Gene Therapy with Interferon Alpha and the Angiogenic Inhibitor, Vasostatin, in Neuroendocrine Tumors of the Digestive System

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Abstract

IFN-α has been applied in medical treatment of various neuroendocrine (NE) tumors, either alone or combination with somatostatin analogues. They can improve clinical symptoms in 50-70% of patients but a significant tumor reduction is only observed in 5-15% patients. Vasostatin (vaso) is believed to be an angiogenic inhibitor. The aim of this study is to evaluate the feasibility to use IFN-α and vasostatin gene therapy in NE tumors.

We constructed plasmid vectors carrying human IFN-α2 (hIFN-α2) gene and human vasostatin gene, which were transfected into BON I cell to obtain stable gene-expressing cell lines. We found that in animal tumor model and cell experiments gene transfer of vasostatin caused a faster cell growth and tumor development via down-regulation of the tumor suppressor gene and p27. Cell adhesion, spreading, migration and invasion ability were increased in vaso-expressing BON I cells. Transflecting chicken vinculin could reverse the malignant behavior and restored expression of tumor suppressor genes. Moreover, vinculin knockdown could result in a faster cell growth and an increased colony formation.

Condition medium taken from hIFN-α2-expressing BON I cells showed significant antiproliferative effects both on the NE tumor cells, BON I and LCC18, and the endothelial cells, PAE. It also suppressed cell adhesion and cell invasion and inhibited angiogenesis on CAM assay. Mice implanted with a mixture of WT BON cells and hIFN-α2-expressing BON cells (1:1) demonstrated significantly lower tumor incidence and longer tumor doubling time. Furthermore, long-acting IFN-α2b (PEGIntron®) demonstrated a better anti-tumor effect in contrast with IFN-α2b (IntronA®). Intratumoral injection of hIFN-α2 plasmids significantly inhibited NE tumor growth and caused tumor regression.

We concluded that gene transfer of vasostatin into BON I cells might cause an enhanced malignant tumor behavior. Therefore, vasostatin therapy can not be recommended for patients with NE tumors. Vinculin might play an important role in NE tumor development and growth regulation. Gene therapy by using plasmid DNA carrying hIFN-α2 gene is feasible and promising in NE tumors.

Keywords: neuroendocrine tumor, interferon-α, vasostatin, vinculin, tumor suppressor gene, nude mice, gene therapy

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To my family
List of Papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.


II. **Liu, M. H., Öberg K. and Zhou Y.H.** Reversal of the Malignant Phenotype of Neuroendocrine Tumor Cells by Overexpression of Vinculin. (manuscript)


IV. **Liu, M. H., Öberg K. and Zhou Y.H.** Function of IFN-α gene products in neuroendocrine tumors. (Manuscript)

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Contents

Introduction ...................................................................................................11
Neuroendocrine tumors of the digestive system ..................................11
IFN-α and its application in human cancer treatment ......................11
IFN classification ..................................................................................12
Signal transduction pathway of Type I IFN ........................................12
Effects of IFN-α ..................................................................................14
Application in NE tumor .....................................................................16
Vasostatin ...............................................................................................17
Oncogenes and tumor suppressor genes ..............................................17
Cell cycle ...............................................................................................18
Role of cell adhesion in tumor growth and metastasis ....................20
Tumor metastasis .................................................................................20
Extracellular matrix ...........................................................................21
Cell adhesion and adhesion molecules ..........................................21
Tumor angiogenesis ...........................................................................23
Angiogenesis process .......................................................................23
Angiogenic switch .............................................................................23
Other mechanisms of tumor blood supply .....................................25
Cancer gene therapy ..........................................................................26
Strategies ............................................................................................26
Gene therapy vectors ..........................................................................29
Aims of present investigations .............................................................31
Materials and Methods .........................................................................33
Results and discussions .......................................................................41
Paper I ..................................................................................................41
  Effects of Vasostatin on cell growth, mobility and tumor development
  ........................................................................................................41
Paper II ................................................................................................43
  Function of vinculin on NE tumor development .............................44
Paper III ...............................................................................................46
  Effects of hIFN-α2 on cell proliferation, mobility, angiogenesis and
tumor growth ...................................................................................46
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE tumor</td>
<td>Neuroendocrine tumor</td>
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<tr>
<td>IFN-α</td>
<td>Interferon alpha</td>
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<tr>
<td>JAK</td>
<td>Janus activated kinase</td>
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<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
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<tr>
<td>ISGF3</td>
<td>Interferon-stimulated gene factor 3</td>
</tr>
<tr>
<td>ISRE</td>
<td>Interferon-stimulated response element</td>
</tr>
<tr>
<td>2,5 OAS</td>
<td>2',5'-Oligoadenylate synthetase</td>
</tr>
<tr>
<td>PKR</td>
<td>Interferon-induced protein kinase</td>
</tr>
<tr>
<td>Mx protein</td>
<td>Myxovirus resistance protein</td>
</tr>
<tr>
<td>IRF-7</td>
<td>Interferon regulatory factor 7</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double-stranded RNA</td>
</tr>
<tr>
<td>eIF-2α</td>
<td>α-subunit of eukaryotic initiation factor 2</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>CKI</td>
<td>Cyclin-dependent kinase inhibitor</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
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<tr>
<td>CLD 4</td>
<td>Claudin 4</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>PAE</td>
<td>Porcine aorta endothelial</td>
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<tr>
<td>hIFN-α2</td>
<td>Human interferon alpha2</td>
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<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
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Introduction

Neuroendocrine tumors of the digestive system

Neuroendocrine (NE) tumors, or more properly NE tumors of the digestive system or gastroenteropancreatic (GEP) neuroendocrine tumors, are rare and heterogeneous groups of neoplasms. They derive from a variety of diffuse NE cell types, which usually contain secretory granules and have a capacity to synthesize peptide hormones and biogenic amines. Most of them are slow growing neoplasms but have a malignant property. The NE tumors of the digestive system have attracted great interest because of the variable clinical signs and symptoms associated with the overproduction of hormones, tumor masses or metastases [1, 2]. The histopathological diagnosis of NE tumors includes several tumor markers. The most commonly used are chromogranin A and synaptophysin. Surgery is the only way to cure the NE tumor patients. IFN-α either alone or combination with somatostatin analogues has been applied in medical treatment of various NE tumors. They can improve clinical symptoms in 50-70% of patients but a significant tumor reduction is only observed in 5-15% patients. The other management procedures of NE tumor patients include embolization of liver metastases, chemotherapy and radiotherapy [3].

IFN-α and its application in human cancer treatment

Human IFN-α are proteins belonging to type I IFN. They include at least 13 functional subtypes, which share the same receptors and exhibit similar biological activities [4]. Although recognized as molecules with antiviral and immunomodulatory activities at the beginning, IFN-α has subsequently been found to have a variety of other biological effects including antitumor activity [4]. Today IFN-α is the most used cytokine in the treatment for a number of solid tumors, for example, melanoma, renal carcinoma, Kaposi’s sarcoma and NE tumors and hematological malignancies such as hairy cell leukemia, chronic myeloid leukemia, some B and T cell lymphomas [3, 5].
IFN classification

The IFNs were discovered more than 50 years ago originally identified on the basis of their ability to protect cells against viral infection [6]. The IFNs include two major categories: type I and type II. Type I IFNs primarily include interferon alpha (IFN-α), interferon beta (IFN-β), interferon tau (IFN-τ) and interferon omega (IFN-ω), all of which show homology in their structures. Interferon gamma (IFN-γ) is type II IFN. IFN-α and IFN-β are produced by almost all types of cells, mainly leukocytes and fibroblasts, respectively. They are induced following the exposure of viral infection, double-stranded RNAs, polypeptides and cytokines. IFN-γ is produced mostly in cells which are derived from haematopoietic stem cells, for example, T cells or natural killer cells, at stimulations by antigens or cytokines [7, 8].

Signal transduction pathway of Type I IFN

Studies on the mechanism of signals induced by type I IFNs binding to their receptor complexes have elucidated the importance of the Janus tyrosine kinase/signal transducers and activators of transcription pathway (JAK-STAT) in both type I and type II mediated signals. The classical pathway induced by type I IFN involves interaction with a homologous receptor complex termed IFNAR consisted of at least two distinct subunits: IFNAR1 and IFNAR2 which are associated with tyrosine kinase 2 (TYK2) and Janus activated kinase 1 (JAK1), respectively. The binding of type I IFNs induces the assembly of these two subunits of IFNAR leading to phosphorylation in the intracellular domain and TYK2 and JAK1 phosphorylation, which in turn regulate the activation and phosphorylation of STATs. Then the activated STATs form homodimers or heterodimers that translocate to the nucleus and initiate transcription by binding to specific sites in the promoters of IFN-stimulated genes (ISGs). An important transcriptional complex induced by type I IFNs is the ISG factor 3 (ISGF3) that is composed of the phosphorylated forms of STAT1 and STAT2, together with IRF9. These complexes translocate to the nucleus and bind IFN-stimulated response elements (ISREs) to initiate the transcription of many genes, for example, 2',5'-Oligoadenylate synthetase (2,5 OAS), IFN-inducible protein kinase (PKR), myxovirus resistance proteins (Mx proteins), and interferon regulatory factor 7 (IRF-7) [8]. Other STAT complexes induced by type I IFNs are, for example, STAT1–STAT1, STAT3–STAT3, STAT4–STAT4, STAT5–STAT5 and STAT6–STAT6 homodimers, as well as STAT1–STAT2, STAT1–STAT3, STAT1–STAT4, STAT1–STAT5, STAT2–STAT3 and STAT5–STAT6 heterodimers. Such IFN-induced complexes bind the other type of elements, known as an IFN-γ-activated site (GAS) elements to initiate certain gene transcription [9]. Figure 1 is a scheme of the classical signaling pathway induced by type I IFNs.
**Figure 1. Main features of the IFN-α/β signaling pathway.** Type I IFN transmits signals through its homologous receptor complex IFNAR, which is composed of at least two sub-components, IFNAR-1 and IFNAR-2. Ligand binding results in the formation of a signaling complex with IFNAR-1 and IFNAR-2, which leads to the activation of the receptor-associated Tyk2 and Jak1, respectively. This is followed by the tyrosine phosphorylation of STAT1 and STAT2, leading to formation of the heterotrimeric ISGF3 transcription factor, together with an IRF-family member IRF-9. In addition, a STAT1 homodimer, termed AAF, is also formed. These transcriptional-activator complexes translocate into the nucleus and activate the IFN-stimulated response element (ISRE) or IFN-γ-activated site (GAS) to initiate the transcription of many genes. Figure was adopted from Taniguchi et al., 2001 [8].

The signaling via classic JAK-STAT pathway is the most common one induced by Type I IFNs but there are some other signaling pathways. For example: Phosphorylated STAT3 by binding a conserved sequence in the cytoplasmic tail of the IFNAR1 chain of the receptor acts as an adapter to couple the phosphoinositide kinase-3 (PI3K)-dependent pathway to the IFN receptor [10]. Type I IFN can also activate mitogen-activated protein kinases p38 pathway and extracellular signal-regulated kinase (ERK) pathway which participate in the cellular functions controlled by IFNs [11].
It is believed that JAK-STAT signaling pathway initiated by IFNs is turned off within several hours of IFN treatment despite the presence of IFNs in the extracellular environment. At least three members of proteins, which act at various levels of the JAK/STAT pathway, are involved in the negative control of this signal to ensure an appropriate, controlled cellular response. Suppressors Of Cytokine Signaling (SOCS) proteins can block cytokine signaling by acting as kinase inhibitors of JAK proteins. Protein Inhibitor of Activated STAT (PIAS) interact with activated STAT and inhibit their DNA binding or possess small ubiquitin-like modifier (SUMO) ligase activity to degrade activated STATS. In addition, tyrosine-phosphatases (SHPs, CD45, PTP1B/TC-PTP) dephosphorylate activated JAK, STAT or cytokine receptors [12, 13].

Effects of IFN-α

Antiviral effects

Type I IFNs through transcriptional induction of IFN stimulated genes upregulate a wide range of biological effector molecules, some of which have direct antiviral activities [14]. The best-characterized IFN induced antiviral pathways utilize the PKR, the 2,5 OAS system and the Mx proteins. PKR is an IFN-induced, double-stranded RNA (ds-RNA) activated serine/threonine protein kinase [15]. It is normally inactive, but on binding to dsRNA it undergoes autophosphorylation and subsequent dsRNA-independent phosphorylation of substrates [16]. The antiviral effect of PKR is mainly due to its phosphorylation of the α-subunit of eukaryotic initiation factor 2 (eIF-2). This phosphorylation leads to a general block in both viral and cellular protein synthesis [17]. OAS enzymes are activated by dsRNA to synthesize 2'-5' oligoadenylates, which then bind and activate RNase L, an endoribonuclease that cleaves viral RNA and mRNA, leading to a decrease in protein synthesis and viral replication [18] Human Mx A is an IFN-induced 76 kDa cytoplasmic protein belonging to the dynamin superfamily of large GTPases which are involved in a wide range of intracellular transport processes. Mx A is able to inhibit a broad range of RNA viruses acting at different levels of the virus replication cycle, depending on the viral species and cellular models used. In addition, recent reports have shown that antiviral mechanism of Mx A may involve its direct interaction with viral nucleocapsid and trapped it in cytoplasmic inclusions, thus become unavailable for the generation of new virus particles[19].

Effects on cell proliferation and apoptosis

IFN-α has direct anti-proliferative effects. It can interact with the cell cycle components and thus affect the mitotic cell cycle phases, most commonly with a block in G1 or sometimes by lengthening of all phases, G1, S and G2.
For example, IFN-α treatment in Daudi Burkitt’s lymphoma cells can induce cell cycle arrest by down-regulating cyclin D3 expression which resulted in down-regulated expression of cyclin E, cyclin A and cdc25A and the decreased cyclin D-cdk4 and cyclin-cdk6 kinase activities [21]. It has been found that activated STAT1 up-regulates cyclin-dependent kinase inhibitors, p21 and p27, and down-regulates the activates of CDK2, CDK4 and cyclin E and consequently reduces the phosphorylated pRb delaying the cell cycle process [22, 23]. IFN-α can also induce terminal myeloma cell differentiation resulting in the loss of clonogenicity [24]. IFNs can induce PKR expression which regulates the action of several transcription factors such as NF-kB, IRF-1, p53, STAT1, NF-90 [25]. The regulation of this proteins will allow PKR to regulate several cellular functions such as cell proliferation [26, 27], differentiation [28] and apoptosis [29]. Additionally, IFN-α can induced apoptosis through several signaling pathways, for example, TNF-α related apoptosis inducing ligands (TRAIL/Apo2L), Fas/FasL, caspase-4, caspase-8 and 2,5 OAS [30].

**Immunomodulatory effects**

IFN-α can induce up-regulation of MHC I, II expression in tumor cells to enhance the ability of cytolytic and helper T cells to recognize peptide antigens of tumor cells which are presented by class I or II MHC molecules and therefore may lead to elimination of the cancer cells [31]. IFN-α can inhibit tumor growth by promoting NK cell-mediated cytotoxicity [32]. Type I IFNs directly promote the survival of both T helper cells and cytotoxic T lymphocytes (CTLs), thus induce CTLs mediated long-lasting tumor-specific immunity [33, 34]. Type I IFNs also stimulate differentiation and maturation of dendritic cell to increase its antigen presenting function [35]. Furthermore, Type I IFNs can enhance non-specific immunotherapy by inducing the production of IFN-γ from T lymphocytes and NK cells [36, 37].

**Other effects**

IFNs have the ability to inhibit angiogenesis. For example, gene transfer of IFN-α into breast cancer cells results in a long-lasting inhibition of tumor growth associated with reduced vascularization in nude mice [38]. Intraperitoneal administration of a lentiviral vector encoding IFN-α gene has a remarkable antitumor activity leading to prolongation of survival in the majority of animals. The antitumor effect is associated with an increase in ischemic tumor necrosis and a reduction in microvessel density [39]. Intra-tumoral injection of Ad-IFN-β into bladder cancer (resistant to the antiproliferative effects of IFN-β) can inhibit tumor growth and metastasis partially because of various antiangiogenic effects including endothelial cell apoptosis [40]. The mechanism of anti-angiogenesis are partly mediated by direct anti-migration and anti-proliferation effects on endothelial cells [38], by down-regulating the expression of VEGF [41], b-FGF expression [42] and/
or metalloproteinase-9 (MMP-9) [43]. In addition, IFN-α can affect cell adhesion and invasion thus affect tumor metastasis. For instance, systemic therapy with IFN-α increases expression of E-cadherin which reduces tumor volume and inhibits metastasis of bladder carcinoma [44]. Experiments also show that IFN-α can inhibit invasion and metastasis of human liver cancer cells by inhibiting MMP-2 and MMP-9 activities [45].

**Application in NE tumor**

IFN-α was introduced by Oberg et al. for the biological treatment of patients with carcinoid tumors in 1982 [46]. IFN-α either alone or in combination with somatostatin analogs can improve clinical symptoms in 50-70% of patients and reduce the circulating hormone levels in 40-60% of patients. A significant tumor reduction is observed in 5-15% patients. However some side effects such as flu-like symptoms, chronic fatigue, depression and autoimmune reactions might occur in patients treated by IFN-α [1, 3].

IFNs are multifunctional regulatory cytokines. The anti-tumor effects of IFN-α in NE tumor patients are mediated partly by transducing regulatory signals through the JAK-STAT pathway. In our earlier studies, the STAT1 and STAT2 immunostaining were significantly increased in the NE patients during IFN-α treatment. An increased STATs score correlated with those patients that presented objective response or stable disease but not in those with progressive disease. In a NE tumor cell line, BON I cell line, IFN-α dose-dependently increased both STAT1 and STAT2 expression and their phosphorylation in vitro [47]. Increased PKR, IRF-1, IRF-2, MxA and 2,5 OAS protein or mRNA expressions were also observed in NE tumor patients and (or) NE cell lines after IFN-α treatment [23, 48, 49]. IFN-α directly inhibited BON I cell proliferation and colony formation. It blocked cell cycle in the G1/S phase through up-regulation of the activities of cyclin-dependent kinase inhibitors p21 and p27 and down-regulated the activites of CDK2, CDK4 and cycling E [23]. In addition, IFN-α also exerts an immunomodulatory effect in NE tumor patients. For example, patients with classical midgut carcinoids display low natural killer cell activity, which can be stimulated by IFN-α treatment [3] and the expression of MHC I in mid-gut carcinoid patients can be induced by IFN-α treatment [50]. Furthermore, recent studies showed that IFN-α inhibited transcription of the VEGF gene in NE tumors cells and decreased expression of VEGF and angiogenesis in NE mouse tumor model [41, 51].
Vasostatin

Vasostatin, a fragment of calreticulin including amino acids 1–180, has been shown to be an angiogenesis inhibitor [52-54]. In vitro, recombinant vasostatin can selectively inhibit proliferation of endothelial cells, fetal bovine heart endothelial cells (FBHE) and HUVEC endothelial cells and have a minimal effect on other cell lines, for example, human peripheral blood mononuclear cells, lung carcinoma cells and breast adenocarcinoma cells. In vivo, vasostatin significantly inhibited angiogenesis and reduced growth of human Burkitt lymphoma and human colon carcinoma in mouse tumor models [52]. Vasostatin can specifically inhibit endothelial cell attachment to the extracellular matrix component, laminin, thus subsequently reduce endothelial cell growth induced by basic fibroblast growth factor [55]. Conditioned medium from COS cells transfected by plasmid DNA encoding vasostatin gene is able to inhibit proliferation of human umbilical vein endothelial cells (HUVEC) and mouse endothelial cells (SVEC4-10). Intramuscular administration of vasostatin-plasmid DNA can result in the inhibition of tumor angiogenesis, tumor growth and prolong the survival of Meth A fibrosarcoma and LL/2c Lewis lung cancer tumor-bearing mice [53]. A combination of vasostatin and interleukin-12 (IL-12) can inhibit growth of human Burkitt lymphoma, colon carcinoma, and ovarian carcinoma [54].

Oncogenes and tumor suppressor genes

Cancer is a multi-step process that needs accumulation of numerous genetic mutations in a single cell. Two groups of genes, oncogenes and tumor suppressor genes have been discovered in recent years. These genes play an important role in the control of cell proliferation and the development of malignant phenotype.

*An oncogene* is a gene that normally directs cell growth. When mutated or expressed at abnormally high levels it contributes to the transformation of a normal cell into a cancer cell. Such oncogenes are k-ras, bcl-2, c-Myc and cyclin D1 [56-59].

*A tumor suppressor gene* is a protective gene that encodes a protein involved in controlling cell proliferation, differentiation and apoptosis. A mutation or deletion of such a gene causes to deregulation of cellular growth and maybe lead to cancer [60, 61]. P53 and Rb gene are the most studied genes in this group.

p53 gene encodes Nuclear phosphoprotein p53. It is an efficient inhibitor of cell growth. p53 plays important role in cell cycle checkpoint control. It
induces cell cycle arrest after DNA damage by inducing p21 activity [62, 63]. It also induce apoptosis by activating genes, such as Bax and Fas [64]. A mutant or absent p53 gene occurs in almost 50% of all tumors [65], but rarely mutated in NE tumors [66, 67]. Transfection of wild-type p53 gene into cancer cells with p53 mutations can inhibit cell growth and induces apoptosis [68-71].

The retinoblastoma (Rb) gene family has three members, Rb/p105, p107, and pRb2/p130. Their gene products, pRb, p130, and p107 are structurally and biochemically similar proteins and found mutated in a wide range of human tumors [72]. The active form of Rb protein inhibits the transition of cell into the S phase and serves as a transducer between the cell cycle machinery and promoter-specific transcription factors [73-75].

The nm23 gene, also known as nucleoside diphosphate kinase, NDPK, is regarded as a metastasis suppressor which has been identified in many human cancers [76]. In human breast cancer, several studies have demonstrated a significant inverse relation between the nm23 expression and aggressive tumor behavior [77]. The transfection of nm23 gene into gliomas cells reduces cell migration and the invasiveness ability [78]. In addition, the nm23-H1-transfected human prostate carcinoma lines demonstrates decreased colonization in soft agar and adhesion to extracellular matrix [79].

Cell cycle

Cancer is frequently considered to be a cell cycle disease [80]. Deregulation of cell cycle control is one of the hallmarks of tumorigenesis. Therefore, understanding the molecular mechanisms of the deregulation of cell cycle progression in cancer is quite helpful for the design of cancer treatment strategies.

Cell cycle is a series of events in a eukaryotic cell that drive cell growth and division. It is typically divided into four sequential phases: G1 (Gap1), S (DNA synthesis), G2 (Gap2) and M (mitosis) phase. The most two dramatic phases are S phase, during which DNA replication occurs and M phase in which the replicated genome DNA is divided into two similar daughter cells. S and M phase are separated by two gaps: G1, preparation for S phase, and G2, preparation for M phase. Non-replicating cell resides in the quiescent state, called G0 phase. It may re-enter cell cycle by several growth stimuli [81].

Cell cycle progression is precisely regulated by members of the cyclin-dependent kinase (CDK) family of serine/threonine kinases in a timely man-
ner. After binding to specific regulatory proteins, cyclins, they form active complexes driving the cell forward through the cell cycle [82]. The most well known substrate of CDK4/6-cyclin D complex is the stimulated production of the phosphorylated retinoblastoma tumor suppressor gene (pRb). In the mid-G1 phase, pRb is phosphorylated by CDK4/6-cyclin D complex partly, after which CDK2-cyclin E become active and continually activates pRb. Rb phosphorylation causes the release of various transcription factors, E2F family members, enabling the transcription of numerous of genes for DNA synthesis [75]. The progression through cell cycle is regulated by different CDK/cyclin complex shown in figure 2.

![Figure 2. The stages of the cell cycle.](image)

CDK activity is negatively regulated by interaction with CDK inhibitors, the INK4 (p15, p16, p18, p19) and CIP/KIP families (p21, p27, p57) [83], as shown in table 1.

<table>
<thead>
<tr>
<th>CKI family</th>
<th>Function</th>
<th>Family members</th>
<th>p15</th>
<th>p16</th>
<th>p18</th>
<th>p19</th>
<th>p21</th>
<th>p27</th>
<th>p57</th>
</tr>
</thead>
<tbody>
<tr>
<td>INK4 family</td>
<td>Inactivation of G1 CDK (CDK4, CDK6)</td>
<td>(INK4b)</td>
<td>(INK4a)</td>
<td>(INK4c)</td>
<td>(INK4d)</td>
<td>(Waf1, Cip1)</td>
<td>(Cip2)</td>
<td>(Kip2)</td>
<td></td>
</tr>
<tr>
<td>Cip/Kip</td>
<td>Inactivation of G1 cyclin-CDK complexes and cyclin B-CDK1</td>
<td>(Cip2)</td>
<td>(Cip2)</td>
<td>(Cip2)</td>
<td>(Cip2)</td>
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</table>

Table 1. Cyclin dependent kinases inhibitors (CKI) bind to CDK alone or to the CDK-cyclin complex and regulate CDK activity. p19 (ARF) is also encoded by the INK4 locus, but has no known CKI activity. Table adopted from Katrien et al., 2003 [83]
Cell cycle regulation is also monitored at several positions known as checkpoints that assess DNA integrity, cell size and growth factors. Two of the major cell cycle checkpoints are G1 checkpoint (R point) and G2 checkpoint [84]. At the R point, cell cycle arrest induced by DNA damage is p53-dependent. DNA damage can rapidly induce the activity of p53 which stimulates the transcription of different genes. The key target of p53 is p21, the inhibitor of CDKs. This not only leads to the inability to initiate DNA synthesis, but also preserves the phosphorylation of Rb, thereby causing a sustained G1 blockade [62]. At G2 checkpoint, when DNA damage occurs cells are able to initiate a cell cycle arrest by the p53 pathway which induces an up-regulation of cell cycle inhibitors such as p21, GADD45, 14-3-3 sigma proteins [63] and/or by Cdc25 pathway [85].

Dysregulation of cell cycle activators, inhibitors and checkpoint proteins contributes to the tumor development. For example, pRb, p16 and CDK/cyclin D are functionally interrelated and alterations of at least one of these regulators are found in approximately 90% of human cancers [86]. Increased activity of CDK4 is associated with low p16 expression, which may be involved in the development and progression of epithelial ovarian carcinomas [87]. Goblet cell carcinoids of the appendix have shown a dysregulation of the cell cycle with up-regulation of cyclin D1 and down-regulation of p16 [88]. Overexpression of cyclin E has been found in human breast cancers associated with tumor aggression and mortality [89, 90]. Decreased expression of p27 is frequently found in various human cancers and it is a strong indicator of poor prognosis [91]. Mutations of the p53 gene have been seen in bladder cancer and are correlated with tumor grade and contribute to the malignant progression of the cancer [92].

Role of cell adhesion in tumor growth and metastasis

Tumor metastasis

The most deadly aspect of cancer is its metastatic ability. To begin the process of metastasis, a malignant cell must break away not only from the attachment of cell to extracellular matrix, but also from cells around it, which is determined by cell-cell junctions. After detaching from the primary tumor, cancer cells will breach basement membrane by releasing proteolytic enzymes then penetrate it and invade into blood or lymphatic vessels. Cancer cells may lose anchorage-dependent growth control and escape from the destruction by immune system, which makes it possible to survive in the circulation. Finally the surviving cells arrest and reattach themselves in a target organ, invade into vessel walls, migrate into surrounding tissue and develop into a metastatic tumor at the new site [93].
Extracellular matrix

Extracellular matrix (ECM) is a complex structure surrounding cells that can be different in the normal tissue and tumor tissue. Increased ECM proteins, for example, collagen I, fibronectin, vitronectin and tenascin-C can be found in various tumors [94-98]. Loss of basement membrane proteins, such as collagen IV and laminin, has also been observed in tumor progression [99, 100].

Cell adhesion and adhesion molecules

Cell adhesion to ECM and to neighboring cells are critical for multiple biological functions such as formation and maintenance of tissue structure, cell growth, differentiation, migration, invasion and tumor metastasis [101-103]. Cell adhesion comprise several types, for example, focal adhesions, adherens junctions and tight junctions [104, 105].

Cell contact with ECM is called focal contacts or focal adhesions, where integrin clusters connect the ECM proteins to F-actin via actin-binding proteins such as α-actinin, vinculin, talin and paxillin [106, 107]. Integrins represent a large family of heterodimeric transmembrane receptors that associate as α and β subunits. Changes in the expression patterns of integrins have been observed in some human tumors [108]. Focal adhesion kinase (FAK) has recently been described which play a key role of the signal transduction pathways triggered by integrins [109]. Activation of FAK can lead to several signaling pathways that regulate cell proliferation, survival, migration, and invasion [110]. FAK can regulate cell cycle progression by increasing cyclin D1 expression and/or decreasing the expression of the CDK inhibitor p21 [109]. Over expression of FAK augment cell migration [111] and in human breast and colon tissues expression of FAK is correlated with invasive phenotypes [112].

Cell to cell junctions or adherens junctions are mediated by the cadherin superfamily which are transmembrane proteins. They share an extracellular domain consisting of multiple repeats of a cadherin-specific motif [113]. E-cadherin is a representative member of this family, which is responsible for cell-cell interactions and plays an important role in generation and maintenance of epithelial polarity [114]. The cytoplasmic tail of E-cadherin associates with several proteins termed catenins (α-, β-, γ-catenin and p120ctn) which link the cadherin to actin cytoskeleton [115]. In recent years, several studies have revealed that loss of E-cadherin function is often found during development of some human cancers [116-118]. In addition, down-regulation of downstream effector elements, such as α-catenin, vinculin, α-actinin, talin, and FAK in the cadherin or integrin junction cascade may also
disrupt cell-cell or cell-ECM interactions and thereby promote invasion [119].

Tight junctions, another cell-cell adherens structure, form the apical junctional complex of adjacent epithelial cells. It is responsible for sealing cellular sheets, thus controlling paracellular ion flux and maintaining tissue homeostasis [120]. Tight junctions are composed of three major membrane proteins, occludin, claudins, and junctional adhesion molecules. The transmembrane proteins, the claudin family, are comprised of 23 closely related members with tissue specific expression pattern [121]. Studies have shown that occludin is often down-regulated in gastrointestinal tumors [122] and altered expression of claudins have been found in several cancers. For example, claudin-1 has been found to be down-regulated in breast cancer [123] and reduced expression of claudin-7 has been found in head and neck cancer [124]. Claudin 4 are frequently overexpressed in several neoplasias, including ovarian, breast, pancreatic, and prostate cancers [121] and it maybe related to cancer invasion and metastasis. One study has shown that overexpression of claudin-4 in SUIT-2 pancreatic cancer cells inhibits colony formation in soft agar assays and reduces invasive potential of cancer cells in vitro and metastatic formation in vivo [120].

Vinculin localizes at the cytoplasmic face of both focal adhesions and adherens junctions [125]. Focal adhesion complexes are formed by actin filaments binding α-actinin, which in turn can bind to integrins either directly or via vinculin and talin [126]. In adherens junctions, actin filaments might be linked to α-catenin via α-actinin or via vinculin; or actin filaments might directly bind to α-catenin. The α/β catenin complex then binds to the transmembrane cadherins [127]. Down-regulation of vinculin and some other actin-anchoring protein has been seen in invasive breast carcinoma [119]. Early studies have shown that cells lacking vinculin are highly metastatic and motile [128]. Transfection with an antisense vinculin cDNA construct, 3T3 cells acquire increased motility and produce larger colonies in soft agar [129]. In some tumor cells, overexpression of vinculin can result in a decrease of tumorigenic and metastatic potential [130]. In addition, it is reported that apoptotic cells overexpress vinculin [131] and vinculin is regarded as a tumor suppressor [130].

The α-catenin, one of actin anchor proteins, has a similar structure as vinculin. It is also regarded as a tumor suppressor [130]. The anti-tumor function of α-catenin is associated with its function in the formation of cell-cell adherens junctions where α-catenin connects the E-cadherin and β-catenin complex to actin filaments. In gallbladder carcinomas decreased expression of α and β-catenin is observed [132]. Restoration of inducible expression of the wild-type α-catenin protein in cells with mutant form of the α-catenin
protein retard their growth in vitro and attenuate the ability of these cells to form tumors in nude mice [133].

Tumor angiogenesis

Angiogenesis, the formation of new capillary blood vessels from the pre-existing vasculature, is crucial for the growth of primary solid tumors and also their metastases. Increasing evidences suggest that solid tumor growth is seldom beyond 2 or 3 mm³ without new blood supply. In order to provide essential oxygen and nutrients for the demand of continuously growing solid tumors, angiogenesis is required [134]. In addition, in order to form metastases, cancer cells must have chance to invade into circulation and therefore need neovascularization of the primary tumors [135].

Angiogenesis process

Angiogenesis is a complex and multi-step process which involves degradation of the basement membrane by proteinases, migration, invasion and proliferation of endothelial cells towards the angiogenic stimulators. Endothelial cells then adhere to each other to form a lumen of a new capillary tube. Finally, the sprouting tubes fuse into loops and the blood circulates in the newly vascularized region [136].

Angiogenic switch

A net balance between pro- and anti-angiogenic molecules in the microenvironment determines every step of angiogenesis. When this balance is broken and is in the favor of angiogenic stimulators, the angiogenesis starts. Many factors can trigger angiogenic switch from off to on, for example, hypoxia, high pressure generated by tumor cell proliferation, immune response caused by infiltration of inflammatory cells in the tissues and genetic mutations related to angiogenesis regulators [137]. The most important growth factors driving angiogenesis are vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) [138]. The way of angiogenesis triggered by hypoxia is shown in figure 3.
Figure 3. Increased tumor growth results in hypoxia which promotes the secretion of VEGF. VEGF induces endothelial cell proliferation, survival and induces increased blood vessel permeability which further allows the creation of an extravascular fibrin gel that supports endothelial cell growth [139-141]. The newly formed blood vessels will support continuing tumor growth. (Figure adopted from Claudio et al., 2002 [142])

VEGF are highly expressed in most malignant tumors. VEGF-VEGF receptor complexes (VEGFA-FLT1 and VEGFA-KDR) play a critical role in tumor angiogenesis [143]. Experiments have shown that transfection of VEGF121cDNA into human colon cancer cell line enhances angiogenesis, tumor growth and metastasis [144]. Conditioned medium from head and neck squamous cell carcinoma (HNSCC) treated by VEGF antisense results in decrease of endothelial cell proliferation and migration. In vivo, after treatment with VEGF antisense oligonucleotide, HNSCC has a reduction of VEGF protein expression and a significant growth suppression [145].

bFGF and their receptors are overexpressed in various types of cancers. bFGF is a survival factor of endothelial cells [146]. It stimulates endothelial cells migration and stimulates endothelial cells to produce plasminogens activators (PA) and collagenase that can degrade basement membrane [147]. Adenoviral-mediated antisense bFGF gene therapy to a human bladder cancer model in nude mice enhances endothelial cell apoptosis, reduces microvessel density and effectively inhibits tumor growth [148].

Introduction of angiogenesis inhibitors can decrease vascular number and inhibit tumor growth and metastasis. For example, adeno-associated viral-mediated stable expression of angiostatin inhibits melanoma and Lewis lung carcinoma tumor growth and prolongs survival of tumor-bearing mice [149]. Intramuscular administration of a plasmid DNA encoding vasostatin can reduce microvessel formation and tumor growth [53]. Adenovirus-mediated gene transfer of endostatin results in the inhibition of breast carcinoma
growth and metastases accompanied by decreased number of blood vessels[150].

A list of molecules secreted by endothelial cells, tumor cells and by the surrounding stroma which are involved in angiogenesis. (Table 2 and Table 3). Tables were adopted from Tiziana et al., 2003 [151]

Table 2. Known angiogenic factors

| 1-Butyryl glycerol          | Interleukin 8 (IL-8) |
| Acyl fibroblast growth factor | laminin         |
| Adenosine                   | Leptin            |
| Angiogenin                  | Midkine           |
| Angiopoietin-1 (Ang1)       | Nicotinamide      |
| Collagen                    | Perlecan          |
| Del-1                       | Phospholipids (SPP, LPA) |
| Ectactin                    | Placental growth factor |
| Epidermal growth factor     | Platelet-derived endothelial growth factor (PDGF) |
| Ephrins                     | Pleiotropin       |
| Fibroblast growth factor: acid (aFGF) and basic (bFGF) | Proliferin       |
| Fibronectin                 | Prostaglandins E1 and E2 |
| Follistatin                 | Scatter factor (SF) |
| Granulocyte colony-stimulating factor (G-CSF) | Transforming growth factor-alpha (TGF-a) and -beta (TGF-b) |
| Heparin/heparan sulfate     | Tumor necrosis factor-alpha (TNF-a) |
| Hepatocyte growth factor (HGF) | Vascular endothelial growth factor (VEGF) |

Table 3 Known antiangiogenic factors

| 2-Methoxy-estradiol          | Maspin          |
| 1,25-Dihydroxyvitamin D3     | Metalloproteinase inhibitor (TIMPs) |
| ADAMTS-1                    | METH-1          |
| Angiopoietin-2              | PEDF            |
| Angiotatin                  | Pex             |
| Antiangiogenic antithrombin III | Pigment-epithelium-derived factor |
| Calreticulin                | Placental ribonuclease inhibitor |
| Canstatin                   | Plasminogen fragment Kringle 5 |
| Cartilage-derived inhibitor (CDI) | Platelet factor 4 (PF4) |
| CD59 complement fragment    | Prolactin 16 kDa fragment |
| Decorin                     | Proliferin-related protein (PRP) |
| Endostatin                  | Retinoids       |
| Fibronectin fragment        | Soluble VEGF receptor |
| Gro-β                       | Tetrahydrocortisol-5 |
| Heparinases                 | Thrombospordin-1 (TSP-1) and -2 (TSP-2) |
| Heparin hexasaccharide fragment | Tissue inhibitor of metalloproteinases-1 (TIMP-1) |
| Human chorionic gonadotropin (hCG) | Tissue inhibitor of metalloproteinases-2 (TIMP-2) |
| Interferons α, β, γ         | Tissue inhibitor of metalloproteinases-3 (TIMP-3) |
| Interferon-inducible protein (IP-10) | Vascular endothelial growth inhibitors (VEGI) |
| Interleukin-1 (IL-1), -4 (IL-4), -12 (IL-12) | Vasculostatin |
| Ligands of PPARγ             | Vasostatin      |

Other mechanisms of tumor blood supply

In addition, angiogenesis is not the only way of blood supply for tumor growth. Depending on tumor type, stage of the tumor development and loca-
tion it may be via different mechanisms. Vessel co-option, use of pre-existing vessels, is described in brain tumor models [152, 153]. Circulating endothelial progenitor cells (EPCs) from bone marrow in the adult species have been shown incorporated in formation of new blood vessels (vasculogenesis), particularly at tumor periphery [154]. The term, vasculogenic mimicry, describe the generation of microvascular channels by genetically deregulated, aggressive tumor cells without participation by endothelial cells. This structure is observed in some tumors, for example uveal melanomas, inflammatory breast cancer and prostatic carcinoma. The blood vessels are involved in the microcirculation of the tumor and serve to feed the tumors [155, 156].

Cancer gene therapy

Gene therapy is a method to use genetic material such as DNA or RNA to prevent or cure a variety of diseases, both infectious and genetically based diseases.

The first clinical trial of gene therapy was approval in 1990 in children with adenosine deaminase deficiency, a severe combined immunodeficiency syndrome [157]. Until 2005, there are 1020 gene therapy studies in clinical trials and 675 of them are cancer related [158]. Cancer gene therapy is becoming most important application of this strategy and also offers a new opportunity to treat this fatal disease.

Cancer gene therapy has several advantages: 1) Low, continuous expressions of gene products have a potential to overcome limitation of certain drug application caused by half-life or systemic toxicity; 2) Production of functional proteins can be quite expensive so the availability of recombinant proteins may become scarce; 3) Gene therapy offers the opportunity for patients to become his/ her own source of production, release from repeated injection; 4) It can be combined with radiation therapy, chemotherapy or other therapies.

Strategies

Cancer is known as a genetic disease. Mutations of oncogenes and tumor suppressor genes cause the formation of a tumor cell population. Unusual expression of growth factors can further increase the tumor cell mass and induce angiogenesis. The accumulation of new gene mutations and modifications leads to more and more heterogeneous phenotype of cancer cells. Due to the uniqueness of each tumor, gene therapy includes many strategies.
The current cancer gene therapy strategies include: oncogene inactivation, tumor suppressor gene replacement, transfection of suicide genes, immunomodulation and inhibition of angiogenesis.

1) Oncogene inactivation
The biological activities of oncogenes can be modulated at DNA, RNA or protein level.

Antigene oligonucleotides, short single-strand DNA sequences, which are designed to bind to specific oncogene promoter regions can form triple helix structures to prevent oncogene translation. For example, (99m)technetium-conjugated bcl-2 antigen is found to form a stable triplex and to inhibit bcl-2 gene transcription in vitro [159]. Bis-peptide nucleic acids specifically targeting the HER-2/neu oncogene promoter prevents HER-2/neu transcription [160].

Antisense oligonucleotides that bind in a complementary manner to mRNA form the double-stranded RNA complexes. Those complexes can be destroyed by intracellular enzymes. So it reduces oncogene expression at mRNA level. C-Myc antisense oligonucleotides treatment reduces gene expression and growth rate of melanoma cell lines [161].

Transportation of the oncoprotein to its target can be blocked by intracellular antibodies directed against the oncoprotein. Intrabodies against cyclin E protein inhibits the growth of a breast cancer cell line [162].

2) Tumor suppressor gene replacement
Mutant tumor suppressor gene can be replaced by transfection of wild type tumor suppression genes back into cancer cells. That has been effectively performed by using p53, Rb and p16 gene replacement [71, 163, 164].

3) Suicide gene therapy
Cancer cells are transfected by genes encoding an enzyme that converts a systemically delivered nontoxic prodrug into a toxic agent in situ. The toxin should kill the cancer cells expressing the genes as well as the surrounding cells not expressing that gene (bystander effect). One of the most investigated suicide gene-prodrug system is the herpes simplex virus thymidine kinase (HSV-tk)/ganciclovir (GCV) system [165].

4) Immunomodulation
This strategy can be achieved by different approaches. One is the transfer of immune stimulating genes, for example, intratumoral injection of inter-
leukin-12 cDNA which can reduce tumor growth or regression mediated by augmentation of tumor-specific cytotoxic T cells, natural killer cells and enhancement of IFN-γ production [166, 167]. The other one is gene vaccine: a. In vitro modification of antigen presenting cells, dendritic cells, with tumor antigen or transfection with an expression vector containing tumor antigen gene that can boost their antigen presentation function, thus promoting tumor specific immune responses [168, 169]; b. Gene vaccine can be achieved through injecting antigen-coding vectors by intradermal or intramuscular routes directly. After antigen-coding vectors enter local cells, they will produce and secrete antigens. Then antigens are captured by antigen presenting cells and stimulate desired immuno-responses [170].

5) Inhibition of angiogenesis

Angiogenesis is a complex process regulated by multiple positive and negative angiogenic factors secreted by tumor cells, endothelial cells and surrounding stromal cells. Although human cancers are heterogenous, most of solid tumor growth and metastasis require neovascularization [134, 135]. Therefore, targeting tumor angiogenesis may form an effective anticancer approach.

Gene therapy-mediated anti-angiogenesis therapy has several advantages: 1) It has potential for sustained expression of angiogenesis inhibitors. 2) Endothelial cells are rapidly proliferative but they are much more genetically stable than tumor cells and as a result less likely to accumulate mutations that lead to early drug resistance [171]. 3) Angiogenesis inhibitors seem to preferentially target vessel endothelial cells in tumors rather than in normal tissues. This may be because a. endothelial cells proliferate more rapidly in tumors than in normal tissues; b. tumor endothelial cells show higher expression of certain surface markers than normal endothelial cells; c. tumor vasculature is chaotic with interrupted basement membrane which is seen across most human tumors [172, 173].

Many strategies of anti-angiogenesis gene therapy are aimed to destroy the endothelial cells, to suppress angiogenic activators and to enhance angiogenic inhibitors.

1) Inhibition of VEGF activities. Intratumoral injection of human gliomas in nude mice with a recombinant adenovirus encoding antisense VEGF cDNA inhibited tumor growth [174]. Intravenous administration of a recombinant adenovirus carrying the soluble FLT-1 gene reduces tumor burden in mice with preexisting lung or liver metastases [175];
2) Endogenous inhibitors. Experiments have shown that transfection of en-
dostatin, angiostatin and vasostatin by viral and non-viral vectors have a
sustained gene expression and tumor growth inhibition [53, 149, 150].

3) Tissue inhibitors of metalloproteinases (TIMPs). MMPs play a critical
role in ECM and basement membrane degradation that is the first step of
angiogenesis [176]. Its function is inhibited by TIMPs. It has been shown
that adeno-associated viruses mediated delivery of TIMP-1 gene inhibits
tumor growth in a murine tumor model [177].

4) IFN (α,β). IFNs have multiple anti-tumor effects, including inhibition of
angiogenesis. Intraperitoneal administration of a lentiviral vector encoding
murine IFN-α gene has a remarkable antitumor activity, leading to prolonga-
tion of survival in the majority of animals and antitumor effects are associ-
ated with an increase in ischemic tumor necrosis and a reduction in mi-
crovessel density [39]. Intratumoral injection of Ad-IFN-β into bladder can-
cer (resistant to the antiproliferative effects of IFN-β) can inhibit tumor
growth and metastasis partially because of various antiangiogenic effects
including apoptosis of endothelial cell [40].

Gene therapy vectors

One of the main challenges of cancer gene therapy is development of spe-
cific, safe and efficient gene carries to deliver the genetic materials to remote
tumors and metastases. Gene therapy vectors consist of two main classes,
viral based and non-viral based.

Viral based vectors, such as retroviruses, adenoviruses, adeno-associated
viruses, lentiviruses and herpes simplex virus used in cancer gene therapy
are modified in the laboratory to eliminate their pathogenicities and to keep
their high transfection efficiency [178]. However, there still remain some
shortages, for example: safety concerns caused by viral immunogenicity
[179-181] or disruptions of the expression of a tumor suppressor gene or
activation of oncogenes leaded by ectopic chromosomal integration of viral
DNA [182], limitation of the size of inserted genetic materials and the diffi-
culty to production of the viruses [183], which may limit their application in
clinical situations.

The alternative choice of viral vector system is non-viral vector based gene
transfer including the use of naked DNA, cationic lipid-DNA complexes and
cationic polymer-DNA complexes. They are maybe safe, but the efficiency
of gene transfer may be lower than using viral vectors.
The use of naked plasmid DNA represents the simplest possible delivery system. Although the systemic administration of naked DNA is in general much less efficient because of the rapid degradation by serum nucleases, direct injection of naked DNA into certain tissues, particularly muscle where low levels of nuclease activity exist [184, 185], has been shown to produce surprisingly high levels of gene expression leading to inhibition of tumor growth [53, 186]. Moreover several positive results have been obtained by intratumoral administration of naked plasmid DNA encoding tumor suppressor genes or cytokines [187-189]. This method is capable of not only providing persistent production and achieving high local concentration of the gene products within microenvironment but also having minimal side effects. In addition, the non-viral gene delivery system may be applied by using physical devices such as gene guns [190] and electrical pulses [191].

Cationic lipid and cationic polymers interact with the negatively charged DNA through electrostatic interactions. Moreover, the total charge maintains a positive net value. This will allow the carriers to interact with the negatively charged cell membrane and penetrate into cells, mainly through the endocytosis [192]. The formed lipoplexes and polyplexes can protect DNA from degradation by nucleases and also help them to enter into cells. This may improve the transfection efficiency of non-viral vectors. Cationic liposome-mediated intravenous DNA delivery can result in enhanced expression of the chloramphenicol acetyltransferase gene in most tissues examined [193]. Systemic administration of liposome-plasmid complexes coding angiostatin gene can suppress the growth of melanoma tumors in mice [194]. The combination of intratumoral administration of bax cDNA plasmid complexed with a cationic lipopolyamine and anticancer drugs, 5-fluorouracil (5-FU) and cisplatin (CDDP), enhances their therapeutic efficacy in gastric cancer [195]. Intratumoral injection of the complexes of VEGFsi plasmid and polyethylenimine significantly inhibits Ewing’s sarcoma tumor growth in mice and prolongs the survival time [196]. The lipid and polymer vectors can also be linked with targeting ligand which can result in an enhanced targeted gene delivery [197, 198].
Aims of present investigations

To explore the feasibility to use the angiogenic inhibitor, vasostatin in NE tumor gene therapy.

To explore the role of vinculin in NE tumor development.

To evaluate the bioactivity of hIFN-α2 secreted from hIFN-α2-expressing plasmids.

To evaluate possible anti-tumor effect of different ratio of transfection with hIFN-α2-expressing plasmids.

To explore the possibility to use hIFN-α2-plasmids in NE tumor treatment.

To explore the anti-tumor efficiency of Pegylated IFN-α on NE tumor cells.

To understand intracellular signaling pathways induced by IFN-α.
Materials and Methods

Cell culture (paper I-IV)
BON I cell line is derived from a human pancreatic carcinoid tumor [199] and LCC18 is from a human NE colonic tumor [200]. Cells were cultured as described earlier [48]. Porcine aorta endothelial (PAE) cell line was kindly provided by Prof. Lena Claesson-Welsh (Rudbeck Laboratory, Uppsala University, Sweden). Mouse lymphoid endothelial cell line, SVEC4, Burkitt lymphoma cell line, CA46, and colorectal adenocarcinoma cell line, SW480, were purchased from ATCC (LGC Nordic AB, Boras, Sweden). Cells were cultured in complete media according to the information of manufacturers.

Antibodies and reagents (paper I-IV)
Monoclonal anti-c-Myc (9E10), anti-Stat1 (C-136), anti-Stat2 (A-7), anti-p27, anti-p53 (DO-1), anti-Rb (IF8), anti-vinculin (H-10), anti-β catenin (G-11), anti-GFP (B2) and anti-Fibronectin (EP-5) as well as polyclonal anti-GAPDH, anti-CD31 (sc-1506), anti-α-catenin (H-297), Goat anti-calreticulin antibodies, anti-FAK (C-20), anti-Vitronectin (H270) and anti-Mx A (D-14) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Monoclonal anti-PKR antibody was purchased from Transduction Laboratories (Intermedica, Stockholm, Sweden). Monoclonal anti-β-tubulin antibody was purchased from Upstate Biotechnology (Lake Placia, NY, U.S.A.). Monoclonal anti-calreticulin (SPA-601) was obtained from BioSite (BioSite, Täby, Sweden). Monoclonal anti-nm23(Ab-1) was bought from Oncogene Science Corp. (Cambridge, Mass., USA). Monoclonal anti-Claudin 4 antibody was bought from Zymed Laboratories Inc. (Invitrogen, Sweden). Monoclonal anti-human OAS2 antibody was bought from R&D Systems Europe Ltd. (Abingdon, United Kingdom). Rabbit anti-phospho-Stat1 (Tyr701) was purchased from Cell Signaling Technology, Inc. (Danvers, MA 01923, U.S.A.). Human recombinant IFN-α2b (3.0 million IU per milliliter) and Peg IFN-α (PEGIntron, 100 μg per milliliter) were bought from Schering-Plough Corp. (New Jersey, U.S.A.). Anti-mouse and anti-rabbit horseradish peroxidase-linked secondary antibodies were purchased from Amersham (Amersham, U.K.). MBP-vasostatin (MBP-vaso) was a generous gift from Dr. Giovanna Tosat (Experimental Transplantation and Immunology Branch, Center for Cancer Research, NCI, NIH, Bethesda, Md., USA). All other reagents were from Sigma (Sigma-Aldrich Sweden AB, Stockholm, Sweden) unless otherwise indicated.
Plasmid DNA constructs and transfection (paper I-III) and vinculin siRNA transfection (paper II)

hIFN-α2 (human interferon alpha 2 gene) including all open reading frames was amplified with PCR using a set of primers, 5’-GAAGCTTGATGGCCCTTGACCTTTGCTTTACTGG-3’ and 5’-AGAGCTCGCTCTTCTTTAACTTCTTGCG-3’ and cDNA of normal human leukocytes as the template. The amplified 582 bp Hind III-hIFN-α2-Xho I (underlined sequence for enzyme cutting sizes) fragment was cloned into the pcDNA3.1 vector (Invitrogen), later subcloned into pSec-Tag2A (Invitrogen) at the Hind III-Xho I sites.

Human vasostatin was amplified with PCR using human placenta cDNA library (Stratagen) as the template and a set of primers, 5’-GAAGCTTGATGGCTGCTATCCGTGCCG-3’ and 5’-GAGCTCGGGTTGTCTGCGCCAATCAG-3’. The amplified 558 bp Hind III-Vaso-Xho I (underlined sequence for enzyme cutting sizes) fragment was cloned into the pcDNA3.1 vector (Invitrogen). The vasostatin DNA fragment was then subcloned into pSecTag2A at the Hind III-Xho I sites and was named pSecTag2A-vaso. The expression vector without coding regions served as empty control (vector). Plasmids were extracted using Qiagen Mega Endo-Free prep kit (Qiagen, U.S.A.) and the full-length constructs were confirmed by DNA sequencing.

BON I cells (~1 ×10^5) were seeded in six-well tissue culture plates. Cells were transfected after 18-24 hrs culture using FuGene 6 transfection reagent (Roche Molecular Biochemicals, France) according to the manufacture’s instructions.

GFP-vinculin plasmid (pEGFP-C1V1-1066) was kindly provided by Susan W, Craig (John Hopkins University, Baltimore, Maryland 21205, U.S.A). Plasmids were manufactured and transfected into vaso-transformed BON I cells [201] using FuGene 6 transfection reagent (Roche Molecular Biochemicals, France) as described above. After antibiotic selection, stable clones were selected for the study.

siRNA against human vinculin was bought from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Transfection was carried out according to the manufacture’s instructions. Cells were collected for the analysis of protein and mRNA expression at designated time.

Purification and confirmation of recombinant protein (paper I, III)

Serum-free conditioned media were concentrated using Centricons YM-10 column (Bio-Rad) and purified on Ni-NTA resin column (Invitrogen). The recombinant proteins were eluted from Ni-NTA resin with 50-500 mM imi-
dazole, changed buffer (desalted) with Econo-Pac 10 DG columns (Bio-
Rad), and concentrated by centrifugation at 4,000 × g for about 1 hr (~10× concentrated) by using Centricons YM-10 column. The samples were then analyzed by 4-15% SDS-PAGE gels for determining the purity of the recombinant proteins. The band of recombinant protein in Coomassie blue-
stained SDS-PAGE gels showed that preparation of recombinant protein was more than 90% pure. The yield of the purified hIFN-α2 and Vasostatin protein was about 0.23 mg per liter and 1 mg per liter respectively.

Preparation of total protein (paper I-IV)
Total protein was prepared by using buffer (1% SDS, 1.0 m M sodium orthovanadate, 10 mM Tris pH 7.4) and quantified by using protein assay kit (Pierce, Novakemi AB, USA) following the manufacturer’s instructions.

Cell fraction preparation (paper I)
Cell fractions were prepared as described earlier [202] with some modification. Briefly, cells were washed twice with ice-cold PBS and gently scraped in homogenization buffer (250 mM sucrose, 10 mM Hepes, 1 mM EDTA-
NaOH, pH 7.2) containing protease inhibitor cocktail tablets (Roche Diag-
nostics, Germany) by using a rubber policeman. Thereafter, cells were crushed by passages through a 25-gauge needle 12-14 times. The lysate was centrifuged for 15 min at 4 °C (800 g). The supernatant was microcentri-
fuged for 15 min at 4 °C (15,000 g). The resulting supernatant served as the cytoplasmic fraction. The pellet (nuclei) was resuspended in nuclear protein extraction buffer (20 mM Hepes, pH 7.9, 25% glycerol, 0.6 M KCl, 0.2 mM EDTA, 0.5 mM DTT) containing protease inhibitor cocktail tablets, ho-
Mogenized, and extracted by shaking at 4 °C for 1 h. The resulting solution was microcentrifuged for 15 min at 4 °C (15,000 g) and the supernatant (nu-
clear protein) was divided in aliquots and kept in -70 °C freezer for later use.

Measurement of hIFN-α2 production (paper III)
IFN-α-expressing BON I cells were seeded in a flask or on multiple cultural plates. Cells were allowed to grow to ~70% confluence. Cultural media were then replaced with serum-free media. After 24 hrs incubation, cells were counted using a particle counter (Beckman Culture AB, Florida, U.S.A.) according to the manufacturer’s instruction. The concentration of produced hIFN-α2 in the conditioned media was then measured. The secretory product of hIFN-α2 was expressed as ng/million cells (Mil)/day.

Cell proliferation assay (paper I-III)
Different cells, both tumor and endothelial cells, were seeded at 5 × 10³ cells/well in 48-well tissue culture plates. Cells were cultured for 24 hrs when media were changed with fresh media plus different conditioned cul-
tural media or purified recombinant protein. For the analysis of vinculin-
expressing cells, 500 cells/well were seeded in 96-well tissue culture plates. At indicated time points, cells were treated with trypsin-EDTA in PBS for 15 min at 37 °C and counted using a particle counter. For si-RNA study, Vaso-transformed BON I cells were transfected with vinculin siRNA or siRNA control for 24 hrs. After that, cells were trypsinized and seeded. At indicated time points, cells were counted as above. The experiments were performed at least in triplicate and repeated at least three times.

**Clonogenic assay in soft agarose (paper II)**
Colony formation assay was performed as described earlier [23] with modifications. Briefly, Vaso-transformed BON I cells were transfected with vinculin siRNA for 24 hrs, then, different cells (mock or vinculin knockdown BON I cells) were trypsinized and plated in 12-well culture plates (20,000 cells per well). After 14 days of incubation, colonies images were photographed under microscopy with a Canon digital camera (5 x objective plus 3 x digital amplification). Pictures were printed out with A4 papers. Colony formation was scored by counting the number of colonies greater than 5 mm in diameter.

**Adhesion assays (paper I-III)**
Adhesion assays were performed on 48-well plates. Briefly, plates were coated with Fibronectin or conditioned culture media overnight at 4°C. Cells were seeded with \(4 \times 10^4\) cells from each treatment in triplicate wells. After 1 or 2 hrs of incubation at 37°C, unadherent cells were removed by repeated washing in PBS with strong finger tapping to agitate the wells. Attached cells were treated with trypsin-EDTA in PBS for 15 min at 37 °C and counted as described above. The experiments were repeated four times.

**Cell spreading assay (paper I)**
Cell spreading assays were performed as described by Coghill et al. [203] with a modification. Cells were seeded at a density of \(5 \times 10^4\) cells/well onto fibronectin (10 µg/ml)-coated 8-well coverslips. After 24 h of culture, floating cells were washed with PBS. Cells were fixed with 100% methanol and stained with HE. Images were photographed under microscopy with a Canon digital camera (20× objective plus 3× digital amplification). Cells were considered spread if the cytoplasmic surface area was at least twice the nuclear surface area or the cells had flattened morphology with foot formation. At least 200 cells in each well were counted and scored for spreading analysis. The experiments were performed in triplicate.

**Cell migration assay (paper I)**
Cell migration was carried out as described previously [204] with some modifications. Briefly, the inserts (8 µm, pore size) were coated with fibronectin (10 µg/ml). \(1.25 \times 10^5\) cells were seeded and cultured at 37 °C for
3 days. Non-migrating cells on the inner side of the inserts were removed with a cotton swab. Cells that migrated through the porous membrane and adherent to the lower surface of the filters were fixed and stained with HE staining. After staining, filters were removed from the inserts and mounted on slides. Filter images were recorded using a digital camera as described above and the migrating cells were counted at least in four fields per well. Data were expressed as a ratio with WT control values at 1. Experiments were performed in triplicate.

**Invasion assays (paper I, III)**

In vitro invasion assays were performed using Matrigel-coated wells as previously described [205] with modifications. Briefly, the insides of inserts (8 µm, pore size) were first coated with 15 µl of Matrigel, thereafter coated with fibronectin (10 µg/ml) on the Matrigel. BON I and different transfected cells (1.25 x 10^5) were plated onto each well and incubated at 37°C for 3 days. Noninvading cells were removed with a cotton swab. The invading cells were fixed and stained with H&E staining. Cell count and analysis were done as described above. Matrigel assays were repeated three times.

**Cytoskeletal actin observation (paper III)**

WT, vector and hIFN-α2-expressing BON I cells were seeded at 1 x 10^4 cells/well in Human fibronectin-coated 8-Well CultureSlides (Becton Dickenson Labware, Belgium). Cells were cultured for 24 hrs and fixed with 3.7% formaldehyde in PBS for 15 min at room temperature. The samples were washed with PBS, and permeabilized for 5 min in 0.2% Triton X in PBS. TRITC-labeled phalloidin was added for 20 min (1:2500 in PBS) after washing with PBS (three times). The samples were then washed three times with PBS and once with H2O. Finally sample were mounted and viewed by fluorescence microscope (Zeiss Axioplan 2 imaging).

**RT-PCR (paper I-III)**

Total RNA and cDNA were prepared as described earlier [48]. Cycler conditions were: 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 58°C for 1 min 30 s, 72°C for 2 min, with an extra extension of 10 min at 72°C. Amplified products were first analyzed by 1% agarose gel electrophoresis containing ethidium bromide under UV light. The anticipated target bands were cut, purified and inserted into pcDNA3.1 plasmid vector. PCR fragments were confirmed by sequencing. For mRNA expression analysis, gels were recorded by digital camera. The image was then analyzed using Quantity One Software (Bio-Rad).

**Western blot (paper I-IV)**
Total protein (10–25 µg) was loaded and separated in 4-15% SDS-polyacrylamide gradient gels, transferred to nitrocellulose membranes (Bio-Rad, California, U.S.A.). Western blots were done as described earlier [48]. Monoclonal anti-β-tubulin antibody was used as internal protein loading control. The membranes were blotted with different specific antibodies.

For identifying purified recombinant protein, the gels stained with Coomassie blue R-250 and then dried under vacuum. The films or dried gels were scanned into the computer. The images were then analyzed using Quantity One Software as described above.

**Chicken chorioallantoic membrane (CAM) assay (paper III)**

The CAM assay was performed essentially as previously described [206] with minor modification. A window in the shell of a white Leghorn egg was cut on day 0 of embryo incubation and eggs were incubated in a humidified atmosphere at 38°C. A provisional matrix consisting of fibrin (5 mg/ml), rat tail collagen I (4 mg/ml; Upstate Biotechnology), thrombin (2 IU/ml), streptomycin (4.5 mg/ml), penicillin (4.5 mg/ml), amphotericin B (11.25 µg/ml), FGF-2 (5 µg/ml), and aprotinin (10,000 IU/ml, pH 7.5; local pharmacy) was prepared and 100 µl of the solution was applied into a 0.5-cm-high plastic tube glued on a 1.5 × 1.5 cm piece of a nylon mesh (mesh size of 28 µm). The gel was covered with 25 µl of sunflower oil and 5 µl of hIFN-α2 (~11 ng). Constructs were allowed to polymerize overnight at 37°C in a humidified atmosphere. Single construct was placed on the CAM at day 13 of embryo development and eggs were incubated for 5 days. The entire eggs were then fixed in methanol and the constructs cut out from the CAM. The plastic tube was removed, leaving the fibrin/collagen gel on the nylon mesh. The gel was made transparent in benzyl benzoate/benzyl alcohol (1:1) and the underlying chorioallantoic membrane was removed to visualize ingrown vessels. Images were then photographed and vessel lengths were measured by using The Image Processing Tool Kit software (Version 4.0).

**Animals and mouse tumor model (paper I, III, IV)**

Briefly, BALB/c nu/nu mice were bred and maintained in a standard condition. Before inoculation BON I and different transfected BON I cells were cultured until exponential growth stage. Cells were detached with trypsin-EDTA and resuspended in 1 × PBS at a concentration of 1× 10^7 cells/ml. To generate tumors, mice were subcutaneously implanted in the back of the mice with 100 µl of a single-cell suspension containing 1 × 10^6 cells (both left and right sides). The volume of tumors was measured once or twice weekly. Tumor volume was calculated by using the formula: V = 4/3 πr^3, where V = volume, r = radius, π = constant, expressed in cubic centimeters (cm³). Final volume (V_f) was calculated using the equation V_f = (V_1 + V_2)/2,
where $V_1$ and $V_2$ come from two greatest perpendicular diameters of tumors. After growing the tumor for 14 weeks or the tumor size was >1 cm in diameter, mice were sacrificed and tumor tissues were collected for histopathology and molecular analysis. Each group included 10 mice in study I and III and 6 mice in study IV. All animal experiments were conducted with the approval of the Local Ethical Committee for animal studies.

**PEGIntron treatment of mice tumors (paper IV)**

Two weeks after implantation of tumor cells (WT BON I cells) tumors were recognized (3-5 mm in diameter). Mice were subcutaneously injected with PEGIntron (0.1 µg/mouse, diluted in 50 µl of phosphate buffered saline (PBS)) or PBS weekly. The volume of tumors was measured weekly. After four weeks mice were sacrificed as described above.

**hIFN-α2 plasmid gene treatment of mice tumors (paper IV)**

Mice tumors were generated as described above. After the tumors had grown to 0.3-0.5 cm in diameter after tumor inoculation, the mice were treated with either 10 µg of hIFN-α2 plasmid liposome or 10 µg of vector (weekly) by i.t. injection for 3 weeks. The volume of tumors was measured weekly. After four weeks mice were sacrificed as described above.

**Immunoblot analysis of hIFN-Į2 protein expression in mice tumor (paper III)**

Mice tumors (WT, vector and hIFN-Į2 group) were rinsed in PBS. Proteins were extracted with a homogenizer in protein extract buffer. Lysate were transferred to microcentrifuge tubes and centrifuged at 13000 RPM. Protein concentration was measured as described above. Total protein (100 µg or 50 µg) was loaded for Western Blot analysis. Polyclonal anti-GAPDH antibody was used as internal protein loading control.

**Immunohistochemistry (paper I, III)**

Mice tumors were harvested and stored at -70 °C. Immunohistochemistry was performed on 6 µm. Briefly, endogenous peroxidase activity was blocked with 1x PBS containing 0.3% hydrogen peroxide. Nonspecific binding was blocked by incubation with blocking serum. Thereafter the section was incubated with primary antibody. Then tissues were incubated with biotinylated secondary antibody. After rinsed in PBS, the tissues were incubated with Elite complex (Elite ABC Kit, Vector Laboratories, CA, U.S.A). The antigen-antibody reaction was visualized with 3-amino-9-ethylcarbazole/H$_2$O$_2$. Sections were counterstained with hematoxylin and mounted. Negative controls consisted of the same tissue sections using 1% BSA/PBS without primary antibody. All tumor tissue sections were included in the same batch to ensure the standardization of staining intensity. The samples were examined blindly.
**Immunofluorescence Microscopy (paper II)**

Cells were plated on human fibronectin-coated 8-well Culture-Slides and were immunolabeled with a monoclonal antibody directed against CLD4. Binding sites of the primary antibodies were detected by using a secondary anti-mouse FITC-labeled antibody (green). TRITC-labeled phalloidin (red) was used for F-acting observation. Experiments were performed as earlier described [207] with modifications. Briefly, cells were cultured for 16 h and fixed with 3.7% formaldehyde in PBS for 20 min. After washing (PBS x3) the samples were permeabilized for 5 min in 0.2% Triton X in PBS and blocked 1 h with 5% FCS in PBS. Then primary antibody was added, incubated for 1h. Secondary antibody and TRITC-labeled phalloidin were incubated for 1h (1:50 and 1:1500 in PBS, respectively). Finally samples were mounted and viewed by fluorescence microscope (Zeiss Axioplan 2 imaging).

**Statistical analysis (paper I-IV)**

All data are expressed as the means ± SEM of at least three independent experiments using two determinations for each sample. CAM assay, incidence of tumor, doubling time of the tumor and microvessel density was evaluated by t test. mRNA and protein expression, cell adhesion, proliferation, cell migration, invasion assays and colony formation were estimated by paired t test. P values ≤ 0.05 were considered as significant.
Results and discussions

IFN-α has been successfully applied in the treatment of a range of tumor types including NE tumors [3, 5]. The anti-tumor mechanisms are suggested to include anti-proliferation [47], anti-angiogenesis [41, 51], apoptosis induction [208] and immune regulation [31, 34, 36, 209, 210]. However, IFN-α seems not to be strong enough to totally eradicate tumor growth. Side effects such as flu-like symptoms, chronic fatigue, depression and autoimmune reactions are caused by IFN-α treatment [3] and therefore limit the treatment possibilities.

Vasostatin is the N-terminal domain of Calreticulin (CRT) including amino acids 1–180. Several studies have reported that it has anti-proliferative effects on endothelial cells and presents anti-tumor growth effects by inhibition of angiogenesis in mice tumor models [52-54]. It is regarded as an angiogenesis inhibitor and human NE tumors are often highly vascularized neoplasms [41]. Thereby vasostatin might be a therapeutic option.

Cancer gene therapy is taken into account because it will allow a low and continuous secretion of therapeutic cytokines or increased local concentration of the cytokines in the target sites by direct injection. This may improve the therapeutic effects and reduce systemic side effects. Therefore, we selected IFN-α and vasostatin for our development of gene therapy in NE tumors based on their documented anti-proliferative and antiangiogenic effects.

Paper I
Effects of Vasostatin on cell growth, mobility and tumor development

The effect of vasostatin on cell growth was studied by conditioned culture medium taken from vasostatin-expressing (vaso-expressing) BON I cells. We observed that vasostatin only slightly inhibited the growth of endothelial cell, PAE but not SVEC4 cells. On the contrary, it seemed to promote BON
I cell growth. A purified MBP-vaso protein, a generous gift from Dr. Giovanna Tosat in USA, promoted the growth of BON I cell as well. In addition, we also noticed that vaso-expressing BON I cell had a faster growth compared with wild type BON I cells as well as vector control BON I cells (data not shown). Vaso-expressing BON I cells demonstrated a low level of p53, Rb, p27, vinculin, nm23, human NKG2D ligands, MICA and MICB, and PKR protein. Moreover, we found that conditioned culture medium taken from vaso-expressing BON I cells promoted BON I cell adhesion. Vaso-expressing BON I cells had an enhanced spreading, migration and invasion ability. Furthermore, mice implanted with vaso-expressing BON I cells, developed tumors earlier and have a shorter tumor doubling time (4.96±0.67 days) compared with tumors in WT group (11.98±1.13 days) and vector group (6.25±0.93 days), p<0.0001 and p<0.001, respectively.

Deregulation of the cell cycle control is one of the hallmarks of human cancers [75, 83]. p53 and Rb gene are two of the most studied tumor suppressor genes. They play a key role in the regulation of normal cell cycle processes [62, 68-71, 73-75]. Mutations of p53 and Rb gene have been found in a wide range of human cancers [65, 72]. p27, an cyclin-dependent kinase inhibitor, inhibits cell-cycle progression by interacting with CDK complexes [104]. In our previous studies, we found that p27 could inhibit the activities of CDK2 and cyclin E in the NE tumor cell line, BON I cell line [23]. In vaso-expressing BON I cells the expressions of p53, Rb and p27 were down regulated. These results may explain why vaso-expressing BON I cells grow faster than WT BON I cells.

Cell adhesion, migration and invasion are important for the tumor development and metastasis [211-213]. Vinculin, an actin-anchoring protein, is involved in adherence junctions and focal adhesion [214]. Cells lacking vinculin are highly metastatic and motile [128]. In some tumor cells, overexpression of vinculin can result in a decrease of tumorigenic and metastatic potential [130]. Thus, vinculin is regarded as a tumor suppressor. nm23 is also regarded as a tumor suppressor which has been identified in many human cancers [76, 78, 79]. We noticed reduced levels of vinculin and nm23 in our study which might have contributed to the faster growth of vaso-expressing BON I cells.

NKG2D was first described in human natural killer cells and plays an important role in tumor cell surveillance [215]. Human NKG2D ligands/receptors include MICA, MICB, and ULBP1,2,3 [215, 216]. Induction of NKG2D ligand expression in tumor cells may inhibit tumor cell migration, invasiveness and abrogate tumorigenicity [217]. Inversely, blocking NKG2D can inhibit natural cytotoxicity of all tumor cells tested that express ligands for the receptor [218]. Tumor cells themselves may reduce or lose expression of
NKG2D ligands to evade NKG2D-mediated immunity [219]. Therefore, down-regulation of the mRNA expression of MICA and MICB may be involved in the transformation and tumor growth in vaso-expressing BON I cells.

In this study, both mRNA and protein level of PKR were significantly down-regulated in vaso-expressing BON I cells and we also could not detect PKR protein expression in tumor tissue from mice implanted with vaso-expressing BON I cells. PKR is an IFN-induced, double-stranded RNA (dsRNA) activated serine/threonine protein kinase [15]. The best studied substrate of PKR activity is the α-subunit of eukaryotic initiation factor 2 (eIF-2α) [220]. Phosphorylation of eIF-2α by PKR leads to a general block in protein synthesis [17]. Additionally, PKR also regulates action of several other transcription factors such as NF-kB, IRF-1, p53, STAT1, NF-90 [25]. Modulation of these different targets allows PKR to regulate several cellular functions such as cell proliferation [26, 27], differentiation [28] and apoptosis [221, 222]. Therefore PKR might function as a tumor suppressor and the down-regulation of PKR expression seen in vaso-expressing BON I tumor cells might further explain the rapid growth in these cells.

Paper II

Cell adhesions to extracellular matrix (ECM) and cell to cell adhesions are critical for multiple biological functions including cell growth, differentiation, migration and tumor metastasis [101-103]. Vinculin, an actin anchor protein, exists at both focal adhesions and adherens junctions [125-127]. It is regarded as a tumor suppressor and believed to play an important role in the control of cell adhesion and cell motility, thus in the control of tumor metastasis [119, 128-131].

In my first study, we found that transfer of vasostatin gene into neuroendocrine tumor cells, BON I cells, resulted in an enhanced mobility and increased growth rate of vaso-expressing BON I cells and faster vaso-expressing BON tumor development in mice. One important finding was that vinculin had been significantly down-regulated in vasostatin-expressing BON I cells [201]. Therefore, we assumed that the malignant behavior of vaso-transformed BON I cells might be at least in part related to down-regulation of vinculin. In order to further investigate the function of vinculin in NE tumors, we decided to transfer vinculin gene back into vaso-expressing BON I cells.
Function of vinculin on NE tumor development

Effects of vinculin overexpression on vaso-expressing BON I cell growth, adhesion and morphology

We analyzed three vector control clones and four different vinculin-expressing clones to evaluate the effects of vinculin overexpression on tumor cell growth. We found that vinculin overexpression significantly slowed down growth of vaso-expressing BON I cells.

Cell adhesion was analyzed in vinculin-expressing BON I Cell (clone 11) and vector control (clone 17). We found that in vinculin-expressing BON I cells, cell adhesion ability was significantly lower compared with vector control cells.

Overexpression of vinculin induced a clear morphological change. Vaso-expressing BON I cells (Vaso) were characterized by prominent cell adhesion protrusions. Some cells demonstrated angular or elongated even fibroblastoid phenotype. In contrast, vin-expressing cells (Vin) restored to cobblestone morphology which is similar to the parental WT BON I cells as shown in figure 4.

Effects of vinculin overexpression on tumor suppressor gene and cell adhesion related protein expression in vaso-expressing BON I cells

In our earlier studies, we found that several tumor suppressor genes had been down-regulated in vaso-expressing BON I cells [201]. In this study, expression of these tumor suppressor genes was investigated after vinculin gene transfection. We could show that Rb, nm23 and p53 had been restored after vinculin transfection, and that cell cycle regulating protein, p27, was up-regulated as well. However, the expression of PKR protein was not restored.

Expression of extracellular matrix glycoproteins are considered to be involved in tumorigenesis [94-100]. Vitronectin, a glioma-derived extracellular matrix protein, protects tumor glioma cells from apoptotic death [97]. Studies of cell adhesion and tumor progression in mouse skin carcinogenesis
have shown that increased synthesis and organization of fibronectin is associated with the undifferentiated spindle phenotype [95]. We further explored the expression of vibronectin and fibronectin in vaso-expressing BON I cells. The expression of these proteins was not significantly changed after vinculin transfection.

Focal adhesion kinase (FAK) has been thought to play a key role in the signal transduction pathways triggered by integrins [109]. Activation of FAK can trigger several signaling pathways that regulate cell proliferation, survival, migration and invasion [110]. In this study, we investigated the expression and the activity of FAK. Vinculin transfection did not change the expression level of FAK protein. In addition, we did not find detectable levels of phosphorylated FAK in the cells.

Recently, a group of tight junction proteins, claudin proteins, have been reported to be abnormally regulated in several human cancers [121]. Among them, a protein called claudin 4 (CLD4) maybe related to cancer invasion and metastasis [120, 223]. We noticed that vaso-expressing BON I cells had a low expression of CLD4 protein, whereas after vinculin transfection cells expressed high levels of CLD4 protein. It was mainly located at cell-cell junctions.

**Effects of vinculin siRNA on WT BON I cell growth**

Vinculin overexpression can restore cell behavior and the expression of tumor suppressor genes in vaso-expressing BON I cells as described above. To further explore the functions of vinculin in NE tumors, vinculin was knocked down by siRNA technology in WT BON I cells. We found that cell growth became significantly faster as compared with control cells. A significantly increased colony formation was also noticed. Furthermore, the expression of CLD4 protein was reduced after vinculin knockdown.

Claudin 4, a component in tight junction, is found to be overexpressed in several neoplasias, including ovarian, breast, pancreatic and prostate cancers [121]. Our results have shown that vaso-expressing BON I cells had a low expression of CLD4 protein. After transfection of vinculin genes cells expressed a high level of CLD4 protein and the growth rate of those cells was reduced. Moreover, vinculin knockdown resulted in the decreased expression of CLD4 and faster growth of BON I cells. These data indicated that the change of malignant phenotype of BON I cells seems to be related to CLD4 expression in some way. CLD4 may play a role of controlling NE tumor cell behavior together with vinculin. This finding is supported by a recent study in which overexpression of claudin-4 in SUIT-2 pancreatic cancer cells inhibit colony formation in soft agar assays and reduced the invasive potential of cancer cells. These effects were paralleled by increased cell contact for-
mation. In addition, tail vein-injected claudin-4 overexpressing cells formed significantly less pulmonary metastases compared with mock-transfected cells in vivo. Claudin-4 may be regarded as a potent inhibitor of invasion and metastatic abilities of pancreatic cancer cells [120].

Paper III
Effects of hIFN-α2 on cell proliferation, mobility, angiogenesis and tumor growth

Effects of hIFN-α2 on cell growth were studied by applying conditioned culture medium taken from hIFN-α2-expressing BON I cells. Our results showed that hIFN-α2 significantly inhibited the growth of NE tumor cells, BON I and LCC18, other tumor cells, CA46 and SW480 and endothelial cell PAE. In addition, we further observed that cell adhesion and invasion abilities of hIFN-α2-expressing BON I cells were reduced. The reduced abilities were inversely correlated with the secretory levels of hIFN-α2. Moreover, recombinant IFN-α2b dose-dependently inhibited cell adhesion. We also found that most of hIFN-α2-expressing BON I cells showed a rounding morphology with a substantial reduction in the number of actin stress fibers. Furthermore, concentrated conditioned medium taken from hIFN-α2-expressing BON I cells significantly inhibited angiogenesis in a CAM assay.

Effects of hIFN-α2 on tumor growth was further studied by subcutaneously injecting BON I cells, vector control BON I cells and hIFN-α2-expressing BON I cells into the back of nude mice. We observed that mice in WT and vector group started to present tumors on week 3 after tumor cell implantation, but in hIFN-α2 group (implanted with hIFN-α2-expressing BON I cells) tumors developed on week 6 and showed a lower incidence and longer tumor doubling time compared to both WT and vector group. In addition, we noticed that mice implanted with a mixture of WT and hIFN-α2-expressing BON I cells (1:1) had significantly lower incidence of tumors and even longer doubling time compared to the hIFN-α2 group, as shown in table 4. These mice developed tumors later than hIFN-α2 group (week 7 vs. week 6). As expected there were high levels of expression of PKR protein in tumor tissues of hIFN-α2 group and 1:1 mixed group. Furthermore, immunohistochemical studies from our mouse tumor tissues showed that microvessel density was significantly reduced in the tumor tissues of hIFN-α2 group.
Table 4. In vivo tumorigenesis analysis

<table>
<thead>
<tr>
<th>Group</th>
<th>Incidence of tumor, %</th>
<th>Doubling time of tumor, day</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>10/11</td>
<td>11.98 ± 1.13</td>
<td>20</td>
</tr>
<tr>
<td>vector</td>
<td>10/10</td>
<td>6.25 ± 0.93&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20</td>
</tr>
<tr>
<td>hIFN-α2</td>
<td>7/10</td>
<td>22.04 ± 2.06&lt;sup&gt;a, b&lt;/sup&gt;</td>
<td>14</td>
</tr>
<tr>
<td>mix&lt;sup&gt;1&lt;/sup&gt;</td>
<td>5/10</td>
<td>27.20 ± 8.37&lt;sup&gt;a, b&lt;/sup&gt;</td>
<td>10</td>
</tr>
</tbody>
</table>

<sup>a</sup> p<0.0001 to compare with WT; <sup>b</sup> p<0.0001 to compare with vector. t test. <sup>1</sup> WT and hIFN-α2-expressing BON I cells (1:1).

In our previous studies, IFN-α has been shown to present a direct anti-proliferation effect on BON I tumor cells [47]. This function is partly mediated by up-regulation of the activities of cyclin-dependent kinase inhibitors p21 and p27 and down-regulation of the activities of CDK2, CDK4 and cyclin E, which will induce a release of unphosphorylated Rb protein leading to cell cycle block in the G1/S phase [23]. These effects of IFN-α on cell cycle regulatory protein will contribute the anti-proliferative function of IFN-α on NE tumor cells.

As described earlier, PKR is an IFN-induced protein kinase [15]. It regulates several cellular functions such as cell proliferation [26, 27], differentiation [28] and apoptosis [221, 222]. In one of our previous studies, we demonstrated that expression of PKR have prognostic significance in NE tumors [48]. Therefore, up-regulation of PKR expression maybe one reason of the anti-tumor effects of IFN-α in NE tumors.

Cell adhesion, migration and the invasion ability contribute to tumor development and metastasis [211-213]. The ability of cell adhesion is determined by cytoskeletal organization and thus by cellular morphology [224]. In our study, we noticed that ~84% of hIFN-α2-expressing BON I cells were rounded. In a recent study, the cytoskeletal organization of hairy cells was changed after treatment with IFN-α and the hairy cell reversed into a rounded morphology which was coupled by up-regulation of the Rho family of GTPases [225]. In addition, Salgia R et al. reported that IFN-α reduced cell motility and pseudopod formation of BCR/ABL-expressing cells on a fibronectin-coated surface and increased the number of cells with spherical morphology [226]. Altered morphology and adhesion of NE cells treated by IFN-α may also contribute its anti-tumor function.

Solid tumor growth, invasion and metastasis are angiogenesis dependent [134, 135]. VEGF and bFGF are most important growth factors driving angiogenesis [138, 144, 145]. In our study, we found that IFN-α can inhibit endothelial cell, PAE, proliferation and inhibit angiogenesis on CAM assay.
In addition, others have shown that IFN-α inhibit VEGF gene transcription which lead to a decreased expression of VEGF, thus inhibiting tumor induced angiogenesis and NE tumor growth [41, 51]. This might explain the antiangiogenic function of IFN-α on NE tumor development.

We noticed that mice implanted with a mixture of WT and hIFN-α2-expressing BON I cells (1:1) had significantly lower tumor incidence and even longer tumor doubling time compared with hIFN-α2 group (100% hIFN-α2 expressing BON I cell). In an earlier study in a mouse tumor model with bladder carcinoma, the daily s.c. administrations of 5,000 or 10,000 units IFN-α produced maximal inhibition of bFGF expression, maximal reduction in tumor vessel density and the best anti-tumor effect. However, daily injection of higher doses of IFN-α failed to produce significant antangiogenic and anti-tumor growth effects [42]. Another study also showed that lower doses of IFN-α were more effective than higher doses in an animal model of neuroblastoma [227]. These data suggest that frequent administration of IFN-α at optimal biological doses may provide better therapeutic effects.

Furthermore, we found that a high level of hIFN-α2 protein was detected in the hIFN-α2-expressing BON tumors. Subcutaneous implantation of BON I cells expressing human IFN-α might be a tumor model with a specific local microenvironment where a constantly high concentration of hIFN-α2 exists. This model may produce minimal systemic side effects and better therapeutic efficiency.

Paper IV
Effects of Pegylated IFN-α, hIFN-α2-expressing BON I cells and hIFN-α2 plasmid on NE tumor development

**Pegylated IFN-α treatment**
Pegylated IFN-α (PEGIntron), a pegylated recombinant IFN-α2b which demonstrate delayed clearance in serum compared with nonpegylated IFN-α2b, has been shown to possess both antiviral and antitumor activity [228]. Data from patients with chronic hepatitis C infection suggest that exposure to Peg IFN-α at a dose of 0.25 µg/kg per week is similar to that observed after administration of IFN-α2b at a dose of 3 million IU three times per week and dose intensification can be achieved safely in patients with CML and solid tumors using PEGIntron, which may potentially improve therapeutic efficacy [229]. In patients with advanced renal cell carcinoma, Pegylated IFN-α showed a higher efficiency and less toxicity than regular Intron A [230]. Pegylated IFN-α possesses many advantages: convenient to adminis-
In a clinical setting, higher efficiency and lower toxicity. Therefore, it should be further evaluated in clinical trials in NE tumors.

In the present study, we want to explore the anti-tumor efficiency of Pegylated IFN-α by weekly subcutaneously injection of PEGIntron at 0.1 µg/mouse in NE mouse tumor models. The results showed that on week two and on week three after start of therapy, the reduction of tumor volumes were 58.6% and 48.2%, respectively. A previous study showed that daily application of IFN-α2b (IntronA) ( 1 MU s.c. every morning ) for 28 days can reduce tumor volume by 32% [231]. Long-active IFN-α, PEGIntron may have a better anti-tumor effect despite lower total weekly dose of IFN-α.

**hIFN-α2-expressing BON I cell line**

In our earlier studies, we found that 100% hIFN-α2-expressing BON tumor cells had a significantly longer doubling time and lower incidence of tumor development than control animals [232]. An interesting results was that tumor cells in a mixture group, hIFN-α2-expressing BON I cells mixed with wild type BON I cells (1:1), have an even longer doubling time of tumor development than those cells in 100% hIFN-α2-expressing BON I cell group (27.20 ± 8.37 vs. 22.04 ± 2.06 (day), respectively. P< 0.0001). The incidence of tumor development was also lower in 1:1 mixture group than 100% hIFN-α2-expressing BON I cell group (50% vs. 70%), respectively. We decided to check whether less hIFN-α2-expressing BON I cells, a ratio of 1:10 (10% of hIFN-α2-expressing BON I cell mixed with 90% of WT BON I) could change the results. We could show that hIFN-α2-expressing BON I cells mixed with WT BON I cells (1:10) could significantly reduce tumor volume by 92.7% (week 4) and 89.6% (week 5), P < 0.0001. However, tumor formation occurred much earlier than 100% and 50% (1:1) groups, week 6 and week 7, respectively. Our data indicated that optimal biological dose or expression levels of hIFN-α2 may provide better therapeutic effects.

**hIFN-α2 plasmid**

The use of naked plasmid DNA represents the simplest and possible gene delivery system [53, 186]. Intratumoral administration of naked plasmid DNA encoding tumor suppressor genes or cytokines have been shown to have strong anti-tumor effects [187-189]. In addition, the complexes of naked DNA and cationic lipid or cationic polymer can protect DNA from degradation by nucleases and also help them to enter into cells. This may improve transfection efficiency of non-viral vectors and improve therapeutic effects [192-196].
IFN-α plasmid has demonstrated anti-tumor effects in several mice tumor models [233, 234]. In the present study we evaluated the anti-tumor effect by directly intratumoral injection of hIFN-α2 plasmid and liposome complex (10 μg/ tumor weekly). hIFN-α2 plasmid reduced tumor volume by 57.2% (week 4, p = 0.0477) and 62.5% (week 5, p = 0.0489) respectively. In addition, we noticed that intratumoral injection of hIFN-α2 plasmid resulted in tumor shrinkage. Tumor volume shrunk by 53.7% (40.37 ± 5.73 mm³ vs. 18.98 ± 3.74 mm³, p < 0.0001, n = 11). These results suggest that hIFN-α2 plasmids gene therapy is feasible and promising.

Effects of IFN-α on the expression of intracellular signaling proteins in NE tumor cells

**Intracellular signaling by hIFN-α2**

We reported previously that hIFN-α2 (conditioned media from hIFN-α2-expressing BON I cells) possessed significant anti-proliferative effect in NE cell lines (BON I and LCC18), other tumor cell lines (CA46 and SW480) and endothelial cells (PAE) [232]. We further analyzed the expression of IFN-inducible genes, STAT1, PKR, MxA and 2,5 OAS in the hIFN-α2 treated BON I cells and the expression of STAT1 in PAE cells.

We found that hIFN-α2 dose-dependently up-regulated protein expression of STAT1 and PKR in BON I cells and dose-dependently up-regulated expression of STAT1 in PAE cells. hIFN-α2 also resulted in phosphorylation of STAT1 protein and up-regulated the proteins of MxA and 2,5 OAS in BON I cells. These data indicated that our recombinant hIFN-α2 possess biological activities.

**Kinetic analysis of IFN-α2b on the expression of STAT1, MxA and 2,5 OAS in BON I cells.**

We noticed that STAT1 phosphorylation started from 0.5 hour up till 8 hours after IFN-α2b exposure and the up-regulation of STAT1 protein started from about 2 hours after IFN-α2b treatment, however, detectable expression of MxA protein appeared a little later. A slightly increased expression of MxA protein was noticed 4 hours after IFN-α2b exposure and a significantly up-regulated MxA protein was observed after 8 hours treatment with IFN-α2b. Up-regulated 2,5 OAS expression appeared also later after stimulation with IFN-α for 26 hours. In addition, we noticed that IFN-α2b dose-dependently up-regulated the phosphorylation of STAT1 and the expression of MxA and 2,5 OAS protein.

It is suggested that the JAK-STAT signaling pathway is turned off within several hours despite the presence of IFNs in the extracellular environment.
Dephosphorylation of STATs is regulated by negative regulators of the signal for example, SOCS proteins (Suppressors Of Cytokine Signaling), PIAS (Protein Inhibitor of Activated STAT) or Tyrosine-phosphatases (SHPs, CD45, PTP1B/TC-PTP) [12, 13]. Leon S et al. reported that IFN-α can induce maximal phosphorylation of STAT1 within 30 min after stimulation in RAMOS cells, a Burkitt lymphoma B cell line and the phosphorylated STAT1 can be observed even after 24 h [235]. The involvement of the negative regulation of JAK-STAT signal is important to ensure an appropriate, controlled cellular response.
General summary and conclusion

Vasostatin gene transfer caused a faster growth of BON I cells and tumor development. In vaso-expressing BON I cells, the expression of tumor suppressor genes, Rb, nm23, p53 and vinculin, cell cycle inhibitor, p27 and NKG2D ligands were down-regulated and the abilities of cell adhesion, spreading, migration and invasion were enhanced. The present data indicate that transfection of BON I cells by the vasostatin gene might cause an enhanced malignant tumor behavior. Gene therapy based on vasostatin can not be recommended in NE tumors.

Vinculin gene transfection reversed the malignant behavior of vaso-expressing BON I cells and up-regulated the expression of tumor suppressor genes. Down-regulation of vinculin expression by siRNA in WT BON I cells caused a faster growth of cells and an increased colony formation. Expression of CLD4 was found to be associated with vinculin expression. Therefore, Vinculin might play an important role in the regulation of NE tumor cell development.

hIFN-α2 exerted significant antiproliferative effects on NE tumor cell lines, BON I and LCC18 and the PAE endothelial cell line. hIFN-α2 suppressed cell adhesion and cell invasion. hIFN-α2 inhibited angiogenesis and BON tumor growth in nude mouse tumor model. Intratumoral injection of plasmids coding hIFN-α2 gene showed a strong anti-tumor effects and might cause tumor regression. These data suggested that hIFN-α2 possessed bio-activities and that gene therapy by using hIFN-α2 plasmid is feasible and maybe a new and efficient treatment for NE tumor patients.

A mixture of WT and hIFN-α2-expressing BON I cells (1:1) demonstrated a prolonged tumor doubling time than those tumors with 100% hIFN-α2-expressing BON I cells. The incidence of tumor development was also lower in the 1:1 mixture group than that in 100% hIFN-α2-expressing BON group. hIFN-α2-expressing BON I cells mixed with WT BON I cells (1:10) also reduced tumor volume. However, tumor formation occurred much earlier than 100% and 50% (1:1) groups. These may indicate that a ratio of 50% transfection of hIFN-α2 may provide better anti-tumor effects.
Subcutaneous injection of Pegylated IFN-α in mouse NE tumor models resulted in reduction of tumor volumes of 58.6% and 48.2%, on week two and three respectively. This is in contrast to the reduction of tumor volume of 32% with the treatment of regular IFN-α2b (IntronA) for 28 days. The long-acting Pegylated IFN-α may have a better anti-tumor effect compared with regular IFN-α2b although the later was given at higher weekly dose. Both the results with Pegylated IFN-α and the experiments with a mixture of hIFN-α2-expressing BON I cells and WT BON I cells (1:1) indicate that it is important to optimized the dosing of IFN-α during treatment.

IFN-α2b dose-dependently up-regulated the phosphorylation of STAT1 protein and the expression of STAT1, PKR, Mx A and 2,5 OAS proteins in NE tumor cells.
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