drug is applied. As it is not possible to precisely reproduce in cultured cells the above mentioned in vivo situation, we evaluated the proliferation inhibitory effects of our siSRF797 under the two extreme conditions represented by starved (see protocol 1 in Material and Methods) and actively proliferating cells (protocol2). Our data indicate that, besides being very active when applied to starved cells siSRF797 is also very effective when delivered to actively growing cells (Fig.72), thus showing its therapeutic potential also in the unfavourable condition of lesions containing variable degrees of replicating cells. Instent restenosis more frequently occurs in diabetic patients (Dibra et al., 2005). The reason for this phenomenon are not completely understood, however, it is known that in vitro VSMCs cultured in high glucose (to simulate the diabetic condition) tend to have an higher proliferation rate that in normal glucose. To test the efficacy of our anti proliferative approach, the effect of SRF depletion was also studied under high glucose concentration (Zanetti et al., 2008).
that a significant reduction of the S-phase cells upon SRF depletion occurred in our VSMCs indicates the potential effectiveness of our approach also in diabetic patients, where restenosis rate are higher compared to non diabetic patients, despite current therapeutic approaches (Fig.73).

Taken together, our results show that SRF is crucially involved in the control of human primary VSMC proliferation which complements the well-established role of SRF in VSMC differentiation and demonstrates the important role of SRF in human primary VSMCs. Moreover, the possibility to down modulate VSMC growth in condition of high glucose potentially makes our approach attractive also for diabetic patients. Finally, the lack of any major toxic effects on VSMCs confers to our approach milder effects on VSMC biology, in contrast to what observed for currently used anti-proliferative drugs. Further studies will clarify whether interfering with SRF function \textit{in vivo} may result in effective strategy to combat hyperproliferative vascular pathologies such as in-stent restenosis.
CONCLUSION

In this work we have identified siRNAs able to efficiently induce the destruction of the mRNAs of different cell cycle related genes, i.e. the transcription factors SRF and E2F1 and the cyclins E1 and E2. The anti-proliferative potential of these siRNAs have been tested in HCC-derived cell lines and in VSMCs. HCC-derived cell lines were used to simulate in vitro HCC cell growth while VSMC were considered to simulate in vitro the over-growth observed in VSMCs of coronary arteries in which in-stent restenosis develops. SRF depletion has been shown to be effective in down modulating the growth of VSMC and the most differentiated HCC cell, i.e. HepG2, where apoptosis was also induced. In the less differentiated HCC cells, JHH6 and HuH7, SRF depletion was only partially achieved probably because of an increased SRF protein stability. This in turn resulted in a poor inhibition of JHH6 and HuH7 growth. In contrast to SRF, E2F1 and cyclin E1/2 depletion was efficiently achieved in all HCC cell lines. Whereas in the most differentiated HepG2 the down modulation of cell expansion was due to both apoptosis induction and proliferation inhibition, in the less differentiated JHH6 and HuH7 only proliferation inhibition was observed. Together these data support the rationale to continue the studies for the development of future novel anti HCC and ISR approaches based on the use of siRNAs.
ACKNOWLEDGEMENTS

This PhD thesis with this dissertation has been extensive and trying, but above all exciting, instructive and fun! Without help, support and encouragement from several person, I would never have been able to finish this work!

First of all I would like to express my sincere gratitude to Prof. Gabriele Grassi, who has been my supervisor since the beginning of my study. He provided me with many helpful suggestions, important advice and constant encouragement during the course of this work. I am very privileged for having Dr. Barbara Dapas as my tutor (but especially as my friend!), whose calmness, caring and unwavering optimism often soothed me!

I sincerely thank my external supervisor Prof. Olaf Heidenreich, for taking interest in this study as well as providing valuable suggestions that improve the quality of this study.

I am grateful to Prof. Gianfranco Guarnieri, head of the Department of “Scienze Cliniche, Morfologiche e Tecnologiche” where my project has been carried out.

Many thanks to the official supervisor of my thesis, prof. Gianni Biolo, who has offered me the opportunity to attend this PhD.

I also wish to express my appreciation to Prof. Gabriele Pozzato, who made many valuable suggestions and gave constructive advice and to Dr. Bruna Scaggiante for providing useful ideas and discussions.

I also thank Dr. Laura Uxa and the laboratory of clinical analysis for allowing us to use their cytofluorimetry.

A special thank to Dr. Cristina Zennaro for her skilful technical assistance in microscopy analysis and for her moral supports.

Another special thank to Dr. Barbara Toffoli for the innumerable chats about the joy and grief of this job.

I would like to thank all people in lab. (especially Anna Maria, Mariella, Paola, Francesco) for making me feel okay about drinking so much coffee!
I thank my family: my parents Carmen and Luciano for educating me, for their unconditional support and encouragement to pursue my interests; a special thank to my brother Gabriele for rendering me the sense and the value of brotherhood and for reminding me that my research should be useful!

And finally, never enough thanks to my husband David, who has encouraged me throughout the whole course of my PhD: thank for your love and patience, thank for your silence and smiles, thank for everything, because without you, I could not live!

THANK YOU!
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