Module 4 Cancer gene therapy

Lecture 23

Cancer gene therapy (part I)

23.1 Introduction:

Uncontrolled division of cells with a solid or fluid filled lesion is called a tumor which in malignant state invades neighbouring tissues and spreads to other parts of the body causing cancer. Benign tumors are restricted to a location. They do not show uncontrolled division and are not involved in invading neighbouring tissues.

Some of the common cancers include urinary bladder cancer, bone cancer, brain cancer, breast cancer, cervical cancer, colon cancer, head & neck cancer, liver cancer, lung cancer, lymphoma, pancreatic cancer, prostate cancer, skin cancer, stomach cancer, ovarian cancer etc.

Classification of cancer on the basis of their origin

- Carcinoma:Cancers that arise from epithelial cells. Eg: cancers of breast, colon, prostate, pancreas etc.
- Sarcoma: Cancers that arise from the connective tissue. Eg: bone sarcoma, hemangiosarcoma etc.
- Lymphoma & Leukemia: Cancers that arise from hematopoietic (blood forming) cells.
- Germ cell tumors: Cancers derived from pluripotent cells, most often presenting in the testicle (seminoma) & the ovary (dysgerminoma).
- Blastoma:Cancers derived from immature "precursor" cells or embryonic tissue. Eg: Retinoblastoma
- Adenoma: Benign tumor of glandular origin.
- Adenocarcinoma: Malignant adenoma.

23.2 Genes associated with cancer can be classified into three categories

- **Genes that induce cellular proliferation** includes

1. Growth factors:*Fibroblast growth factor* (*FGF*), Insulin-like *growth factor*(*IGF*) etc.

2. Growth factor receptors: *fms*- Receptor for colony stimulating factor 1 (CSF-1)*erbB*- Receptor of EGF*neu*- Protein (HER-2) related to EGFR*erbA*- Receptor for thyroid hormone.

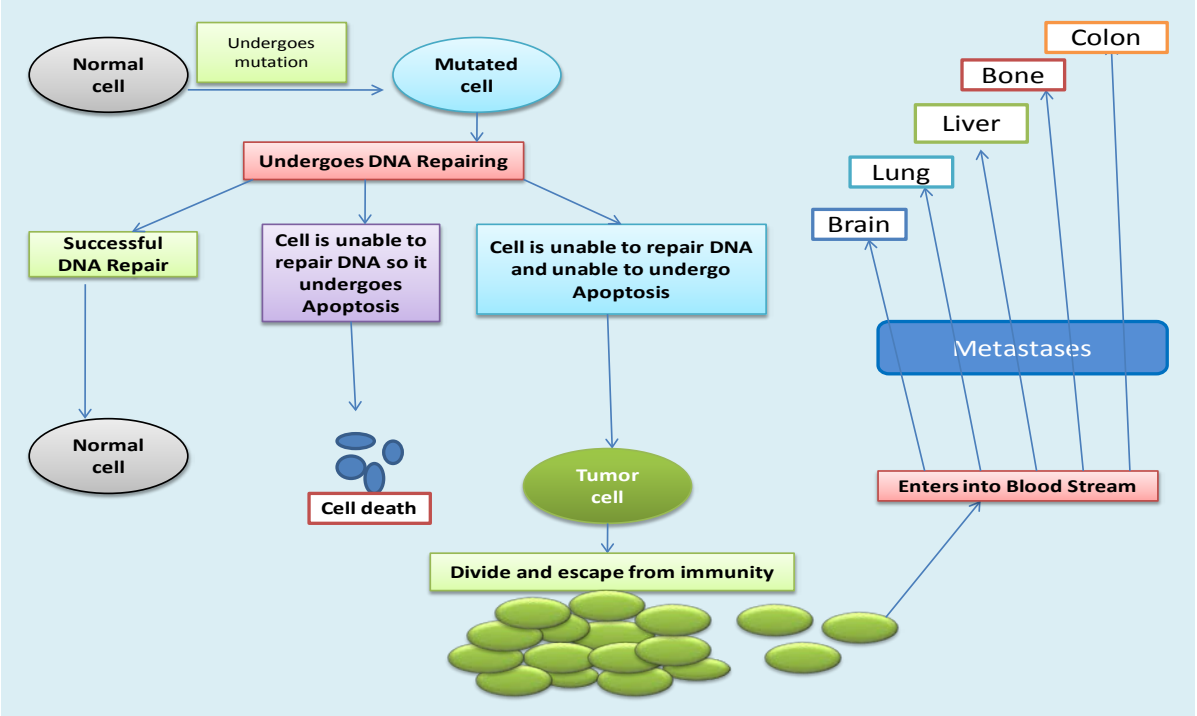
3. Signal transducers:Src (sarcoma)-tyrosine kinase ,Abl (Abelson murine leukemia)-tyrosine kinase, Ras (rat sarcoma)-GTP –binding protein.

4. Transcription factors: Jun- Component of transcription factor AP-1, Fos-(Finkel–Biskis–Jinkins murine osteogenic sarcoma virus), Myc-(myelocytomatosis)

- **Genes that suppresses tumor and inhibits cell proliferation** includes
  Rb: Suppressor of retinoblastoma
  p53: Tumor suppressor and DNA repair
  APC: suppressor of adenomatous polyps
  NF1: suppressor of neurofibromatosis
  WT1: Suppressor of Wilm's tumor

- **Gene involved in regulating programmed cell death** includes Bcl-2 (B cell lymphoma-2) suppressor of apoptosis

Figure 23.1 Schematic representation of development of cancer:



Gene therapy for cancer has generated great interest for more than a decade and intensive experimental and clinical investigations are in progress. By using gene therapy the gene of interest can be targeted to cancer cells or to normal tissue. It finds application for diagnosis as well as for treating the malignancy. The nature of cancer is quite difficult so based on the complex nature of cancer, these technologies involve complex strategies and principles like:

- **Immunomodulation**
- **Prodrug converting enzymes ‘‘suicide strategy’’** (i.e., transfer of the cDNA of a prodrug converting enzyme)
- **Tumor suppressor genes and anti-oncogenes involving gene replacement strategies such as transfer of a tumor suppressor and/or an anti-oncogene**
- **Tumorlysis by recombinant viruses or viral oncolysis**
- **Antiangiogenic and antiproteolytic gene therapy**
- **Drug-resistance genes** (i.e., the delivery of drug-resistant genes into the hematopoietic precursor cells)
- **Marker gene**

Many of these experimental approaches are still being evaluated and more than 1300 trials have been published or are in progress. More than 780 of these trials are directed against cancer. Different types of cancer involve different strategies which are effective under specific clinical settings.

23.3 Gene Therapy principles:

A. Immunomodulation

Several studies have exhibited that tumors show a certain degree of immunogenicity. As a result the human immune system recognises specific tumor antigens and starts mounting humoral and cellular responses. During cancer development, these responses are of very less intensity and for limited duration. The cancer cells use various mechanisms to escape detection by the immune system. These mechanisms have been elucidated thus enabling development of more and more strategies to design an effective antitumor immune response. In the last decade tremendous increase in the knowledge of the immunobiology of cancer has made immunological approaches, like immunomodulation, a very important strategy in cancer gene therapy.

The immunomodulation studies can be categorized on the basis of

- The target cells involved(can be tumor cells, host cells, T-cells, or APC's such as dendritic cells or other cells)
- The mode of gene delivery involved (type of vector used, type of delivery used which maybe *in vivo* or *in vitro* or *ex vivo*)
- The transgenes involved (can be genes coding for cytokines, co-stimulatory molecules, and tumor-associated antigens).

Of the different target cells involved T lymphocyte is one of the most attractive target cell types for genetic modification. The earliest clinical protocols for gene therapy involved the application of cytokine-transduced, tumor-infiltrating T-lymphocytes (TILs). For *ex vivo* genetic modification T-lymphocytes have been targeted. The genetic modification in T-lymphocytes is being done by the transfer of cytokine gene whereas the redirection is done by the tumor antigen specific T-cell receptor genes also called chimeric receptor genes which encodes for the protein with

extracellular domain involved in antigen binding and the intracellular domain involved in the cell signalling. They have also been isolated from genetically modified tumors or their draining lymph nodes. Other approaches enhance T-cell reactivity with antibodies that are targeted directly at the respective receptors on T-cells. These approaches are complemented by different methods to enhance antigen recognition on the surface of tumor cells.

One of the immunological methods to invoke a local inflammatory response is based on the short-range molecular interaction which enables communication between immune and non immune cells. As a result, direct actuation of specific as well as nonspecific immune cells by the cytokine transfected tumor cells or fibroblasts takes place. Therapeutic index increased in this case with the constant local release of cytokines. Similarly transfer of co-stimulatory molecules may also lead to increase in therapeutic index. Down-regulation of surface expression of MHC class I /MHC class II and co-stimulatory molecules on tumor cell surface could be up-regulated by transducing the tumor cells with a wild-type cDNA responsible for reactivating the antigen recognition on the transfected tumor cells. In the past few years antigens derived from tumor have become an attractive target for gene transfer methods. These tumor derived antigens are delivered as naked DNA or by the viral vectors directly to the tumors or via dendritic cells.

Tumor-derived antigens / peptides are being explored as an important target for gene transfer methods and have been extensively defined during the last decade. In order to elicit an immune response against the tumor cells these antigens are being delivered directly, to the tumor cells or through dendritic cells, by using viral vectors or in the form of naked DNA. To detect tumor antigens, other than those already being explored, efficient and promising methods like serological analysis of tumor antigens by recombinant expression cloning also called SEREX as well as microarray technique (which enables the detection of differential gene expression) are being used.

Another important approach worth mentioning in this context is the antibody based immunotherapy because profound benefits in clinical trials have been observed while using recombinant antibodies. The basic concept behind antibody based immunotherapy is to develop monoclonal antibodies which target the tumor antigens that are either soluble, expressed on the malignant cell surface or are present on the tumor stroma.

Lecture 24

Cancer gene therapy (part II)

B. Prodrug-Converting Enzymes (‘‘Suicide Strategy’’)

The ‘‘suicide strategy’’ in cancer gene therapy makes use of the combination of classical cytotoxic chemotherapy with gene transfer technology. The concept behind this strategy is to limit the action of a known cytotoxic drug to the local area of the tumor lesion without affecting the neighbouring normal cells. First, the cDNA of a prodrug-converting enzyme is delivered into the tumor by a suitable vector system followed by regional or systemic application of the corresponding nontoxic prodrug. Once the prodrug has reached the tumor cells it triggers the activity of prodrug converting gene in the tumor cells to express the prodrug-converting enzyme. The prodrug converting enzyme converts the prodrug to the cytotoxic drug. The conventional treatment of cancer by chemotherapy is highly toxic to neighboring cells and tissues and shows side effects which are dose-limiting. In case of suicide gene therapy, the cytotoxic effects of the drug are mainly restricted to the area of tumor, and for the time up to which the cancer cells express the prodrug-converting enzyme.

In addition, the efficacy of the suicide strategy is enhanced by the ‘‘bystander effect’’ that involves the killing of even uninfected tumor cells in the neighborhood of infected cells due to

- Intercellular communication mediated by gap junctions.
- Transfer of toxic metabolites via apoptotic vesicles or direct transmembrane diffusion.
- Local anti tumor immune responses.
- Systemic anti tumor immune responses.

Many prodrug converting enzyme systems are under development, a number of experimental or clinical investigations and validation of the system is in progress. The table below enlists some of these systems.

Table 24.1 Prodrug system used experimentally:

Gene	**Prodrug**	**Converted to Active drug**	**Cytotoxic effect**	**Bystander effect**
HSV-TK (Herpes Simplex Virus-Thymidine Kinase)	GCV (Gancyclovir)	GCV TP (Gancyclovir triphosphate)	Disrupts DNA synthesis	Present
CD (Cytosine Deaminase)	5-FC (5-Fluorocytosine)	5-FU (5-Fluorouracil)	Disrupts DNA/RNA synthesis	Present
VZV-TK (Varicella Zoster Virus-Thymidine Kinase)	AraM (9-(b-D-arabinofuranosyl)-6-methoxy-9*H*-purine)	AraATP (adenine arabinonucleoside triphosphate)	Disrupts DNA synthesis	Low/ Absent
*E.*coli NTR (*Escherichia coli* Nitroreductase)	Dinitrophenylaziridine of CB1954	4-hydroxylamine metabolite of CB1594	Crosslinks DNA, DNA breaks, Disrupts DNA synthesis	Present
CYP2B1 (Cytochrome P450 2B1)	CPA (Cyclophosphamide)	Phosphoramide mustard, Acrolein	DNA alkylation (crosslinks DNA), Crosslinks cellular protein	Present
CPG2 (Carboxypeptidase G2)	CMDA(4-[(2-chloroethyl)(2-mesyloxyethylamino)]	Benzoic acid mustard derivative	DNA alkylation (crosslinks DNA)	Present

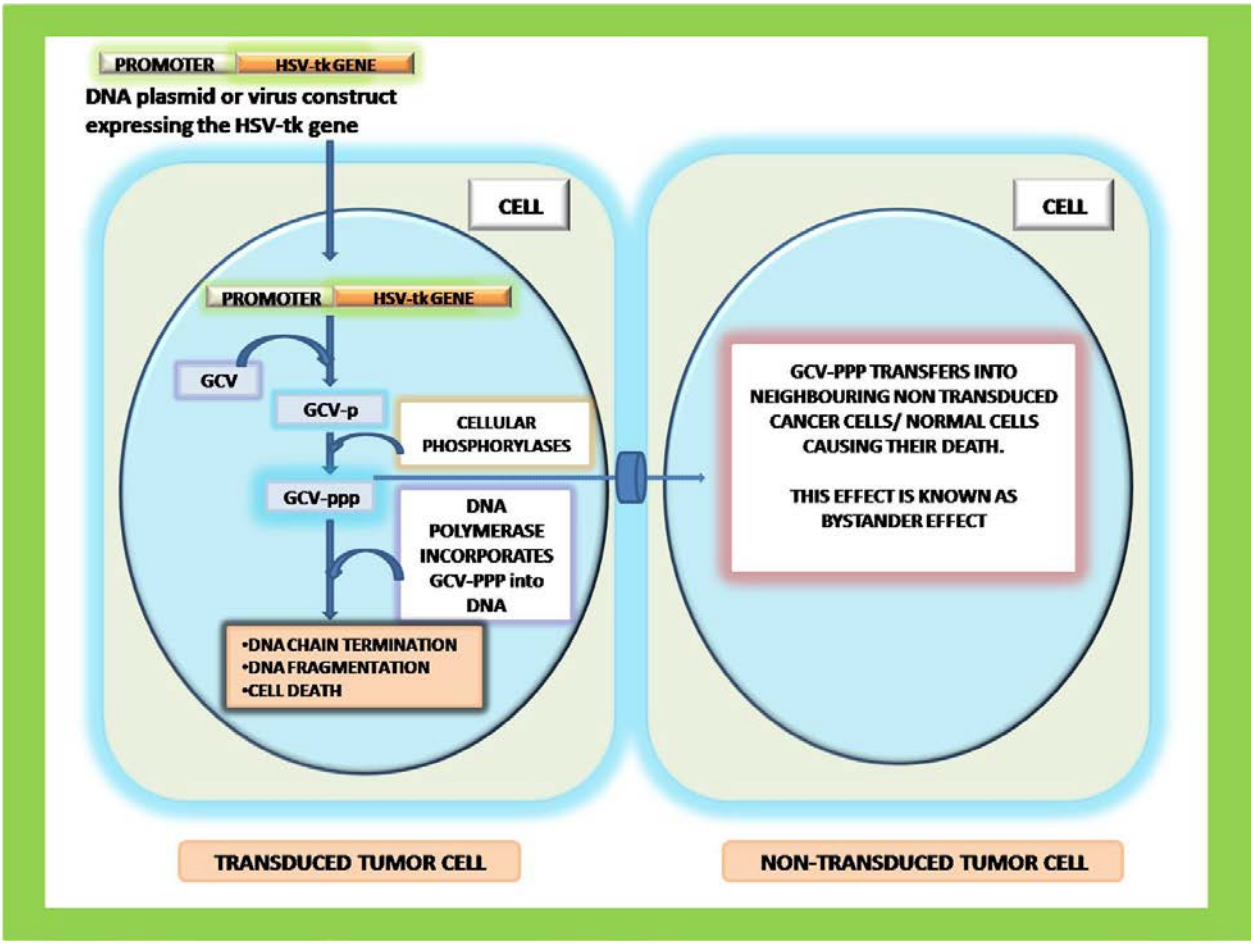
	Bensoyl-L-glutamic acid)			
E.coli-XGPRT (*Escherichia coli*-xanthine guanine phosphoribosyl transferase)	6-TX (6-Thioxanthine)	6-GMP (6-thioguanine monophosphate)	Disrupts DNA synthesis	Assumed to be present
E.coli-PNP (*Escherichia coli*-Purine Nucleoside Phosphorylase)	6-MePdR (6-methyl purine deoxyriboside)	6-MeP (6-methylpurine)	Direct toxin/cellular necrosis	Present
HRP (Horseradish Peroxidase)	IAA (Indole Acetic Acid)	Toxic radical	Free radical formation	Present
CYP1A2	Acetaminophen	NABQI (N-acetyl-benzoquinoneimine)	Protein arylation /oxidation	Present
Mutated tyrosinase	Quinone compounds	Quinone compounds	Unknown	Unknown
E.coli-UPRT (*Escherichia coli*-uracil phosphoribosyl transferase)	5-FU (5-Fluorouridine)	FUMP (5-fluorouridine-5'-monophosphate)	Disrupts DNA/RNA synthesis	Minimal

Methods of Suicide Gene Delivery include:

- **Non viral Transfection**
- **Retrovirus mediated Suicide Gene delivery**
- **Adenoviral Gene transfer**
- **Oncolytic viruses**
- **Other viral vectors being explored for suicide gene delivery includes Adeno associated virus, Herpes Simplex Virus, Human Papilloma Virus, Vaccinia virus, avipox virus and baculovirus.**

Many studies have demonstrated that tumor eradication takes place even on transducing only 10% of the tumor mass, probably due to a very effective bystander effect.

Figure 24.1 Suicidal gene therapy:



Several experiments conducted on immune-compromised animals (using intratumoral application of vector carrying HSV tk gene) showed a reduction of tumor volume of more than 50% of the controls under various experimental conditions and methods. In some cases even complete remissions have been observed. Similar experimental studies on immune-competent animals suggest that the immune system may play a supportive role in the efficacy of this approach. Promising results of tumor growth inhibition using suicidal gene therapy with side effects have been reported in case of the HSV tk system and all other suicidal gene therapy systems

Limitations of suicidal gene therapy includes

- **Low efficacy of *in vivo* gene transfer:** Just like other gene therapy strategies, the capability of the suicide gene therapy to kill cancer cells in experimental and clinical studies is curbed by overall low efficiency of *in vivo* gene transfer.
- **Incomplete transduction of tumors even with most efficient vectors available:** It is being observed that even with the most efficient vector system and direct intratumoral vector transfer technique, only a few tumor cells get transduced with a nonuniform intratumoral vector distribution. Thus, very low antitumor efficacy is seen.
- **Bystander effect is a curse as well as a boon:** It is a limitation when it shows toxicity to normal cells and a boon when it shows toxicity to tumor cells that has not been transfected with the suicide gene.

Limitations pertaining to suicidal gene therapy can be overcome to some extent:

- Vector distribution within the tumor can be improved by **changing the delivery route of the vector and the way it is applied** (route used could be either intravesical, intraperitoneal, intrathecal or intraventricular and the way it is applied could be repeated or bulk-flow).
- By making use of **replication-competent vectors.**
- **By enhancing the bystander effect** through the transfer of connections that increase the number of gap junctions and **by receptor targeted delivery.**

C. Tumor Suppressor Genes and Anti-Oncogenes

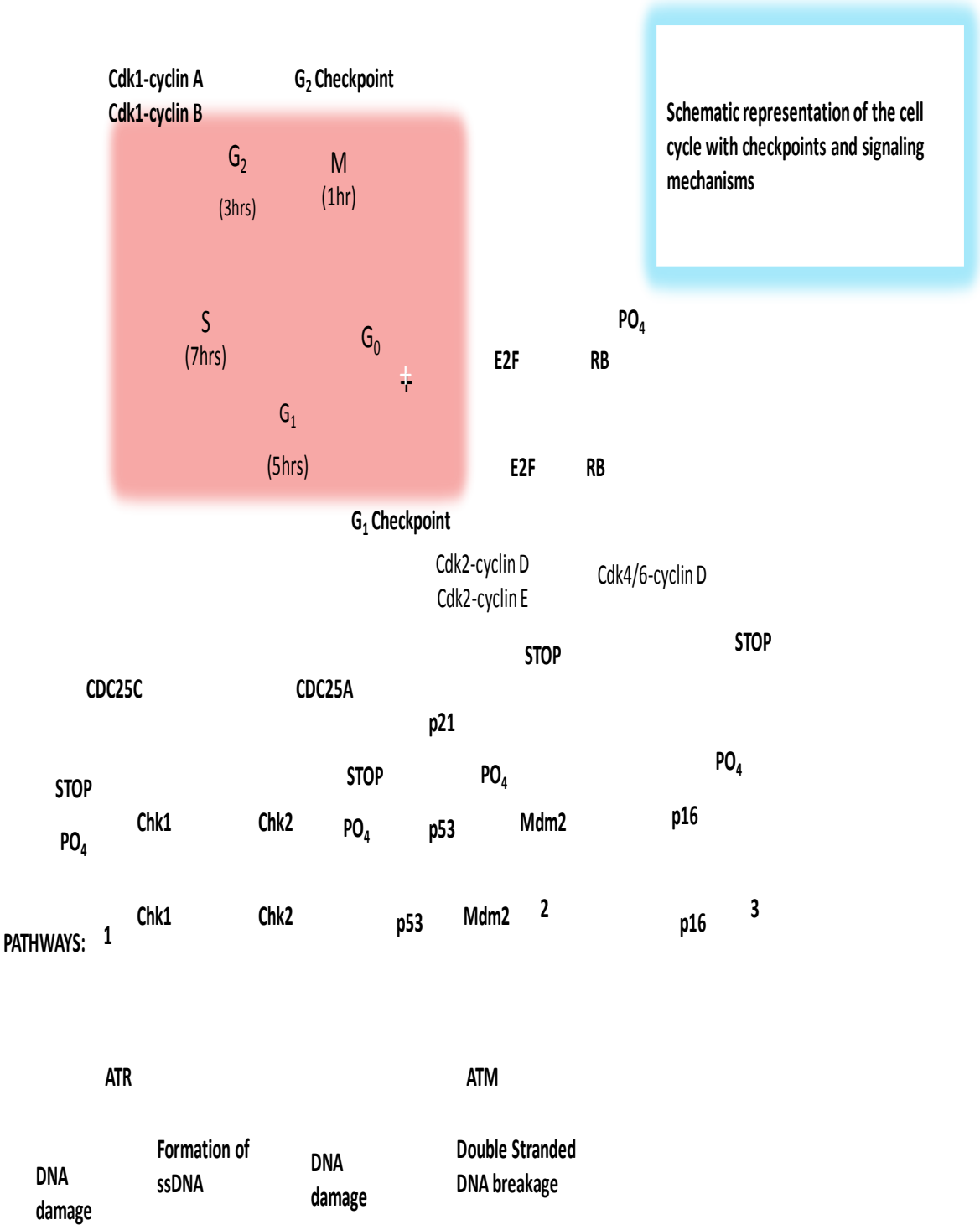
An increasing number of genes (oncogenes and tumor suppressing genes) have been identified so far. These genes get dysregulated during carcinogenesis. Different molecular mechanisms and genetic alteration like gene deletion, mutation, or promoter silencing are the major reasons for gene dysregulation which along with some complex processes leads ultimately to cancer (an example of which has been depicted in supplementary figure 2). These processes of genetic changes and modification cause the deactivation or activation of multiple genes. As a result of these processes the cancer cell proliferates in an uncontrolled fashion and does not enter apoptosis and becomes extremely invasive in nature. Oncogenes get activated as a result of genetic alteration and are involved in promoting carcinogenesis. The tumor suppressing genes become inactivated during carcinogenesis. Current techniques in this field involve the inactivation of over expressed oncogenes by antisense molecules or dominant negative mutants or, alternatively, the reintroduction of tumor-suppressor genes that was lost or mutated. The principle here is not likely the complete reversal of tumor cells back to normal cells (this task is extremely difficult to accomplish as more than one mutation is usually involved in the generation of tumor cells during the transformation of a normal cell to a cancer cell) but the goal instead is to find out the weak point in the cell's regulatory balance which can be exploited and accordingly identify the genes having the highest impact on arresting cell-cycle or involved in apoptosis. Thus, overall understanding of the cellular mechanisms like signal transduction, regulation of cell cycle (as shown in supplementary figure 1), cellular apoptotic processes are extremely important. Targeting those genes which become dysfunctional in the tumor cells is usually the most efficient method to cure cancer. As already known, that mutation in more than one gene is involved in the development of cancer, hence by targeting all those genes specific and efficient treatment of cancer can be achieved. One of the main hurdles to this therapeutic method is the requirement of an efficient high gene-transfer technique. To some extent a weak or moderate bystander effect has been reported with respect to the p53 gene transfer. In those cases where the immune system is weak, an efficient high gene-transfer technique is extremely required to completely eradicate the tumors. With the current vectors available it is not possible to achieve such an efficient high gene-transfer, therefore this highly efficient tumor specific method depends on the

quality of the vector that will be developed in the near future. Out of many tumor suppressor genes that have been identified p53 has shown promise *in vivo* and is being used in clinical investigations and gene therapy for cancer. Many other genes like caspases, PTEN, BRCA1 and proapoptotic genes like bax and bcl-x_s are connected to the regulation of the G1-phase of the cell cycle. All these genes have been evaluated by conducting numerous animal experiments. The entry of cells into S-phase can be prevented by transferring the wild-type and truncated pRb (which in its active form binds to E2F-1, a gene regulatory protein) as well as by keeping pRb in its hypo-phosphorylated active state by cdk/cyclin inhibitors p16 and p21. The efficacy of arresting the cell-cycle for treating established tumors is sufficient or there is a need of a strong apoptotic induction obtained by the transfer of p16 and p27 is yet to be evaluated. Reversal of tumor cells to normal cells by using p53 or p21 can enhance the cells' susceptibility to radiation and chemo-therapy. Tumoricidal effect can be greatly increased by combined activation of a number of tumor suppressor genes as shown for the p53 and p16 genes together. Many clinical trials involving gene therapy directed against oncogenes or apoptosis suppressors like bcl-2 or bcl-xlh are being explored. These anti-oncogene approaches are mainly supported by the recent success of small molecular inhibitors (Gleevec or Imatinib mesylate) of oncogenes which inhibit the EGF receptor, the RAS pathway, and the ABL gene. Gene therapy is efficient in competing with small molecules as small molecules are able to efficiently target enzymes and receptors only, whereas the oncogene can be inhibited by relevant transdominant anti-oncogenes, antisense oligonucleotides, and the highly potent RNA-interfering nucleotides.

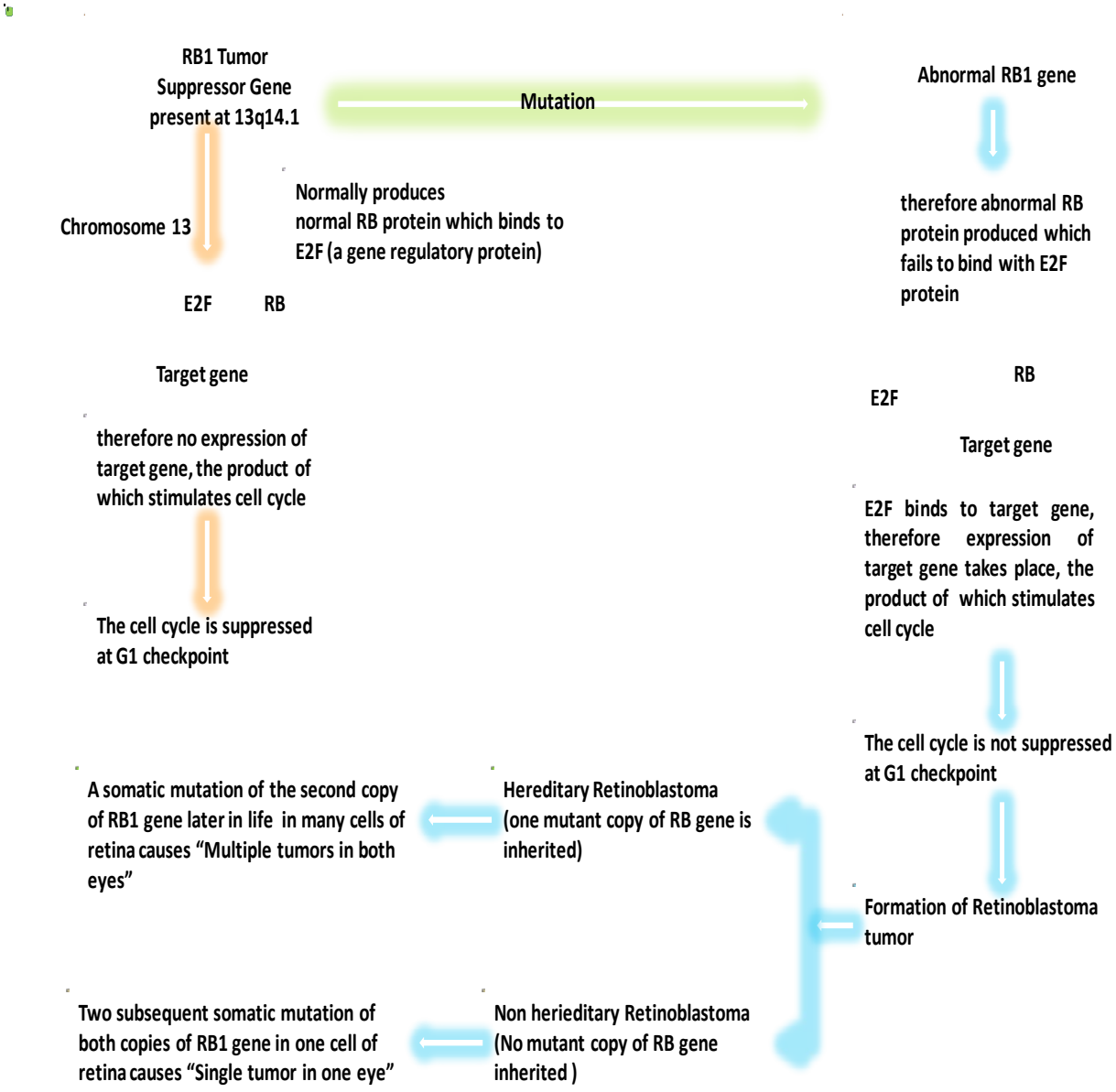
Table 24.2 Tumor –Suppressor Genes:

Tumor-Suppressor-Gene (TSG)	**Protein encoded by the TSG**	**Associated Cancar due to mutation in the TSG**
RB1	Retinoblastoma associated protein $p110^{RB}$	Retinoblastoma, Breast, Prostate, Bladder, and Lung carcinoma
TP53	Tumor protein 53	Li-Fraumeni syndrome, most of the cancers in human being
NF1	Neurofibromin protein	Schwannoma
APC	Adenomatous polyposis coli protein	Familial adenomatous polyposis coli, Colon cancer
WT2	Wilms tumor protein 2	Wilms tumor
VHL	Von Hippel-Lindau disease tumor suppressor protein	Von Hippel-Lindau disease, Retinal and cerebral hemangioblastomas
BRCA1	Breast cancer type 1 susceptibility protein	Breast and ovarian cancer
BRCA2	Breast cancer type 2 susceptibility protein	Breast cancer in both breasts
MLH1	DNA mismatch repair protein MLH1	Hereditary nonpolyposis colon cancer
MSH2	DNA mismatch repair protein MSH2	Hereditary nonpolyposis colon cancer

Supplementary figure 24.2: Schematic representation of cell cycle with the various checkpoints and the signaling mechanisms involved:



Supplementary figure 24.3: Mechanism of action of the tumor suppressor gene: RB1gene:



Lecture 25

Cancer gene therapy (part III)

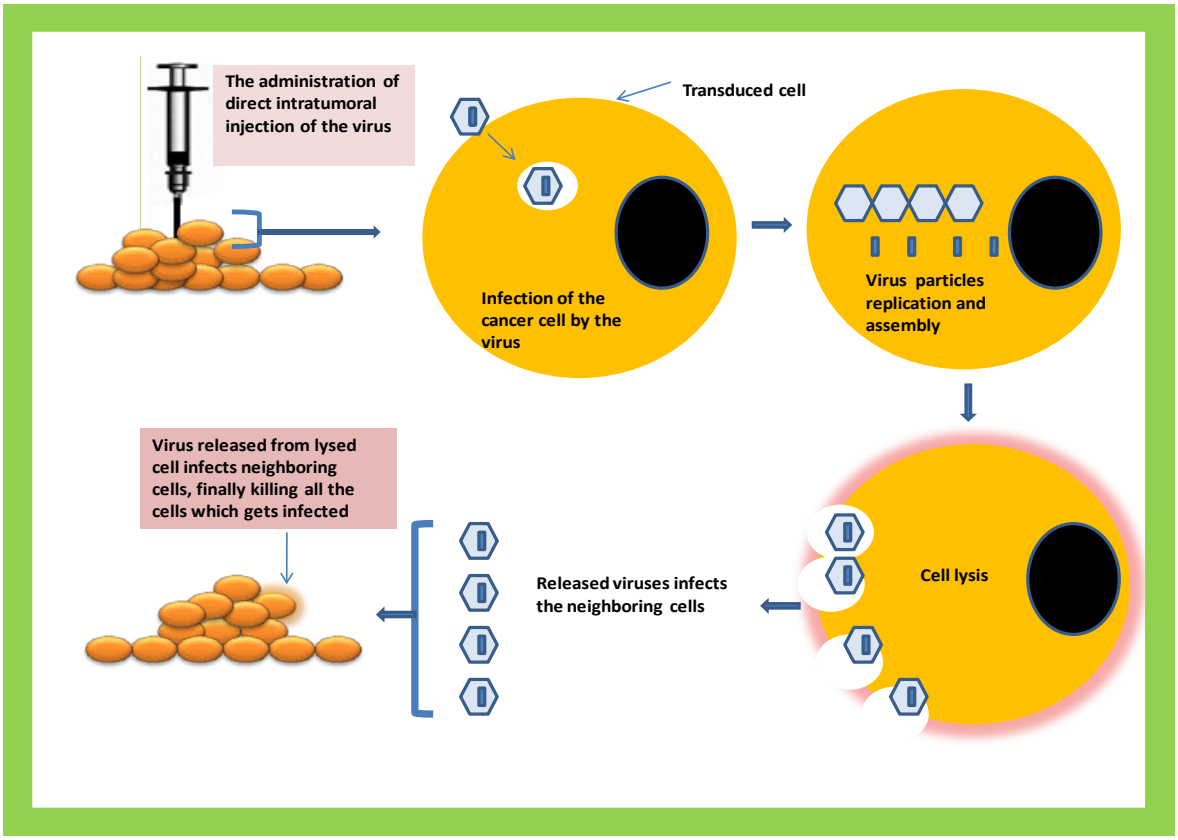
D. Tumor Lysis by Recombinant Viruses

The lysis of tumor by making use of recombinant viruses is one of the most important strategies where viruses may be used as a selective anticancer agent. From the onset of the idea of treating the malignancies of cancer, which was perceived almost in the 1900's by using viruses as an oncolytic agent, it's implementation has been quite challenging. In 1957, adenoviruses were used for the first time in treating cancer.

The basic steps involved in this strategy are as follows:

- The administration of direct intratumoral injection of the virus.
- It leads to the transduction of cancer cells.
- Virus replicates in the transduced cancer cells.
- The transduced cells undergo lysis.
- The viral progeny are released from the lysed cells.
- The released viruses target neighboring cells thus spreading the infection (transfer efficiency is very high).

Figure 25.1. Tumor lysis by recombinant viruses:



The oncolytic virus production and purification techniques are being refined. One of the major concerns with this technique is how to contain the viral replication specific to the tumor site so that much larger amounts of adenovirus can be administered at the tumor site.

A method to enable replication specifically at the targeted tumor has been developed. In this method the adenovirus replication relies on the genetic condition of p53 in the infected host cell. P53 is the best known inducer of apoptosis and it indirectly induces cell cycle arrest. The E1B 55kD gene of the adenovirus blocks p53. When E1B gene is deleted from the adenoviral genome it enables viral replication in p53 lacking tumor cells only but causes apoptosis when viral infection in p53 possessing normal cells takes place. This method was thought to be widely applicable because 50% of common solid tumors lack functional p53. Meanwhile, both the lack of replication in all p53 possessing cells and the potential replication in all p53 lacking cells has been inquired and is little doubtful. Though this approach has few limitations still this concept of conditional replication competent virus is very promising and many

virologists and cancer biologists are exploring all possibilities to develop this approach.

pRB which is a tumor suppressor and an inducer of cell cycle arrest is inactivated by the adenoviral E1A. It helps in adenoviral replication by enabling replication of the cell. Similar to the above method, E1A was mutated and its capability to bind and inactivate pRB was curbed leading to the replication of the virus in tumor cells having defects in the pRb pathway. A successful application of this method both *in vitro* and *in vivo* has been reported.

Another method to attain cancer specific replication of adenoviruses is by using tissue or tumor specific gene expression. For such expression the E1A region of the adenovirus (involved in controlling the replication of the virus through many mechanisms) must be under the control of tissue or cancer specific promoters like probasin promoter, MUC1 promoter, TCF-responsive element etc. rather than under the control of E1A promoter. This approach enabled specific replication in the respective cancer cells and tumors.

Other than adenoviruses, many other oncolytic viruses have been clinically tested in animal models.

Table 25.1 Tissue specific promoter regulation of early region genes is shown below:

Target	**Regulatory element**	**Recombinant virus vector**	**Adenovirus gene regulated**
Prostate	PSA promoter	CN706	E1a
	Kallikrein 2 promoter	CN763, CN764	E1a, E1b
	Rat probasin	CV787, CV739	E1a
	Human PSA	CV739	E1b (E3 region intact)
Prostate and bone metastases	Osteocalcin c	Ad-OC-E1a	E1a
liver	AFP	AvE1A041	E1a

	AFP	GT5610+AdH_B	E1a require helper virus infection
	AFP	CV890	E1a-IRES-E1b-Expression cassette
	AFP	YKL-1001	E1a in a E1b 55K-deleted backbone
	AFP	AdAFPep/Rep	E1a (13s cDNA), E1b 55K-deleted backbone
Neuroblastoma	Midkine	AdMKE1	E1a
Breast	PS2/2-ERE	Ad5ERE2	E1a and E4
	MUC I promoter/enhancer	Ad.DF3-E1	E1a
	3-HRE/5-ERE	AdEHE2F	E1a
	E2F-1	AdEHE2F	E4
Ovary	L-Plastin Truncated protein	Ad-Lp-E1a	E1a
Skin	Tyrosine promoter/enhancer	Ad-Tyr-E1a, Ad-Tyr Δ24, Ad-TyrΔ2Δ24	E1a, E1a Δ24 mutation, E1aΔ2Δ24 mutation
Lung	Surfactant protein B promoter	KD-1-SPB	E4, EIA01/07 backbone, E3-deletion except ADP
Bladder	Uroplakin II promoter	CG8840	E1a-IRES-E1b, E1b-19K deletion
Colon	Tcf-4-binding sites (multiple copies)	vMB19, vCF62	E1a, E1b, E2, E4

Various	E2F-1 promoter	ONYX-411	E1a, E4
Angiogenic endothelial cells	Flk 1 promoter/enhancer	Ad-Flk-1	E1a
	Endoglin promoter	Ad-Flk-1-endo	E1b

Herpes simplex virus (HSV) is a DNA virus that shows tropism to the brain (neurotrophic) and directly lyses the cell while being released. When a 360-bp deletion in the thymidine kinase gene (HSV-tk) was done it prevented HSV replication in normal and quiescent cell but replication in rapidly multiplying cancer cell was observed. This HSV containing a 360-bp deletion in the thymidine kinase gene is called the first generation virus. The second generation viruses were made after incorporating additional mutation that enhanced tumor specific replication and also reduced neurovirulence.

Newcastle Disease virus (NDV) causes very less or no disease in humans. It is being tested for its oncolytic properties and many reports regarding its cytotoxic effects on a number of human tumor cell lines and its resistance towards many human fibroblast lines have been published. The mechanism of its tumor specificity has not been completely understood maybe it is because of an ineffective or failed protective interferon response. The process of killing of tumor cells may be due to virus replication and direct cell lysis or by tumor necrosis factor (TNF) mediated killing (cancer cells are sensitive to stimulated secretion of TNF). High dose intravenous injections of NDV showing good oncolytic activity has been reported.

The future of oncolytic virus strategy to treat cancer is very promising and with an increased knowledge of tumor and virus biology appropriate recombinant oncolytic viruses can be generated. The factors on which the efficiency of the recombinant oncolytic virus based gene therapy for cancer depends are as follows:

- Size and viral genome functionalities.
- Eclipse period.
- Number of infective recombinant virions produced after cell rupture or lysis.
- Mounting of humoral or cellular immune response.

- Tumor characteristics like presence of blood vessels.
- Presence of fibrosis.

E. Antiangiogenic and Antiproteolytic Gene Therapy

There are major differences in this type of gene therapy from the other nonimmunological conventional gene cancer therapeutic methods. The benefits of antiangiogenic and antiproteolytic gene therapy are as follows:

- The resulting products of the genes delivered act outside the cell (extracellular action) because of extracellular action of the gene product majority of tumor cells need not be transduced. Normal tissue are easier to transduce hence may be alternatively targeted.
- These gene therapy methods can be used for long-term treatments and thus can be used to treat monogenetic diseases.
- The requirements to treat monogenetic disease can be satisfied by these gene therapy methods.

The requirement of monogenetic disease treatment includes the following:

- There should be low or no immunogenicity due to the vector and transgene.
- The gene expression should be for long-duration.
- The product of the transgene in the target tissue or in the blood should at least be equal to threshold level.

Inhibition of angiogenesis has become a very promising target for cancer therapy.

Table 25.2 Examples of some of the antiangiogenic genes are shown in the table below:

Vector	**Gene**
Plasmid	Endostatin, Angiostatin
Plasmid transfected cells	Endostatin, Angiostatin, Thrombospondin
Endothelial precursor stem cells	Soluble VEGFR2 (Flk-1)
Adenovirus	Angiostatin, IL-12, Endostatin, Soluble VEGFR2 (Flk-1), Soluble VEGFR2 (Flt-1), TIMPs, TIE-2
Adeno associated virus	VEGF antisense, Angiostatin, Soluble VEGFR2 (Flt-1),
Lentivirus/Stem cell	(TIE-2/TEK) promoter/Thymidine kinase
Moloney murine leukemia virus	Endostatin, Angiostatin, Platelet factor 4, Dominant negative (Flk-1)

The major advantages of targeting the growth of blood vessels in the tumor are as follows:

- We can avoid resistance offered by various mechanisms of the tumor cells.
- Angiogenesis targeting is a broad and highly efficient mechanism since it is the source of nutrition for the proliferating cells.
- Target cells can be easily reached through the vasculature.

Many endogenous inhibitors and stimulators of angiogenesis have been found to act as targets for small inhibitory molecules. It is interesting to note that many chemotherapeutic drugs, being used to treat cancer, target angiogenesis and inhibit vasculature development in cancer cells. Almost 80 antiangiogenic agents are being investigated in several clinical trials. These antiangiogenic agents are small proteins

molecules. Antiangiogenic therapy is expected to be highly efficient during the early stages of the cancer growth.

The antiangiogenic gene therapies have shown promising results under several preclinical settings and are yet to be tested in patients. Among the first genes explored for their efficacy in antiangiogenic gene therapy were Tie2 and FLT-1 genes which encoded for the soluble form of endothelial cell receptor proteins. Functions of these proteins include interaction with the angiogenetic factors angiopoietin-1 and vascular endothelial growth factor (VEGF), respectively. The transfer of the genes Tie2 and FLT-1 was done both *in vivo* and *ex vivo*, it resulted in significant inhibition of tumor growth. Other methods involve the transfer of antiangiogenic proteolytic fragments (example: angiostatin, endostatin), immunomodulatory genes, tissue inhibitors of metalloproteinases (TIMPs), p53, or p16.

Some of the advantages of molecular therapy over the direct application of the gene product or effector molecules are as follows:

- By using targeted vectors the concentration of the antiangiogenic factors within the tumor is enhanced and the potential risk of side effects obstructing processes like wound healing, endometrial maturation, or embryo growth is removed.
- The cost associated with the delivery of gene is lower than that of extended protein therapy.
- For an efficient antiangiogenic therapy a constant inhibitor presence in the blood is desired. This constant inhibitor presence is achievable only by gene therapy and not by effector protein therapy.

Antiangiogenic therapy is in its infancy but its applications are thought to be of immense importance in pre-surgical as well as post-surgical conditions since it makes the tumor static (tumoristatic nature). This strategy along with immunotherapy, chemotherapy and radiotherapy may avoid the reappearance of metastases. The assessment of the progression of cancer can be made from the degradation and restructuring of the extracellular matrix (ECM). The four classes of proteases involved in ECM development are matrix metalloproteinases (MMPs), ADAMs, serine proteinases and the BMP1 (Bone Morphogenic Protein) family. The plasmin

activator inhibitors (PAI) inhibit MMPs, the four known TIMPs and serine proteases. The PAI is an example of inhibitor extensively evaluated for antitumor efficacy and being used for antiproteolytic gene therapy.

Experiments have been conducted in rats with orthotopically implanted tumors and the effect of the adenovirus mediated delivery of TIMP-2, 3 by intravenous and intramuscular injections on blockage of tumor growth has been studied. First generation TIMP-2 adenovirus when directly injected into subcutaneously growing tumors of mammary, colorectal, and bronchial origin caused reduction of tumor size by 60–80% and by 90% in case of metastases of the lung. When TIMP-3 was delivered subcutaneously into growing melanomas by using adenovirus as a vector then also reduction in tumor size by 60-80% was obtained. When Adenovirus mediated TIMP-2 was delivered into the tail vein of immunocompetent mice with orthotopically implanted breast tumors it resulted in blockage of tumor growth by 50%. When intraperitoneal injection of liposomes mediated TIMP-2 for the treatment of spontaneously arising breast tumors was given then a 40% size reduction and a dramatic reduction of the incidence of lung metastases was observed. Thus by varying the approach of delivery based on the target tissue good results can be obtained.

The transfer of anticancer defense genes into the normal noncancerous tissue, is called the ‘‘impregnation’’ approach and has been found to reduce tumor growth by approximately 70-90%. In contrast to the results obtained in above experiments when intramuscular electro-adjuvated injection of naked plasmid with TIMP-4 was administered for the prevention of orthotopically growing breast tumors it resulted in increase in size by 3 times and increase in the occurrence of the primary tumor by 7 times. TIMP-related toxicity has not been reported yet but toxicity related to vector transfer has been observed. PAI-1 is an inhibitor of serine proteases when transfected into tumor cells it was observed to reduce metastasis. Adenovirus mediated PAI-1 gene transfer into tumor cells reduced the growth of uveal primary tumors (tumor in the eye) as well as the occurrence or incidence of metastases. Similar gene transfer of PAI-2 showed tremendous antitumoral effects. In contrast to above results transgenic mice overexpressing PAI-1 developed pulmonary tumor at the same rate as wild-type mice in a melanoma model. PAI-1 knock-out mice however showcased a reduced local invasion and vascularization in the transplanted malignant keratinocytes tumor. When The PAI-1 was restored by adenovirus mediated gene transfer then primary

tumor development and the vascularization of the tumor was seen. These results are indicative of the fact that antiproteolytic therapy with PAIs, and to some extent with TIMPs, can have enhanced antitumor efficiency. Similar to the MMPs and TIMPs, the PAs and PAIs have displayed effects independent from the degradation of the ECM. Therefore, increased knowledge of either protease or inhibitor system would help us understand the underlying mechanisms and apply the relevant therapeutic mechanism for the treatment of cancer.

F. Drug-Resistance Genes

Normal cells can be made resistant towards the toxic effects of chemotherapy thus instead of killing the tumor cells, this strategy can be used to prevent toxic side effects of modern chemotherapy. Using this method, discrimination between proliferating normal and cancer cells by the chemotherapeutic agent can be achieved. The high chemotherapeutic drug dose affects the hematopoietic precursor cells as at high concentration they directly limit or suppress the bone marrow functionalities. To subdue this limitation of over-dose effect many experimental methods or strategies have been designed. One of the strategies is to transport the drug out of the hematopoietic cells by harboring drug resistant genes like transporters which enables extraction and removal of drugs over the plasma membrane. This strategy makes use of several genes like multiple drug-resistance gene 1 (MDR1), the multiple drug-resistance proteins (MRPs) and several enzymes which imparts drug resistance characters to alkylating agents such as glutathione-S-transferase and O^6-alkyl-guanine DNA alkyltransferase or antimetabolites such as dihydrofolate reductase (DHFR). A P-glycoprotein is encoded by the MDR1 gene which acts as a multidrug transporter and causes extrusion of drugs across the cell membrane. The P-glycoprotein is involved in effluxing the amphipathic and hydrophobic compounds from the cells. Several chemotherapeutic drugs widely used in clinical studies are of amphipathic and hydrophobic in nature. The increased resistance of hematopoietic precursor cells to chemotherapy in rodent models have been observed after the *ex vivo* transfer and expression of the MDR1 gene in hematopoietic progenitor cells. A very low level expression of the transferred drug resistant gene was seen most of the time in the early clinical trials but, recent reports of long term expression of the drug resistant gene *in vivo* with hematopoietic cell recovery has been published. Further clinical improvements, by efficient transduction and optimization of drug selection strategies,

can be achieved (especially in case of very immature cells). Clear indications of the feasibility of successful transduction in human hematopoietic stem cells is based on the report of *in vivo* correction of X-linked severe combined immunodeficiency (SCID) with at least 17 out of 20 patients now recovering from this life saving gene therapy.

G. Marker Genes

Marker genes are being used for tracing purpose in almost 6% of all the protocols used for gene therapy clinical trials. It has enabled the practical visualization of the assumption for reappearance of cancer due to the presence of tumor cells as contaminants in the reinfused bone marrow after autologous bone marrow transplantation. Another set of studies were conducted to perceive or understand the long term survival of hematopoietic stem cells or T-cells by marking them with tracer genes and tracing them for long durations.

Lecture 26

RNA-DNA chimera

26.1 Introduction:

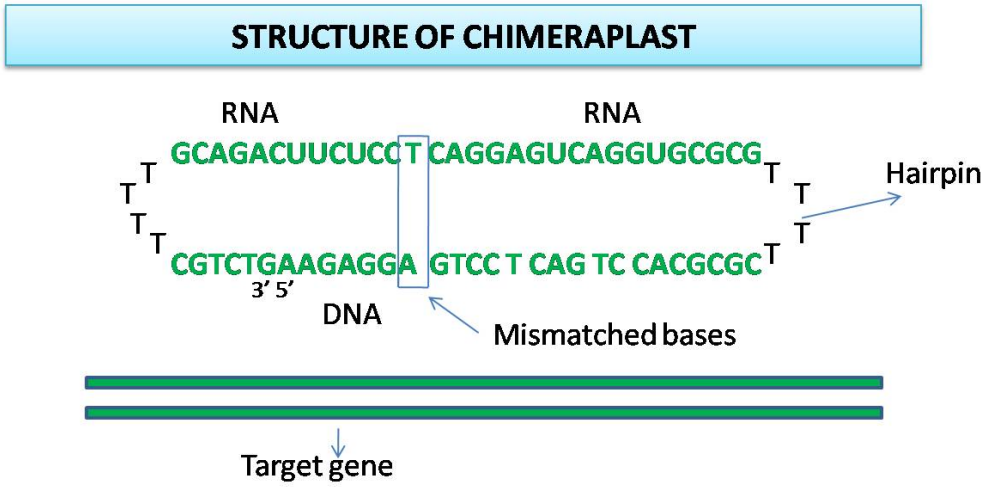
Chimeraplasty is defined as the technique of synthetically creating a hybrid molecule consisting of both RNA and DNA and is being explored as a non viral method for gene therapy. It works in both plants as well as animals. RNA-DNA chimera is used to repair point mutations, deletions, or insertions in DNA. It is used for targeted gene correction.

Dr. Eric Kmiec began with the idea of chimeraplasty while studying homologous recombination where he observed an enhanced recombination rate during transcription. He realized that stability of RNA inside a cell could be increased when coupled with DNA and this RNA/DNA hybrid could be used to invoke gene repair by the endogenous repair mechanism of the cell. His first study was based on producing an oncogenic mutation in *ras* gene by changing thymine to guanine at a critical segment. The chimera he designed consisted of 25 bases of which a 5 bases DNA segment was flanked by two segments of 10 bases RNA. Kyonggeun Yoon in her study corrected point mutation using chimeraplasty in the human alkaline phosphatase gene introduced in CHO cells with efficiency up to 30%. Yoon showed that RNA/DNA chimera was capable of introducing as well as correcting mutation. Later on Kmiec, Yoon, Allyson Cole-Strauss conducted experiments to treat sickle cell anaemia caused by a point mutation. From then onwards a lot of experiments have been conducted some with success and some with failure thus putting a question over the efficacy and limitations of this technology.

26.2 Structure of the Chimeraplast:

A chimeraplast looks like a paper-clip shaped structure as shown in the figure below. It consists of a double-stranded DNA that is flanked with short strands of RNA.

Figure 26.1 Structure of a chimeraplast:



Hybrids of RNA and DNA have been found to actively participate in homologous pairing reactions, based on this concept the structure of chimeraplast was designed. The hairpin cap provides protection to the RNA-DNA chimera from exonucleases and cellular helicases. It also provides stability to the molecule and does not interfere in the base pairing of the RNA-DNA chimera molecule with the target gene. To provide protection to the RNA from cleavage by RNase H the ribose is methylated at the 2'-OH. The short RNA strands serve the purpose of activating the oligonucleotide ("oligo") for homologous recombination.

26.3 Design

To design a chimeric oligonucleotide the following steps are used:

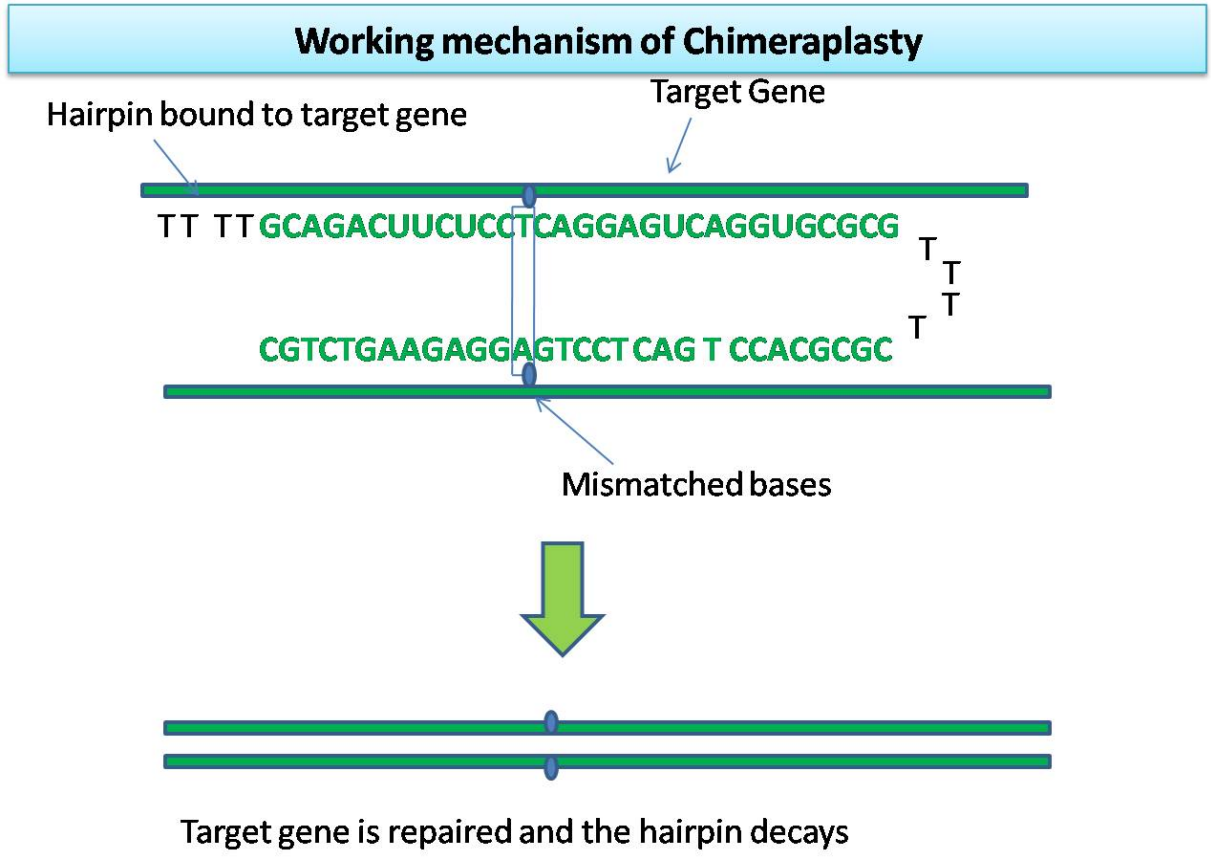
- Replicate a short portion of the base sequence of the target gene surrounding the base pair mutation (it should align perfectly with the gene, except for the one base pair where the mutation occurs).
- Substitute the correct base into the oligo at the place of mutation.

Thus, when the chimera inserts into the target DNA and pairs with the target gene, the mismatched base pair is recognized by the endogenous repair system. The change in sequence takes place either in the chimeraplast where the target DNA acts as a template or in the target DNA where the chimera acts as a template.

26.4 Mechanism of Repair

In the cell free extract, mechanism of repair involves simple homology pairing wherein homology chimeric oligos pair with a plasmid target using DNA pairing enzymes and complexes. The mismatch between the gene and the chimera is recognized by the endogenous repair machinery that uses mismatch repair to correct the wrong nucleotide by using the chimera as the template sequence.

Figure 26.2 Working mechanism of chimeraplasty:



Once the target gene is repaired by the repair machinery, the chimera is no longer needed and decays, leaving only the corrected target DNA.

A successful in vivo repair study was carried out at the University of Arizona in Tucson by Li-Wen Lai by binding oligos to organ-specific ligands. Chimeric molecules can also be delivered to appropriate cells or tissue by liposomes, synthetic polymers and microscopic gold particles coated with the chimeric molecules (in plants). Once delivered inside the cell the oligos enter the nucleus where they specifically bind with the target DNA and invoke the mismatch repair mechanism that is performed by the cell's endogenous repair system. After the point mutation in the target gene is corrected the chimera decays.

As shown in the figure above, the chimeraplast appears like a paper-clip in which the double stranded stretch of DNA is flanked with short strands of RNA. The box shown in the figure has mismatched bases rest all the bases between the chimeraplast and the target gene are correctly matched. The incorporation of the chimera between the two DNA strands is enabled by the binding of the hairpin loop with the target gene. After successful base pairing of the chimeraplast with the target gene, the endogenous repair mechanism recognizes the mismatched bases and changes the bases of the target gene by using chimera as the template.

Thus, the endogenous repair system corrects the mismatch and repairs the target gene. After the correction of the point mutation in the target gene the hairpin of the chimera decays. As a result, the chimera dissociates and gradually decays leaving behind the repaired target gene.

26.5 Applications of Chimeraplasty:

- **Gene Therapy for point mutations:** Chimeraplasty has not been used in human subjects till date due to its unproven credibility and specificity, yet, it has a huge potential to correct genetic diseases like Sickle Cell and Crigler-Najjar Syndrome that occur due to a point mutation. Since, chimeraplasty can successfully alter maximum three consecutive base pairs in a gene hence it is a suitable candidate for treating diseases with point mutations.
- **Gene Therapy for autosomal dominant mutations:** Another promising role of chimeraplasty can be correction of autosomal dominant mutations. Even one mutated gene in dominant mutation is enough to cause the disease because dominant gene expresses itself even in heterozygous condition thus the

addition of a second normal gene by traditional gene therapy method is not capable of overcoming the expression of the defective gene. Chimeraplasty can be used effectively to correct the autosomal dominant defective gene itself.

- **"Knock out" genes:** Chimeraplasty can be used to study the difference between the functional as well as the non-functional state of a gene. It would allow the knocking out of genes from adult tissues and enable designing and studying knock out strategies. Using chimeraplasty scientists can create knock out animal models for various human diseases.

1. Using chimeraplasty only small corrections of maximum three consecutive base pairs in a gene can be performed.

2. One of the main problems faced with chimeraplasty is that the rate at which the gene correction takes place cannot be predicted or determined. Variation in gene correction between 1% and 40% has been reported and thus proves its unpredictable correction rate. The reason of such a variation remains unknown but may be linked to the improper delivery of chimera to the target DNA.

3. Another drawback of chimeraplasty is that the chimeric molecule may accidently act unspecifically causing alteration in other genes that are closely related to the target gene (due to sequence similarity of genes within a family) causing unwanted and harmful effects.

4. The RNA DNA hybrid is not robust enough and has been found to degrade within 48 hours.

Lecture 27

Gene Therapy for Crigler-Najjar Syndrome (part I)

Crigler-Najjar syndrome is (an autosomal recessive trait) a genetic disease, the main characteristics of this disease being accumulation of high levels of free or unconjugated bilirubin in blood also called hyperbilirubinemia. During the process of normal turnover of the red blood cells (RBC), hemoglobin is broken and the waste product that is generated is bilirubin. It is produced from the reticuloendothelial cells (macrophages) when they take aged erythrocytes and degrade the heme moiety present in hemoglobin. Bilirubin is insoluble in water and before excretion in the bile it must be conjugated with a substance called UDP-glucuronic acid. This process takes place in the hepatocytes where, the bilirubin-uridine diphosphoglucuronate glucuronosyltransferase (B-UDPGT) also known as UDP-glucuronosyltransferase 1A1 (UGT1A1) enzyme which is expressed in human liver helps in the conjugation of bilirubin with UDP-glucuronic acid. To understand this process let us go through the metabolism of hemoglobin and glucuronidation reaction (Figure 27.1).

1. RBC has a mean life of 120 days. On completion of this duration it undergoes senescence releasing heme protein.

2. Breakdown of heme to bilirubin takes place in the macrophages of reticuloendothelial system (tissue macrophages, spleen and liver). First, heme is oxidized in the presence of heme oxygenase to biliverdin which is then reduced in the presence of biliverdin reductase to bilirubin.

3. Unconjugated or free bilirubin is complexed or bound with albumin and transported through blood to the liver. Unconjugated or free bilirubin which is not bound to albumin is fat soluble and travels to the brain.

4. Bilirubin is taken up by the hepatocytes through facilitated diffusion. One molecule of bilirubin is conjugated with 2 molecules of UDP-glucuronic acid by the process called “glucuronidation” and is one of the methods used by the cells for detoxification.

Some facts:

UDP-glucuronic acid is synthesized in the cytosol by the UDP-glucuronic acid synthetic pathway in the presence of UDP-glucose dehydrogenase.

5. Glucuronidation is catalyzed by UDP-glucuronosyltransferases (UGTs) which belongs to a super family of endoplasmic reticulum membrane bound enzymes residing on the luminal surface of the endoplasmic reticulum. The UGT activity is highest in liver, kidney and intestine (activity depends on factors like age, gender, hormonal status, genetic factors and environmental exposures). Two large families of UGT, sharing more than 50% amino acid identity, UGT1 and UGT2 exist.

In humans UGT1 exist as 9 isozymes (UGT1A1, UGT1A3-UGT1A10) and in rats it exists as 7 isozymes (UGT1A1-UGT1A3, UGT1A5-UGT1A8). The UGT1 family members contain identical carboxyl terminal sequence since they share four exons located at the 3' end of the UGT1 locus. At the 5'end of the common exons a series of first exons encoding for the complete amino terminal sequence of the UGT1 family are positioned. The amino terminal codons are unique for each member of the UGT1 family. The naming of UGT1 genes is based on the relative position of the first exon with the common exon. The first exon of UGT1A1 is closest to the common exon whereas first exon of UGT1A10 is the farthest from the common exon. Each overlapping gene in the UGT1 gene complex has a unique promoter which enables the gene to independently regulate its activity in tissues in response to different inducing stimuli.

The UGT2 family is encoded by independent genes. In humans UGT2 exist in six forms (UGT2B4, UGT2B7, UGT2B10, UGT2B11, UGT2B15 and UGT2B17). In rats it exists in seven forms (UGT2A1, UGT2B1, UGT2B2, UGT2B3, UGT2B6, UGT2B8, and UGT2B12). In human the UGT2 isoforms are encoded by a cluster of genes present on the chromosome 4.

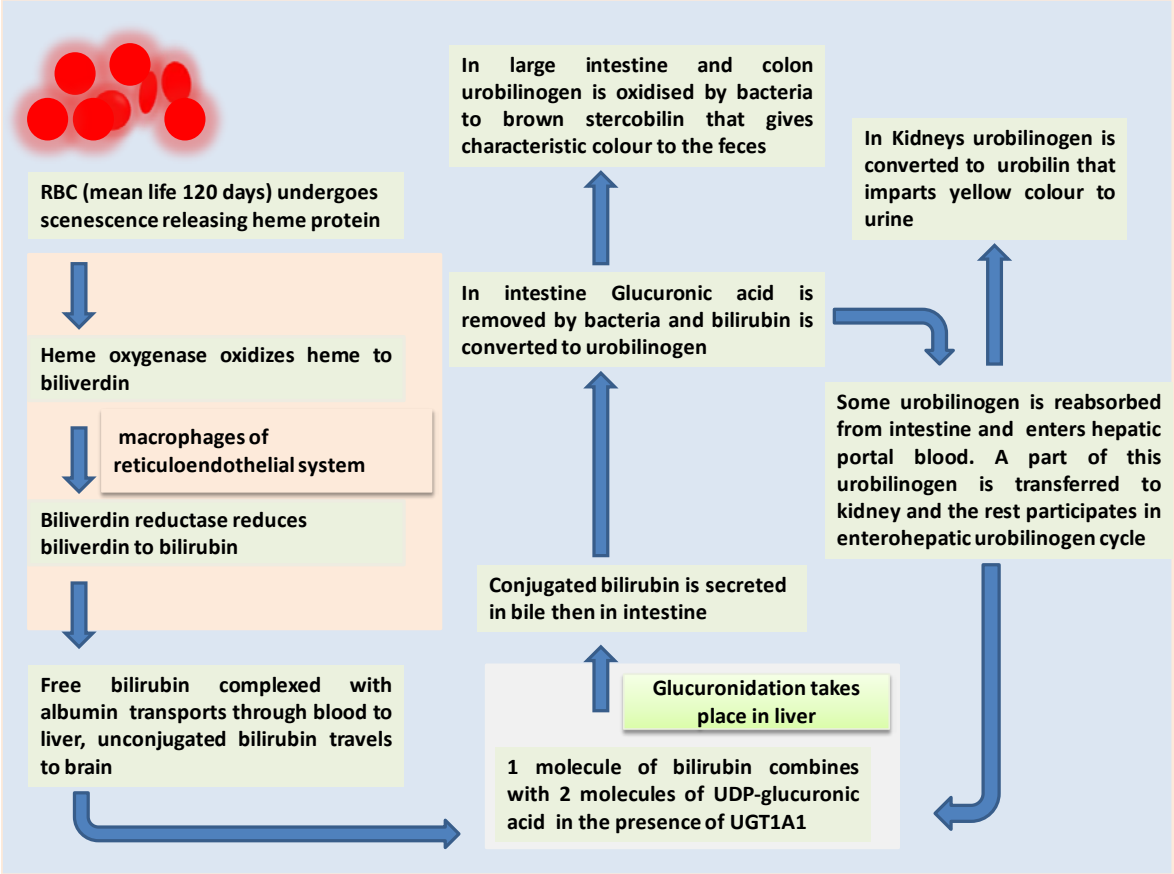
The UGT1A1 is involved in the glucuronidation of bilirubin whereas all others are involved in the glucuronidation of xenobiotics and other endotoxic substances. The full length cDNA of the gene UGT1A1 has been successfully cloned and sequenced.

6. After successful conjugation the conjugated bilirubin is secreted into the bile and then into the intestine.

7. In the intestine the glucuronic acid is removed by the bacteria and the bilirubin is converted to urobilinogen. Some amount of urobilinogen is reabsorbed from the intestine and enters the hepatic portal blood.

8. A part of this urobilinogen participates in the enterohepatic urobilinogen cycle whereas the remaining urobilinogen is transported to the kidneys where it is converted to urobilin that imparts yellow color to the urine. In the large intestine and colon the urobilinogen is oxidized by the bacteria to brown stercobilin that imparts the characteristic color to the feces.

Figure 27.1 Metabolism of hemoglobin and glucuronidation reaction:



Crigler-Najjar syndrome is a rare autosomal recessive disorder with an estimated case of 0.6–1.0 per million live births and is of two types. In type I patients the enzyme UGT1A1 is inactive whereas in type II it is severely reduced. In type I serum bilirubin level is usually above 345 μmol/L (310–755) which is very high as compared to normal serum bilirubin level of 2–14 μmol/L. Type I is difficult to treat as compared to type II. In type I the unconjugated bilirubin cannot be excreted into the bile and remains in the blood causing an elevated level of unconjugated bilirubin in plasma that leads to jaundice and may lead to kernicterus (bilirubin encephalopathy).

Long back when phototherapy was unavailable many children afflicted with Crigler-najjar syndrome died of kernicterus (bilirubin encephalopathy) some who survived till early adulthood developed neurological damage. The therapy includes

- 12h/d phototherapy (phototherapy becomes ineffective at later stage)
- Exchange transfusions of blood in the immediate neonatal period
- Use of heme oxygenase inhibitors
- Administration of oral calcium salts (help in complexes formation with bilirubin in the gastrointestinal tract)
- liver transplantation (Surgical intervention before the onset of brain damage and at later age when phototherapy becomes ineffective)

Lecture 28

Gene Therapy for Crigler-Najjar Syndrome (part II)

28.1 The Gunn rat, a model for Crigler-Najjar syndrome

Gunn rat is named after the scientist C. H. Gunn, who first discovered mutant rats in 1934 (published in 1938) in a Wistar rat colony maintained at the Connaught Laboratory at Toronto in Canada. The rats were observed to have jaundice due to an autosomal recessive characteristic. It took several decades to unravel this deficiency in the Gunn rats at the molecular level. The UGT1A1 gene in this strain bears a premature stop which leads to a subsided UGT1A1 activity. In 1952 the syndrome, explained by John Fielding Crigler and Victor Assad Najjar, was found to correspond with the conditions of the Gunn rat and is said to be the human analogue of the crigler-najjar syndrome.

A mouse knock-out model (Ugt1-/- mice), with a disorganized UGT1 locus, has been built. In these mice the unconjugated levels of bilirubin are very huge and the animals commonly die within 2 weeks after birth.

Crigler-Najjar syndrome appears to be an attractive model disease for the build-up of liver directed gene therapy for several reasons.

- Crigler-Najjar syndrome is a well-defined disease both at the biochemical and molecular level, the pathway of which has been illuminated.
- The restoration of even 5% of the normal UGT1A1 activity decreases serum bilirubin sufficient to lessen the danger on brain damage thus permitting patients under therapy to stop phototherapy treatment.
- Efficacy of the treatment can be easily audited by measurement of bilirubin and conjugates in serum and bile.

28.2 Plasmid mediated gene therapy for Crigler-Najjar syndrome

Examination of plasmid mediated gene therapy for Crigler-Najjar syndrome was done by Wilke et al. Through his experiments he demonstrated that it was a practical approach for *in vitro* transfection of a plasmid encoding human UGT1A1 that recovered bilirubin glucuronidation in Gunn rat hepatocytes. However, the *in vivo* function of this procedure was noticed to be impractical without specific delivery of the plasmid to the liver cells.

An approach for targeting DNA to liver cells *in vivo* is by associating it to **asialoorosomucoid** which is a ligand for the liver specific **asialoglycoprotein** receptor. Due to endosomal degradation of the plasmid DNA the efficiency of this strategy was found to be very low in Gunn rats. Inhibition of the endosomal degradation of DNA by administration of an endosomolytic agent, depolymerization of microtubules or by partial hepatectomy was observed. An efficient liver transduction was accomplished upon combining asialoglycoprotein receptor mediated delivery with the administration of the microtubule inhibitor colchicine prior to plasmid injection. By this method 25-35% decrement in the serum bilirubin level was recorded for several weeks in the Gunn rat.

28.3 Chimeraplasty for Crigler-Najjar syndrome

A single guanosine deletion results in UGT1A1 deficiency in the Gunn rat. The chimaeric RNA/DNA oligonucleotides have been proclaimed to repair this single guanosine deletion. According to Kmiec's theory these so called chimeraplasts which are constructed to absolutely align to the targeted mutation, insert an error that induces DNA repair. The endogenous repair mechanism then later mends the error by homologous recombination. Intravenous administration of such a chimeraplast mixed with polyethyleimine (PEI) or encapsulated in anionic liposomes lead to the repair of 20% of the UGT1A1 genes in the Gunn rat liver as revealed by Kren and coworkers.

However, many articles have raised serious doubts with regard to the efficiency of gene correction obtained by chimeraplasty.

28.4 Adenovirus as a vector for treating Crigler-Najjar syndrome

Advantages of using Adenovirus as a vector for crigler-najjar syndrome are as follows:

- Systemic administration of adenoviral vectors enables their highly selective uptake in the liver. This is one of the major advantages of using adenoviral vector for liver directed gene therapy.
- Adenoviral vectors are competent of mediating high levels of gene expression in liver cells.
- Adenoviral vectors can be generated with high titer stocks.
- Adenovirus genome permits the insertion of big sizes of foreign DNA.
- The adenoviral genome is present in an extra chromosomal (episomal) form in the nucleus and thus does not have a liability for insertional mutagenesis.

Early 1 coding regions are needed for expression of all other adenoviral genes. The first- generation of replication deficient adenoviral vectors were stripped of their early 1 (E1) coding regions. The deletion of the E1 genes inhibits viral replication but it does not entirely silence the expression of adenoviral proteins. The expression of these proteins causes a quick and pronounced immune response against cells transduced by E1 deleted adenoviral vectors. In Gunn rats the significant reduction of serum bilirubin levels obtained with these vectors persisted only up to 7 to 12 weeks after injection due to vector immunogenicity.

Adenovirus will cause harmful effects because of the virulent nature of this virus in patients with impaired immune system resulting in immune rejection. Therefore for such patients the following strategies can be used.

- Induce only a transient block of immune suppression responses using drugs. The effective transient immune suppression was attained with FK506 to allow long-term gene expression by repeated administration of an adenoviral vector.
- An additional approach that seems practical to overcome immune rejection is reinserting the adenoviral immune modulating E3 region.
- Expression of other immune modulating proteins such as cytotoxic T-lymphocyte antigen 4 Ig (CTLA4Ig), which inhibits co-stimulatory

mechanisms between the antigen presenting cells (APC) and the cytotoxic T lymphocytes (CTL).

28.5 Helper-dependent adeno virus as a vector for treating Crigler-Najjar syndrome

One of the major causes of the strong immunogenicity of the first generation adenoviral vector is the residual expression of viral proteins. In order to overcome this, all viral coding sequences from the vector must be eliminated. The helper-dependent Adeno virus (HD-Ad) that lacks all the viral coding sequences and only consists of the adenoviral ITRs (inverted Terminal repeats) and packaging signal sequences is called "gutless" vector. These "gutless" vectors are less immunogenic and less cytotoxic because of the entire absence of viral gene expression. *In vivo* these vectors lead to a longer expression of the encoded therapeutic genes in the liver cells.

It has been stated that the administration of a single injection with an optimum dose of $3x10^{12}$ viral particles per kg of a HD-Ad encoding the human UGT1A1 gene results in an 80% decrease in serum bilirubin levels for up to 2 years. This constant and important reduction makes HD-Ad a potential vector for liver directed gene therapy for Crigler-Najjar syndrome.

28.6 Adeno-associated virus as a vector for treating Crigler-Najjar syndrome

Adeno-Associated viral vectors are highly favorable to be used as a vector for crigler-najjar syndrome.

Advantages of using Adeno-Associated Virus are as follows:

- It is a non-pathogenic virus that cannot replicate independently.
- It has a broad host range.
- It has the ability to transduce non dividing cells.
- It shows low immunogenicity.
- It shows persistent expression and is stable.
- AAV serotypes, such as AAV-5 and AAV-8 have a tropism for the liver.

Major defects associated with AAV are as follows:

- It has a small genome size (4.7kb).
- It exhibits episomal persistence.

The therapeutic potential of different AAV serotypes has been tested in Gunn rats. A lifelong, 70% reduction of serum bilirubin levels has been achieved by injecting $2.5x10^{12}$gc/kg of ssAAV1-hUGT1A1 through portal vein injection in Gunn rats.