CHAPTER II

REVIEW OF THE RELATED LITERATURES

1. Down syndrome

A. History

An English physician, John Langdon Down first characterized Down syndrome as a distinct form of mental retardation in 1862, and in a more widely published report in 1866 entitled "Observations on an ethnic classification of idiots" (*29*). Due to his perception that children with Down syndrome shared physical facial similarities (epicanthal folds) with those of Blumenbach's Mongolian race, Down used terms such as mongolism and mongolian idiocy (*30*). Idiocy was a medical term used at that time to refer to a severe degree of intellectual impairment. Down wrote that mongolism represented "retrogression," the appearance of Mongoloid traits in the children of allegedly more advanced Caucasian parents.

By the 20th century, "mongolian idiocy" had become the most recognizable form of mental retardation. Most people with it were institutionalized. Few of the associated medical problems were treated, and most died in infancy or early adult life.

Until the middle of the 20th century, the cause of Down syndrome remained unknown, although the presence in all races, the association with older maternal age, and the rarity of recurrence had been noticed. Standard medical texts assumed it was due to a combination of inheritable factors which had not been identified. Other theories focused on injuries sustained during birth (*31*). With the discovery of karyotype techniques in the 1950s, it became possible to identify abnormalities of chromosomal numbers or shapes. In 1959, Professor Jérôme Lejeune discovered that Down syndrome

resulted from an extra chromosome (*32*). The extra chromosome was subsequently labeled as the 21st, and the condition as trisomy 21.

In 1961, a group of nineteen geneticists wrote to the editor of The Lancet suggesting that mongolian idiocy had "misleading connotations," had become "an embarrassing term," and should be changed (*33*). The Lancet supported Down's Syndrome. The World Health Organization (WHO) officially dropped references to *mongolism* in 1965 after a request by the Mongolian delegate (*34*).

In 1975, the United States National Institute of Health convened a conference to standardize the nomenclature of malformations. They recommended eliminating the possessive form: "The possessive use of an eponym should be discontinued, since the author neither had nor owned the disorder (*33*). While both the possessive and non-possessive forms are used in the general population, Down syndrome is the accepted term among professionals in the USA, Canada and other countries, while Down's syndrome continues to be used in the United Kingdom and other areas (*35*).

B. Clinical features

Down syndrome, a particular combination of phenotypic features that includes mental retardation (IQ 50–70) to moderate range (IQ 35–50), and characteristic facies is caused by trisomy 21, one of the most common chromosomal abnormalities in liveborn children.

It has long been recognized that the risk of having a child with trisomy 21 increases with maternal age. For example, the risk of having a liveborn with Down syndrome at maternal age 30 is one in 1,000 and at maternal age 40 is nine in 1,000. Although the risk increases with maternal age, 80% of children with Down syndrome are born to women under the age of 35 reflecting the overall fertility of that age group. Other than maternal age, no other risk factors are known. However, in up to 12% of trisomy 21 cases, the extra chromosome comes from the paternal gamete (*36*). There does not appear to be a paternal age effect.

Many standard prenatal screens can discover Down syndrome. Genetic counseling along with genetic testing, such as amniocentesis, chorionic villus sampling (CVS), or percutaneous umbilical blood sampling (PUBS) are usually offered to families who may have an increased chance of having a child with Down syndrome, or where normal prenatal exams indicate possible problems. Genetic screens are often performed on pregnant women older than 30 or 35.

Patients with Down syndrome develop the neuropathologic hallmarks of Alzheimer disease at a much earlier age than individuals with Alzheimer disease without trisomy 21 (*37*). Characteristic senile plaques and neurofibrillary tangles are present in the brain of all individuals with Down syndrome over the age of 40 years (*37*). The triplication of the amyloid precursor protein gene (APP) may be the cause of this phenomenon. Several mutations in the APP gene have been described in families with early-onset Alzheimer disease without trisomy 21.

C. Cytogenetics

Most Down syndrome individuals (95%) with trisomy 21 have three free copies of chromosome 21; in about 5% of patients, 1 copy is translocated to another acrocentric chromosome, most often chromosome 14 or 21 (*38,39*). In 2 to 4% of cases with free trisomy 21, there is recognizable mosaicism for a trisomic and a normal cell line (*40*).

- *Origin of Free Trisomy 21*

The availability of highly informative DNA markers has allowed the parental origin of the extra chromosome 21 and the meiotic/mitotic origin to be determined. More than 400 families have been studied (*41*) and the results are as follows : 1) Errors in meiosis that lead to trisomy 21 are overwhelmingly of maternal origin; only about 5% occur during spermatogenesis. 2) Most errors in maternal meiosis occur in meiosis I and the mean maternal age associated with these is 32 years (the mean maternal age of the general population is approximately 27 years). Thus, meiosis I errors account for 76 to

80% of maternal meiotic errors and 67 to 73% of all instances of free trisomy 21. 3) Maternal meiosis II errors constitute 20 to 24% of maternal errors and 18 to 20% of all cases of free trisomy 21. The mean maternal age is also advanced and is 31.4 in one study and 34.1 in another. 4) In rare families in which there is paternal nondisjunction, most of the errors occur in meiosis II. The mean maternal and paternal ages are similar to the mean reproductive age in western societies. 5) In 5% of trisomic individuals, the supernumerary chromosome 21 appears to result from an error in mitosis. In these cases there is no advanced maternal age and there is no preference for which chromosome 21 is duplicated in the mitotic error.

- Origin of Translocation Trisomy 21

All de novo t(14;21) trisomies studied have originated in maternal germ cells (*42,43*). The mean maternal age was 29.2 years. In de novo t(21;21) Down syndrome the situation is different (*43, 44, 45*). In most cases (14 out of 17) the t(21;21) is an isochromosome (dup21q) rather than the result of a Robertsonian translocation caused by a fusion between 2 heterologous chromatids. About half were of paternal and half of maternal origin. In the 3 de novo t(21;21) true Robertsonian trisomy 21 cases, the extra chromosome 21 was maternal.

D. Mapping

- Down Syndrome Critical Region

Mapping of the chromosomal region, if triplicated, results in the phenotypic characteristics of Down syndrome has been facilitated by the use of DNA samples from individuals who have partial trisomy 21 with or without features of the Down syndrome phenotype (*46, 47, 48, 49, 50*). Although detailed analysis of these DNAs is still under way, an area of approximately 5 Mb between loci D21S58 and D21S42 has been identified that is associated with mental retardation and most of the facial features of the syndrome. In particular, a subregion that includes D21S55 and MX1 (interferon-induced protein p78), the latter being located in band 21q22.3, has been associated with mental retardation and several morphologic features, including oblique eye fissure, epicanthus,

flat nasal bridge, protruding tongue, short broad hands, clinodactyly of the fifth finger, gap between first and second toes, hypotonia, short stature, Brushfield spots, and characteristic dermatoglyphics (*49*). Additional phenotypic characteristics may map outside the minimum critical region (symbolized DCR). Material from other rare patients who have features of Down syndrome but no visible chromosomal abnormality may help to narrow down the critical region. In several such studies, however, no triplicated region has been identified (*47, 49*). It is possible that these patients do not have any chromosome 21 abnormality and their phenotype is a phenocopy of Down syndrome.

By analysis of a 3-generation Japanese family containing 4 Down syndrome individuals with partial trisomy 21 Korenberg (*48*) and Ohira (*51*) defined a 1.6-Mb region between LA68 and ERG in 21q22 as the Down syndrome critical region. They constructed a contig map covering more than 95% of this 1.6-Mb region.

E. Molecular genetics

- *Genes Within the Down Syndrome Critical Region*

Extensive studies on DS patients with partial trisomy 21 have allowed researchers to narrow the search for genes associated with the phenotypic features of DS to a segment of chromosome 21, the DS region (*2*).

Fuentes et al. (*3*) cloned a gene (*DSCR1*) from the Down syndrome critical region that is highly expressed in brain and heart, and suggested it as a candidate for involvement in the pathogenesis of DS, in particular mental retardation and/or cardiac defects.

Nakamura et al. (*52*) identified *DSCR4* as 2 ESTs that map to the 1.6-Mb Down syndrome critical region. *DSCR4* is predominantly expressed in placenta.

Vidal-Taboada et al. (*53*) identified *DSCR2* within the Down syndrome critical region 2 between DNA marker D21S55 and MX1. *DSCR2* gene related to cell proliferation (*54*).

Nakamura et al. (*55*) identified *DSCR3* within the Down syndrome critical region.

Among the genes located in the DS region is the DS critical region 1 (*DSCR1*) gene that associates with mental retardation and mediates learning and long-term memory in *Drosophila* (*56*). *DSCR1* belongs to a highly conserved calcineurin inhibitor family called calcipressin1. Calcineurin has a role in the transition from short- to long-term memory in mice (*57*). *DSCR1* is normally expressed in the central nervous system and the heart, but overexpressed in fetal DS brain (3,4) *DSCR1* belongs to a highly conserved calcineurin inhibitor family called calcipressin, which contains RCN1P in yeast (5), Nebula in *Drosophila*, and *Dscr1* in mouse (*58*).

F. Treatment

Despite continued hope, no significant medical treatments for mental retardation associated with Down syndrome have been forthcoming. However, the dramatic improvements in medical care described here have greatly improved the quality of life and increased life expectancy.

2. *DSCR1* gene structure and function

DSCR1 (Down syndrome candidate region 1), a gene located in the region 21q22.1–q22.2, encodes a protein that has an acidic domain, a serine–proline motif, a putative DNA-binding domain, and a proline-rich region with the characteristics of a SH3 domain ligand. *DSCR1* highly expressed in the central nervous system and heart (*3*). *DSCR1* expressed as four protein isoforms through the use of four alternative first exons (*59*). Although all the different *DSCR1* spliced transcripts expressed in heart and skeletal muscle, only the exon 1-containing *DSCR1* transcript can be detected in fetal and adult brain, and only the exon 4-containing mRNA is detected in placenta and kidney. These two *DSCR1* transcripts encode polypeptides of 197 amino acids which differ in their N-terminal 29 amino acids.

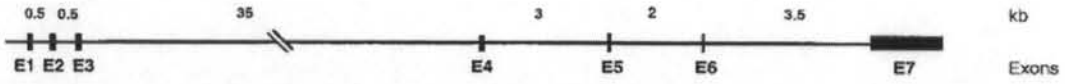

Figure 1 *DSCR1* gene structure

Calcium is used by the cell to modulate gene expression programs by various means. Examples include direct binding of calcium by the transcriptional repressor DREAM, which leads to a reduction in its DNA activity (*60*), or Ca^{2+}/calmodulin kinase IV phosphorylation of the transcription factor CREB allowing its binding to the co-activator p300 (*61*). The best known example of calcium regulation through phosphatases is the NF-AT (Nuclear factor of activated T cells) transcription factor. In this case, an increase in intracellular Ca^{2+} leads to the activation of calcineurin, which then dephosphorylates cytosolic NF-AT family members. This modification unmasks their nuclear localization signals and promotes translocation into the nucleus where they bind cooperatively to DNA with other transcription factors such as AP-1, c-MAF or GATA4. This signaling pathway is important for the immune response (*62*), cardiac and skeletal muscle hypertrophy (*63,64,65*), slow fiber differentiation in skeletal muscle (*66*), cardiac valve development (*67,68*) and the differentiation of a pre-adipocyte cell line to adipocytes in culture (*69*).

Calcineurin is the only serine/threonine protein phosphatase under the control of Ca^{2+}/calmodulin (*70*). It functions as a heterodimer composed of a catalytic A subunit (CaNA) and a calcium binding regulatory B subunit (CaNB). In mammals, there are three different CaNA genes, α, ß and γ; highly similar through their entire sequence but with different tissue distributions. CaNA displays a multidomain structure with a catalytic domain at its N-terminus, similar to that of protein phosphatase 1 (*71,72*), followed by the binding regions for the B subunit and calmodulin (CaM). An autoinhibitory domain is located near the C-terminus, which is thought to be displaced upon CaM binding. The immunosuppressive drugs FK506 and cyclosporin, when complexed to specific immunophilins (FKBP12 and cyclophilin A, respectively), bind calcineurin at multiple sites, including the N-terminus of the CaNB binding helix, the CaNB subunit and the catalytic domain of CaNA (*71*), inhibiting calcineurin activity.

DSCR1 is a calcineurin catalytic A subunit binding protein. The interaction domain in CaNA to the linker region between the catalytic domain and the CaNB binding domain. The overexpression of *DSCR1* can repress the transcriptional activation of a NF-AT-dependent promoter in response to phorbol 12-myristate 13-acetate (PMA) and ionophore by inhibiting the nuclear translocation of NF-AT. Therefore *DSCR1*, and the other related proteins in humans, may represent endogenous inhibitors for calcineurin and define a negative regulatory pathway in calcineurin signaling in mammals.

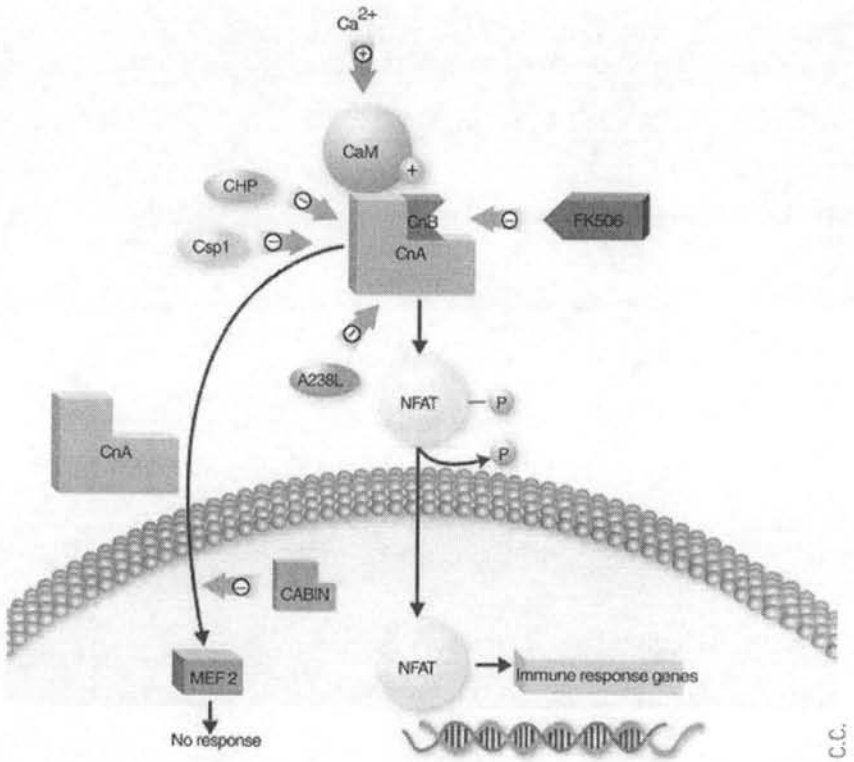

Figure 2. Calcineurin pathway

(From: www.nature.com)

DSCR1, the product of a chromosome 21 gene highly expressed in brain, heart and skeletal muscle, is overexpressed in the brain of Down syndrome fetuses, and interacts physically and functionally with calcineurin A, the catalytic subunit of the Ca2+/calmodulin-dependent protein phosphatase PP2B. *DSCR1* belongs to a family of

evolutionarily conserved proteins with three members in humans: *DSCR1*, *ZAKI-4* and *DSCR1L2*.

3. RNAi/siRNA technology

RNA interference (RNAi) is a naturally occurring cellular mechanism that induces post-transcriptional gene silencing in diverse cell types and has attracted much attention as a potential therapeutic strategy(*12*). Small interfering RNAs (siRNA) are short duplexes of RNA (21–23nt) that are the key intermediaries in this process. Since its discovery in the late 1990s (*15*), RNAi/siRNA technology has made a tremendous impact in molecular biology and has the potential to revolutionise future healthcare. The siRNA/RNAi technology has broad applications in target validation, probing gene function (transgenomics), and may serve as a potential therapeutic strategy for the inhibition of disease-associated genes. Indeed, many now consider RNAi/siRNA technology as the simplest, most effective gene silencing tool that has largely superceded its predecessors such as antisense oligonucleotides, ribozymes and DNAzymes (*24, 25, 74*).

A. EMERGENCE OF RNAi FOR POSTTRANSCRIPTIONAL GENE SILENCING

The technology of siRNA was borne out of work to elucidate the process of RNAi and post-transcriptional gene silencing initially pioneered in plants (*77*). In the early 1990s it was known that the introduction of transgenes into plants led to inhibition of the endogenous gene and the transgene itself—a phenomenon termed as "co-supression". It was also known that gene silencing occurred post-transcriptionally as transcripts from both genes were produced but were then degraded rapidly in the cytosol; hence, the term "post-transcriptional gene silencing". The subsequent identification of small (21–23nt) fragments of double stranded (ds) RNA in plants strongly implied the involvement of dsRNA in this process (*13, 14*). This led to the pioneering finding in the nematode, Caenorhabditis elegans, that long term gene silencing could indeed be achieved through the injection of dsRNA (*15*). Soon after, similar findings were described in other

organisms: fruit flies (*16*), frogs (*17*), mice (*18*) and now human cells (*19*) implying that the natural RNAi process is conserved amongst a variety of invertebrate and vertebrate species.

RNAi operates by cleaving dsRNA with Dicer, an RNAse III nuclease, to form siRNA (*12*); the siRNA then causes significant down-regulation of homologous messenger (m) RNA. The mechanism of action (covered in more detail below) is thought to differ between organisms, thus research is currently elucidating common pathways and key molecules in the process, which among other putative roles is commonly believed to be an antiviral defence mechanism (*15, 20, 74, 75*). For example, the introduction of long dsRNA (greater than 30nts in length) into mammalian cells leads to a global inhibition of gene expression via a protein kinasedependent- mediated interferon response. However, siRNAs (less than 30nts) do not elicit this toxic immune response and cellular administration of these molecules can yield potent gene silencing. Thus, administration of siRNAs to cells offers a convenient way of activating the intracellular RNAi machinery against a specific gene of interest. The power of this application was only realized after Tuschl's laboratory showed that exogenously applied siRNAs can be used in cultured mammalian cells for targeted inhibition of gene expression (*19, 20*). Their discovery lead to an explosion of activity in this area and currently it is one of the fastest developing fields in the academic and commercial sectors.

Although RNAi represents one of the most powerful biological tools ever to be introduced, recent literature reports have also highlighted several challenges facing this technology including issues relating to appropriate design, effective delivery, toxicity and specificity of action. Some of these issues appear similar to those encountered by conventional anti-mRNA approaches such as antisense oligonucleotides, ribozymes and deoxyribozymes (*26, 27, 28*). In this article, we will focus on reviewing the recent studies aimed at improving the design and exogenous (non-viral vector-mediated) delivery of siRNAs. The endogenous expression of dsRNA or short hairpinRNAs using viral vectors for cellular gene silencing is not covered and the reader is referred to other texts (*23*).

B. MECHANISMS OF RNAi ACTION

RNAi can be mediated by exogenous delivery of siRNA or micro RNA (miRNA), the latter is not discussed in detail here. The siRNAs are a class of molecules usually comprising of 21–23 nucleotide (nt) duplexes characteristically housing two nucleotide overhangs at each 30 terminus; Fig. 3 schematically presents the anatomy of an exogenous synthetic siRNA duplex. Inside cells, these duplexes are recognized by the RNA-induced silencing complex (RISC) present in the cytosol (*76*), whereby the antisense RNA strand is then guided by this complex to the complementary sequence in target mRNA. The RISC complex then, by mechanisms that are not fully understood, initiates its hydrolysis and thus, prevents or "silences" gene expression (summarised in Fig. 4). Work is ongoing to unravel the detailed mechanism of RNAi, for instance a cytosolic siRNase enzyme was recently identified and implicated in the regulation of siRNA/RNAi through degradation of dsRNA and siRNA (*77*).

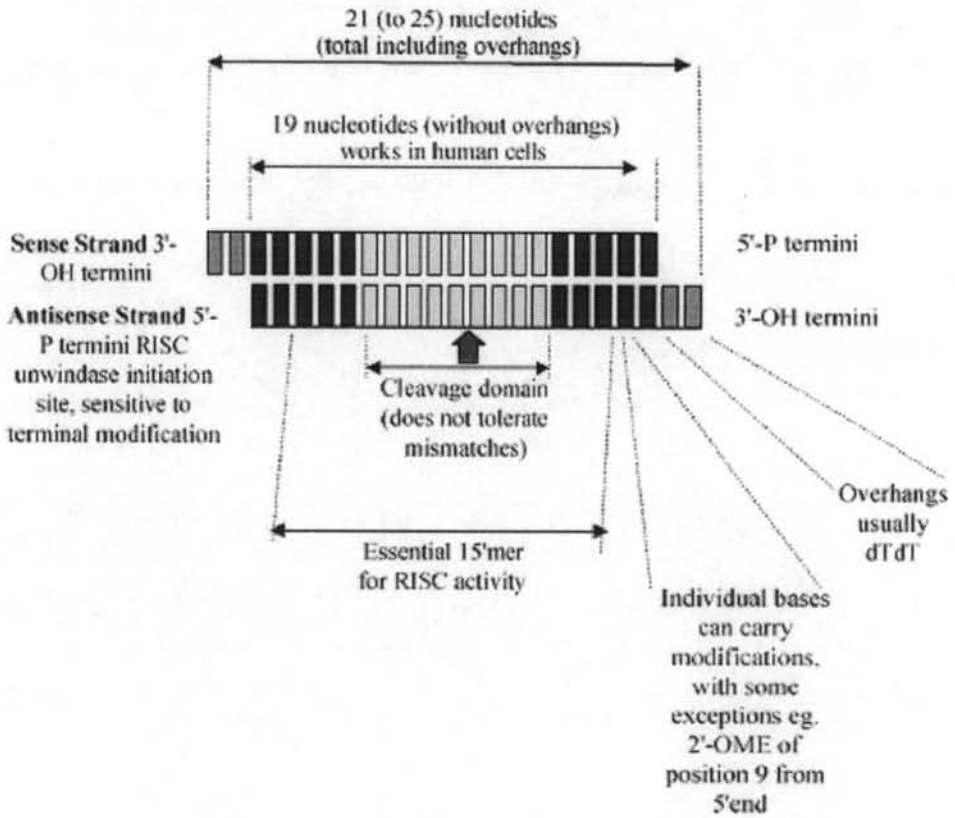

Figure 3. Anatomy of a synthetic siRNA duplex.

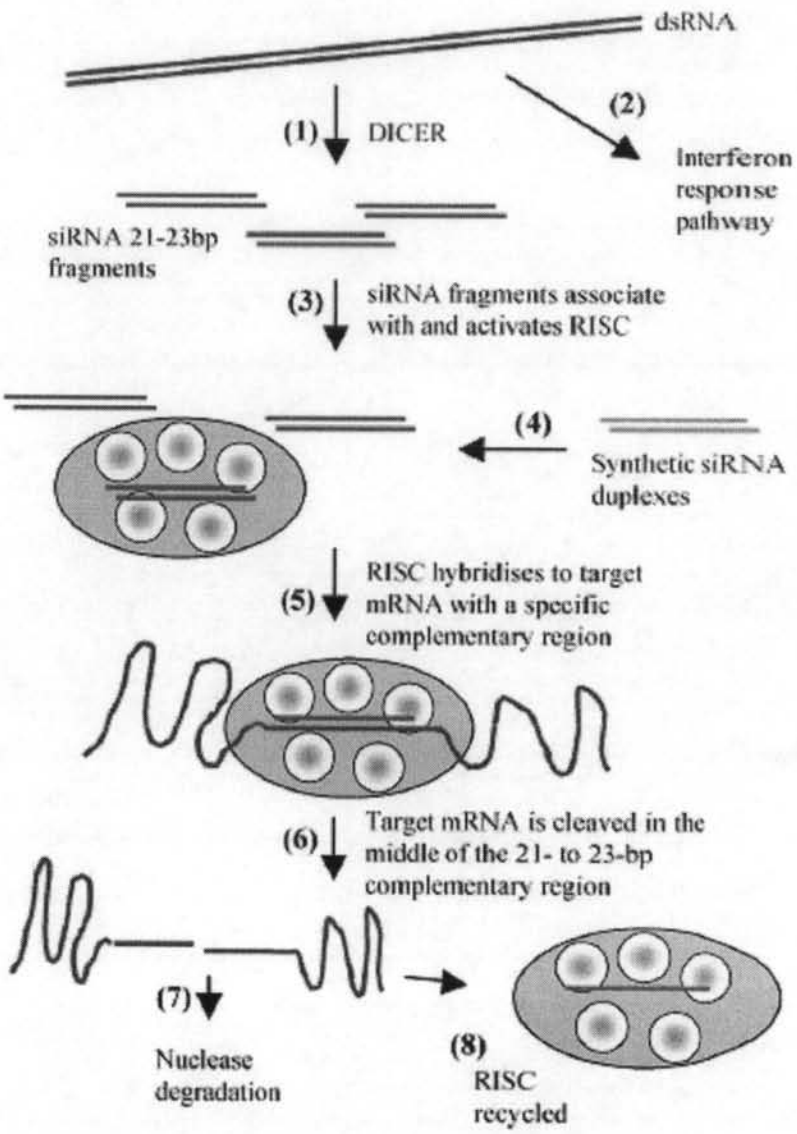

Figure 4. The RNAi/siRNA pathway for gene silencing.

(From: Journal of Drug Targeting, July 2004 Vol. 12 (6), pp. 317)

C. RNA-INDUCED SILENCING COMPLEX (RISC)

The RISC is a multi-component magnesium dependent nuclease that utilizes siRNA as a guide to target specific mRNA degradation (*78*). The exact composition of RISC remains unclear, although gradually its components are being verified. For instance, the components of RISC identified to date in *Drosophila* include Argonaute2 (AGO2) (*79*), fragile X protein (dFXR), Vasa intronic gene protein (VIG) (*80*), Tudor SN (staphylococcal nuclease) (*81*) and siRNA (*74*). In human cells, however, much less has been

determined, with the only identified components being single stranded siRNA, the Argonaute proteins eIF2C1 and eIF2C2 in HeLa cells (*82*) and Tudor SN (*81*). The putative RISC components and interactions are schematically summarised in Fig. 5. It has been demonstrated that an ATP dependent helicase unwinding of double stranded siRNA is required before formation of the RISC complex (*83*). The unwindase action is proposed to be a DEAD box helicase p68 (*84*). The antisense strand of this unwound siRNA duplex directs the sequence-specific cleavage of the mRNA.

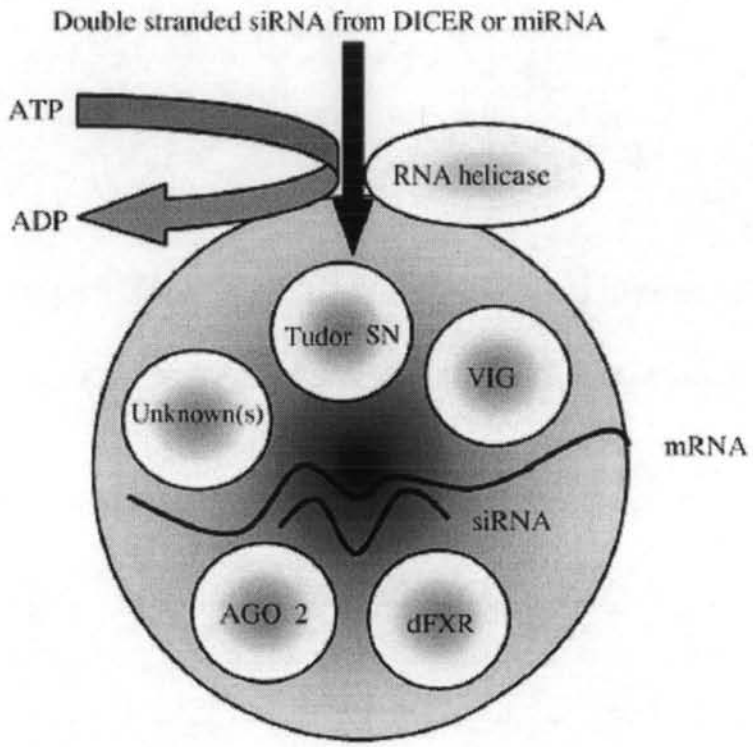

Figure 5. Current model for RISC complex in *Drosophila*.

It is clear that a number of key questions remain to be answered about the RISC complex. What is the total composition of the RISC complex? What is the interaction between the known components? Does RISC form as a response to the presence of siRNA or is it a preformed complex into which siRNA is incorporated? Is RISC localised to one area of the cell? Incrementally, our understanding of RISC is improving, and elucidating the answer to these and other questions that will enable a greater

understanding of the mechanisms involved and a better exploitation of such knowledge both in terms of the cytosolic targeting of siRNA delivery vehicles and in the design of RISC favoured siRNAs.

RNAi in vertebrate cells relies on a cellular post-transcriptional gene regulatory mechanism that uses endogenously encoded, ~22-nucleotide (nt), single-stranded RNAs to guide a ribonucleoprotein complex, the RNA inducd silencing complex (RISC), to target mRNAs(*1,4*). At least 300 of these microRNAs (miRNAs) are found in the human genome; they are believed to have a key role in regulation vertebrate differentiation and development. miRNAs are initially transcribed by RNA polymerase II (Pol II) as part of a long primary miRNA (pri-miRNA) precursor (Fig 6). Mature miRNAs form part of one arm of an ~85-nt RNA stem-loop in the pri-miRNA. The first step in miRNA processing is mediated by the RNase III enzyme Drosha, which cleaves the stem ~22-nt away from the terminal loop to generate an ~65-nt pre-miRNA hairpin intermediate. Drosha cleavage defines one end of the mature miRNA and leaves a characteristic 2-nt 3' overhang. The pre-miRNA is transported to the cytoplasm by the nuclear export factor Exportin-5, where it interacts with a second RNase III enzyme called Dicer. Dicer binds the 2-nt 3' overhang found at the base of the pre-miRNA hairpin and cleaves ~22 nt away from the base, removing the terminal loop and leaving another 2-nt 3' overhang. The resultant duplex intermediate interacts with RISC components, including Argonaute-2, which selectively incorporate the RNA strand whose 5' end is less tightly base-paired. Once programmed, RISC can downregulate the expression of homologous mRNAs.

The miRNA biogenesis pathway includes three distinct RNA intermediates: the initial pri-miRNA transcript, the pre-miRNA hairpin and the miRNA duplex. All can be used as entry points to allow programming of RISC with artificial miRNAs called small interfering RNAs (siRNAs). The first approach to vertebrate RNAi to be described used transfection of synthetic siRNA duplexes, identical in structure to miRNA duplex intermediates, into cultured cells. This approach efficiently downregulates target mRNAs but has the disadvantage of being both transient and expensive. A second RNAi approach uses short hairpin RNAs (shRNAs) transcribed

under the control of RNA polymerase III (Pol III). shRNAs are similar to pre-miRNA hairpins, and shRNA expression vectors can be used to establish stable RNAi responses in culture or in transgenic mice. The problem with the shRNA approach is essentially Pol III promoters, unlike Pol II promoters, do not lend themselves to regulation. A third approach to the induction of a specific RNAi response in mammalian cells relies on the transcription, by Pol II or Pol III, of artificial pri-miRNA transcripts that undergo the same processing steps as natural pri-miRNAs but result in the production of siRNA. Silva *et al.* (*86*) and Dickens *et al.* (*87*) show that such artificial pri-miRNAs not only induce an exceptionally potent RNAi reponse but also allow regulated RNAi when using Pol II-dependent promoters to drive pri-miRNA expression.

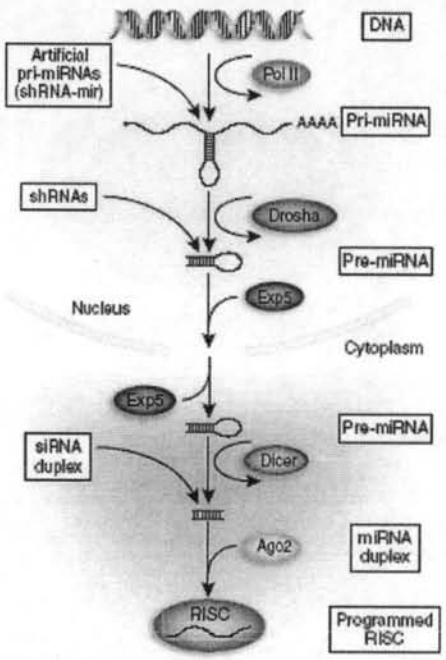

Figure 6. The miRNA biogenesis pathway in vertebrate cells.

(From: www.nature.com)

D. siRNA Construction

In vivo expression of siRNAs can be effectively achieved by generating DNA cassettes that contain a U6 promoter, siRNA template and transcription termination sequences and then transfecting these cassettes into eukaryotics cells. In cells, RNA

molecules, and U6 promoters have been used successfully to generate siRNAs in mammalian cells. Several approaches for RNA interference studies use this model. In one approach, individual promoters direct the transcription of sense and anti-sense strands of the siRNAs. The DNA units are generated by PCR and can be directly transfected into the cells or subcloned into appropriated vectors prior to transfection (*88*).

The siLentGene™-2 U6 Hairpin Cloning Systems use an approach in which siRNAs are expressed as fold-back stem-loop structures and are transcribed from the U6 promoter (*88, 89*). A DNA cassette containing a U6 promoter, a hairpin siRNA target sequence and the transcription termination sequence are generated by a single PCR amplification. The resulting PCR product can be directly transfected into human cells for rapid screening of optimal target sequences or may be subcloned into the provided dephosphorylated psiLentGene™-Basic Vector for transient suppression of the gene of interest. The systems also provide vectors with antibiotic resistance markers, neomycin, for long-term stable RNAi analysis.

- Hairpin DNA Cassette

To generate a DNA cassette containing hairpin structures, needed 1) A U6 cassette template containing the human U6 promoter; 2) An upstream primer that is complementary to the 5′ end of the U6 promoter region, and 3) A downstream primer that is complementary to the 3′ end of the promoter. The downstream primer contains the hairpin siRNA target sequence and a region that is complementary to the 3′ end of the U6 promoter.

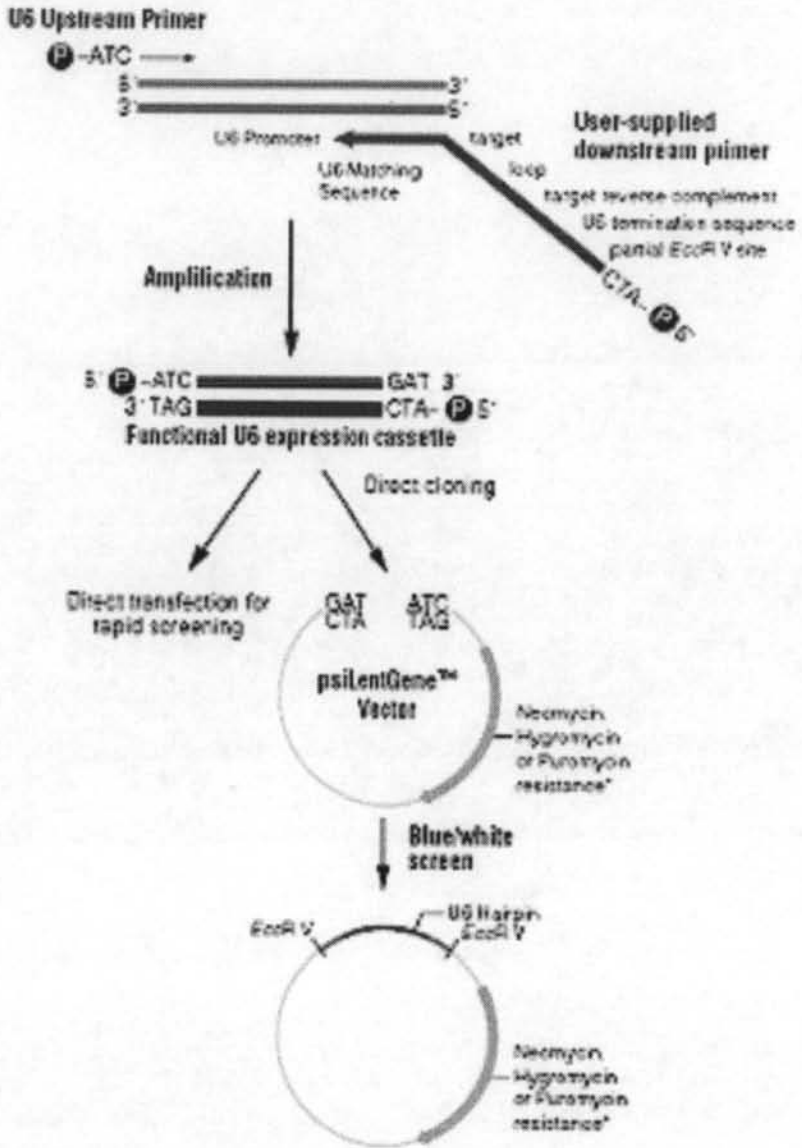

Figure7. Overview of the siLentGene™-2 U6 Hairpin Cloning Systems procedure.

(From: www.promega.com)

4. Relative Quantification

The two most commonly used methods to analyze data from real-time, quantitative PCR experiment are absolute quantification and relative quantification. Absolute quantification determines the input copy number, usually by relating the PCR signal to a standard curve. Relative quantification relates the PCR signal of the target

transcript in a case to that of another sample such as an unrelated control. The $2^{-\Delta\Delta Ct}$ method is a convenient way to analyze the relative changes in gene expression for realtime quantitative PCR experiments (*90, 91*).

Basic terms used in real-time PCR

A number of basic terms are used in real-time PCR analyses and are briefly described in the following section. Data are displayed as sigmoidal-shaped amplification plots (when using a linear scale), in which the fluorescence is plotted against the number of cycles (Fig 8).

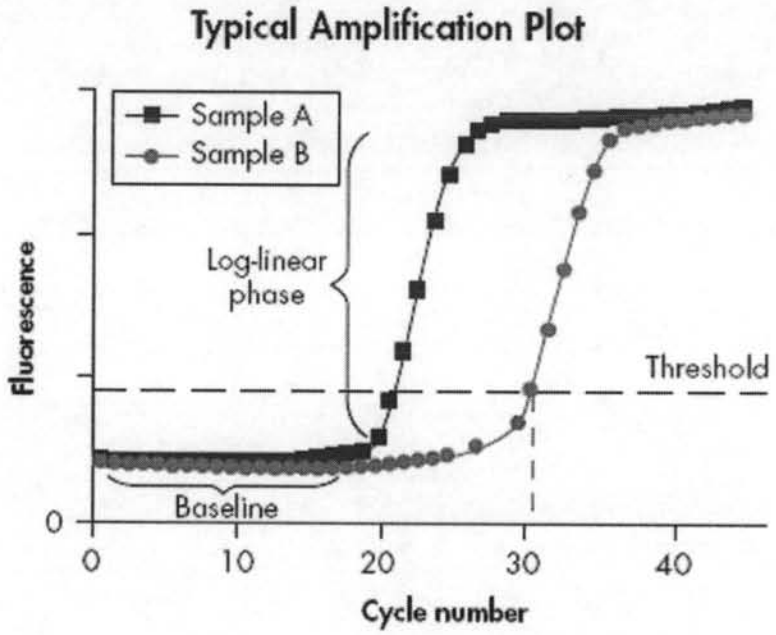

Figure 8. Amplification plots showing increases in fluorescence from 2 samples (A and B). Sample A contains a higher amount of starting template than sample B.

Baseline: The baseline is the noise level in early cycles, typically measured between cycles 3 and 15, where there is no detectable increase in fluorescence due to amplification products. The number of cycles used to calculate the baseline can be changed and should be reduced if high template amounts are used or if the expression

level of the target gene is high. To set the baseline, view the fluorescence data in the linear scale amplification plot. Set the baseline so that growth of the amplification plot begins at a cycle number greater than the highest baseline cycle number. The baseline needs to be set individually for each target sequence. The average fluorescence value detected within the early cycles is subtracted from the fluorescence value obtained from amplification products.

Background: This refers to nonspecific fluorescence in the reaction, for example, due to inefficient quenching of the fluorophore or the presence of large amounts of double-stranded DNA template when using SYBR Green I. The background component of the signal is mathematically removed by the software algorithm of the real-time cycler.

Threshold: The threshold is adjusted to a value above the background and significantly below the plateau of an amplification plot. It must be placed within the linear region of the amplification curve, which represents the detectable log-linear range of the PCR. The threshold value should be set within the logarithmic amplification plot view to enable easy identification of the log-linear phase of the PCR. If several targets are used in the real-time experiment, the threshold must be set for each target.

Threshold cycle (CT) or crossing point: This is the cycle at which the amplification plot crosses the threshold, i.e., at which there is a significant detectable increase in fluorescence. The CT serves as a tool for calculation of the starting template amount in each sample.

ΔCT value: The ΔCT value describes the difference between the CT value of the target gene and the CT value of the corresponding endogenous reference gene, such as a housekeeping gene:

ΔCT = CT (target gene) – CT (endogenous reference gene)

$\Delta\Delta$CT value: The $\Delta\Delta$CT value describes the difference between the average ΔCT value of the sample of interest and the average ΔCT value of a reference sample.The reference sample is also known as the calibrator sample and all other samples will be normalized to this when performing relative quantification:

$\Delta\Delta$CT = ΔCT (sample of interest) –ΔCT (calibrator sample)

Endogenous reference gene: This is a gene whose expression level should not differ between samples, such as a housekeeping or maintenance gene. Comparison of the CT value of a target gene with that of the endogenous reference gene allows the gene expression level of the target gene to be normalized to the amount of input RNA or cDNA. This is done without determining the exact amount of template used in the reaction. The use of an endogenous reference gene corrects for variation in RNA content, variation in reverse-transcription efficiency, possible RNA degradation or presence of inhibitors in the RNA sample, variation in nucleic acid recovery, and differences in sample handling.

Calibrator sample: This is a reference sample used in relative quantification. For example, RNA isolated from a cell line or tissue, to which all other samples are compared to determine the relative expression level of a gene. The calibrator sample usually has a stable expression ratio of target gene to endogenous reference gene.

If the PCR efficiencies of the target gene and endogenous reference gene are comparable, the normalized level of target gene expression is calculated by using the formula:

$$\text{Normalized target gene expression level in sample} = 2^{-\Delta\Delta_{Ct}}$$

In this study, the normalized target gene expression level in all sample are using the average of $\Delta\Delta_{C_t}$ from 5 controls sample as calibrators.