GENE SILENCING IN CANCER CELLS USING siRNA:

GENETIC AND FUNCTIONAL STUDIES

A Dissertation

by

MA'EN AHMAD ABDEL RAHIM

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2004

Major Subject: Toxicology

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ABSTRACT

Gene Silencing in Cancer Cells Using siRNA: Genetic and Functional Studies.

(May 2004)

Ma'en Ahmad Abdel Rahim, B.S., Al-Isra University

Chair of Advisory Committee: Dr. Stephen H. Safe

Sequence-specific small interfering RNA (siRNA) duplexes can be used for gene silencing in mammalian cells and as mechanistic probes for determining gene function. Transfection of siRNA for specificity protein 1 (Sp1) in MCF-7 or ZR-75 cells decreased Sp1 protein in nuclear extracts, and immunohistochemical analysis showed that Sp1 protein in transfected MCF-7 cells was barely detectable. Decreased Sp1 protein in MCF-7 was accompanied by a decrease in basal and estrogen-induced transactivation and cell cycle progression. These results clearly demonstrate the key role of Sp1 protein in regulating growth and gene expression of breast cancer cells.

The aryl hydrocarbon (AhR) is a ligand-activated nuclear transcription factor. siRNA for the AhR decreased TCDD-induced CYP1A1 protein, CYP1A1-dependent activity, and luciferase activity in cells transfected with an Ah-responsive construct. 17β-Estradiol (E2) induces proliferation of MCF-7 cells, and this response is inhibited in cells cotreated with E2 plus TCDD. The effects of TCDD on E2-induced cell cycle progression were partially blocked in MCF-7

cells transfected with siRNA for AhR. The decrease in AhR protein in MCF-7 cells was also accompanied by increased $G_0/G_1 \rightarrow S$ phase progression. Surprisingly, TCDD alone induced $G_0/G_1 \rightarrow S$ phase progression and exhibited estrogenic activity in MCF-7 cells transfected with siRNA for the AhR. In contrast, degradation of the AhR in HepG2 liver cancer cells resulted in decreased $G_0/G_1 \rightarrow S$ phase progression, and this was accompanied by decreased expression of cyclin D1, cyclin E, cdk2 and cdk4. In the absence of ligand, the AhR exhibits growth inhibitory (MCF-7) and growth promoting (HepG2) activity that is cell context-dependent.

Sp family proteins play a complex role in regulation of pancreatic cancer cells growth and expression of genes required for growth, angiogenesis and apoptosis. Sp1, Sp3 and Sp4 cooperatively activate VEGF promoter constructs in these cells; however, only Sp3 regulates cell proliferation. siRNA for Sp3 inhibits phosphorylation of retinoblastoma protein, blocks $G_0/G_1 \rightarrow S$ phase progression of Panc-1 cells, and upregulates p27 protein/promoter activity. Thus, Sp3 plays a critical role in angiogenesis (VEGF upregulation) and the proliferation of Panc-1 cells by a novel mechanism of Sp3-dependent suppression of the cyclin-dependent kinase inhibitor p27.

DEDICATION

In the name of Allah, the Beneficent, the Merciful

"Yet among His servants only the men of knowledge fear Allah; surely Allah is Mighty, Forgiving". The Holy Quran, Al-fatir

To my beloved parents, for their prayer and love.

To my brothers Marwan and Mamoun, for without their support and help I would not be writing this.

To May and Khalid, for the good time we had in College Station.

To Shreen and Adham, for all the early years of friendship and fun.

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INTRODUCTION

RNA INTERFERENCE

The phenomenon of sequence-specific gene silencing with RNA interference (RNAi) was first discovered in the nematode worm *Caenorhabditis elegans* (*C. elegans*) as a response to double-stranded RNA (dsRNA) (1). Antisense RNA has been extensively used to inhibit gene expression and it has also been reported that sense RNA was as effective as antisense RNA for suppressing gene expression in worms (2). Fire and coworkers (1) were the first to show the synergy of sense and antisense RNAs by demonstrating that dsRNA was at least ten-fold more potent for gene silencing in the worm than were sense or antisense RNAs alone. Silencing genes by dsRNAs exhibited a number of remarkable properties: RNAi could be observed by feeding the worm with either dsRNA itself or by using bacteria which expressed the dsRNA (3). Exposure of the parental animal to only a few molecules of dsRNA per cell triggered gene silencing throughout the treated animal (systemic silencing) and in its F_1 (first generation) progeny. This discovery suggested that a number of previously characterized, homology-dependent gene-silencing (HDGS) mechanisms might share a common biological root.

HDGS was first discovered with the introduction of transgenes coding for

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This dissertation follows the style of the *Journal of Biological Chemistry*.

chalcone synthase into petunia plants. Although the expectation was increased flower pigmentation, in many of the plants, the result was in fact the opposite, with white or variegated petunia petals. This observation, suggested not only that the introduced transgenes were inactive but also that exogenous genetic elements affected the constitutive chalcone synthase locus. This apparent communication between unlinked but homologous loci was termed cosuppression (4). It is now recognized that HDGS is a commonly observed outcome of transgenesis in plants. Communication can occur between transgenes and endogenous genes (5), between two related transgenes (6), and even between silenced and active endogenous loci (7). A similar phenomenon, called paramutation, describes an interaction between two endogenous alleles, in which an active locus is repressed by exposure to a silenced locus in a manner that is stable even after alleles are separated by subsequent genetic crosses (8). Communication occurring solely between endogenous loci has also been observed in *Drosophila*. For example, one study showed that crossing flies containing a silenced copy of an I element (a transposon similar to mammalian LINE elements) to flies containing active I elements repressed transposition. Furthermore, such repression was heritable (9). It is possible that paramutation and cosuppression are mechanistically related phenomena that differ only in the source of the silencing trigger. The first report of transgene cosuppression in the animal kingdom was observed in *Drosophila* (10). Introduction of repeated white-Adh fusion transgenes into *Drosophila* lead to considerable repression of the

transgene and also of endogenous Adh expression. The degree of silencing was proportional to transgene copy number.

Transitive RNAi. Systemic silencing phenomena have been observed in *C. elegans* where RNAi can spread throughout the organism, even when triggered by minute quantities of dsRNA (1). Plants also exhibit systemic silencing which can be observed throughout the plant or be transferred to a naive grafted scion (11). These phenomena require a system that transmit signals between cells and amplifies the signal. Recently, a phenomenon termed “transitive RNAi” has provided some useful insights regarding this process. Transitive RNAi refers to the movement of the silencing signal along a particular gene. In *C. elegans*, targeting the 3' portion of a transcript results in suppression of that specific mRNA and in produces siRNAs homologous to the targeted region. In addition, siRNAs complementary to regions of the transcript upstream from the area targeted directly by the silencing trigger also appear and accumulate (12). In plants, the ability of the silencing agent to move within the plant is called Systemic Acquired Silencing (SAS) (13). For example tobacco plants transgenic for the green fluorescent protein (GFP) can be infiltrated with *Agrobacterium tumefaciens* carrying a GFP reporter construct. This results in rapid suppression at the infiltration zone, and, by 18 days postinfiltration, the upper leaves of the plant also silence the GFP transgene (14).

Systemic transmission of silencing was perhaps most strikingly demonstrated by the grafting of a nonsuppressed scion (the upper vegetative

tissues) onto a cosuppressed stock (lower tissues and the root system), which resulted in the scion becoming cosuppressed. In fact, in a three-way graft, silencing can be passed between a silenced stock and an engrafted scion through a central stock that completely lacks sequences corresponding to the targeted gene (11). In both plants and *C. elegans*, dsRNA-induced silencing requires proteins similar in sequence to a tomato RNA-directed RNA polymerase (RdRP) (15), which could be involved in amplifying the RNAi signal. However, only the tomato enzyme has been shown to possess polymerase activity, and biochemical studies will be required to definitively establish the role of these proteins in RNAi.

A model for transitive RNAi in which siRNAs might prime the synthesis of additional dsRNA by RdRPs has been predicted from genetic studies. RdRP activity has been reported recently in *Drosophila* embryo extracts (16), whereas transitive RNAi has not yet been observed in flies. While numerous experiments suggest that an RdRP is not required for RNAi in *Drosophila* extracts, the possibility remains that such an enzyme might act, for example, in triggering RNAi by the production of dsRNA from dispersed, multicopy transgenes. In plants, transitive RNAi travels in both 3'→5' and 5'→3' directions (17), which is inconsistent with the simple notion of siRNAs priming dsRNA synthesis. It is believed that genomic loci may serve as a reservoir for silencing. In some systems, alterations in chromatin structure can be predicted from exposure to dsRNA, which could lead to the production of 'aberrant' mRNAs that are

substrates for conversion to dsRNA by RdRPs. This model would permit bi-directional spread and expansion of altered chromatin structure is an established phenomenon (17). Moreover, a similar model could explain co-suppression that is occasionally triggered by single-copy, dispersed transgenes. This model would be consistent with transitive effects that have been observed for both transcriptional and post-transcriptional silencing in *Drosophila*, which operate in the absence of any homology in the transcribed RNA, and thus differ from 'transitive RNAi' in *C. elegans* (10,18).

Two types of transmission must be considered in plants. The first is short-range, cell-to-cell transmission. Plant cells are intimately connected through cytoplasmic bridges known as plasmodesmata. Movement of RNA and proteins via these cell–cell junctions is well known, and it is likely that either long dsRNA or siRNAs could be passed through these connections. However, the silencing signal must also be passed over a longer range through the plant vasculature (19). Evidence against siRNAs being critical for systemic silencing in plants has been provided from studies of a viral silencing inhibitor. Hc-Pro suppresses silencing and also interferes with the production of siRNAs from dsRNA triggers (20). Expression of Hc-Pro does not interfere with transgene methylation. This methylation could leads to transcriptional gene silencing (TGS) or may contribute to post-transcriptional gene silencing (PTGS) depending on the location of methylation. Hc-Pro expression in a silenced rootstock relieves silencing and inhibits siRNA production, but a systemic signal can still be passed

from this rootstock to an engrafted scion lacking Hc-Pro expression. A protein has been identified in *C. elegans* that is required for systemic silencing. This transmembrane protein that may act as a channel for import of the silencing signal is encoded by sid-1. Expression of sid-1 is largely lacking from neuronal cells, explaining initial observations that *C. elegans* neurons were resistant to systemic RNAi (21). SID-1 homologs are absent from *Drosophila*, consistent with a lack of systemic transmission of silencing in flies. These homologs are present in mammals, raising the possibility that some aspects of RNAi may act non-cell autonomously in mammals.

Mechanism of dsRNA-induced silencing. Exposure to dsRNAs in *C. elegans* resulted in loss of corresponding messenger RNAs (mRNAs). Promoter and intronic sequences were largely ineffective as silencing triggers and this observation is consistent with dsRNA-induced silencing operating at the post-transcriptional level (1). A post-transcriptional model also explains data from plant systems in which exposure to dsRNA (22), for example in the form of an RNA virus, triggered depletion of mRNA sequences without an apparent effect on the rate of transcription (23). Indeed, viral transcripts themselves were targeted, despite the fact that these were synthesized in the cytoplasm by transcription of RNA genomes (24). These studies support the hypothesis that RNAi induces degradation of homologous mRNAs, and this has been validated by biochemical analysis. On the other hands, RNAi machinery affects gene expression through additional mechanisms (Fig. 1). For example it is clear from

studies in plant systems that phenomena related to RNAi [Viral Induced Gene Silencing (VIGS) and cosuppression] also produce effects at the transcriptional level. Interactions between dsRNA and the genome could serve as the basis for such silencing phenomena.

DNA methylation - Production of dsRNA in plant cells induces methylation of homologous DNA sequences. RNA-directed DNA methylation (RdDM) was first discovered in plants infected with recombinant viroids (25). It has been found that genomic targets with as few as 30 bp of sequence complementary to the viroid RNA are methylated during infection (26). In fact, genomic methylation commonly accompanies PTGS. However, if cells are exposed to dsRNA that is homologous to the promoter region, rather than the expressed region of the gene, methylation is also evident and silencing occurs at the transcriptional level. In plants, virus-induced PTGS is not heritable, but TGS is heritable and is correlated with the inheritance of methylation (23). Methylation of the targeted gene in response to dsRNA did not require MET1 (the major maintainance methylase of Arabidopsis); however, both heritable silencing and maintenance of methylation in progeny required an intact MET1 gene (23). These findings suggest a model in which dsRNA initiates PTGS, and independently, methylation of the genome in a MET1-independent manner. Heritable silencing occurs when methylases, such as MET1, maintain the methylated state following DNA replication through preferential recognition and modification of hemi-methylated DNA.

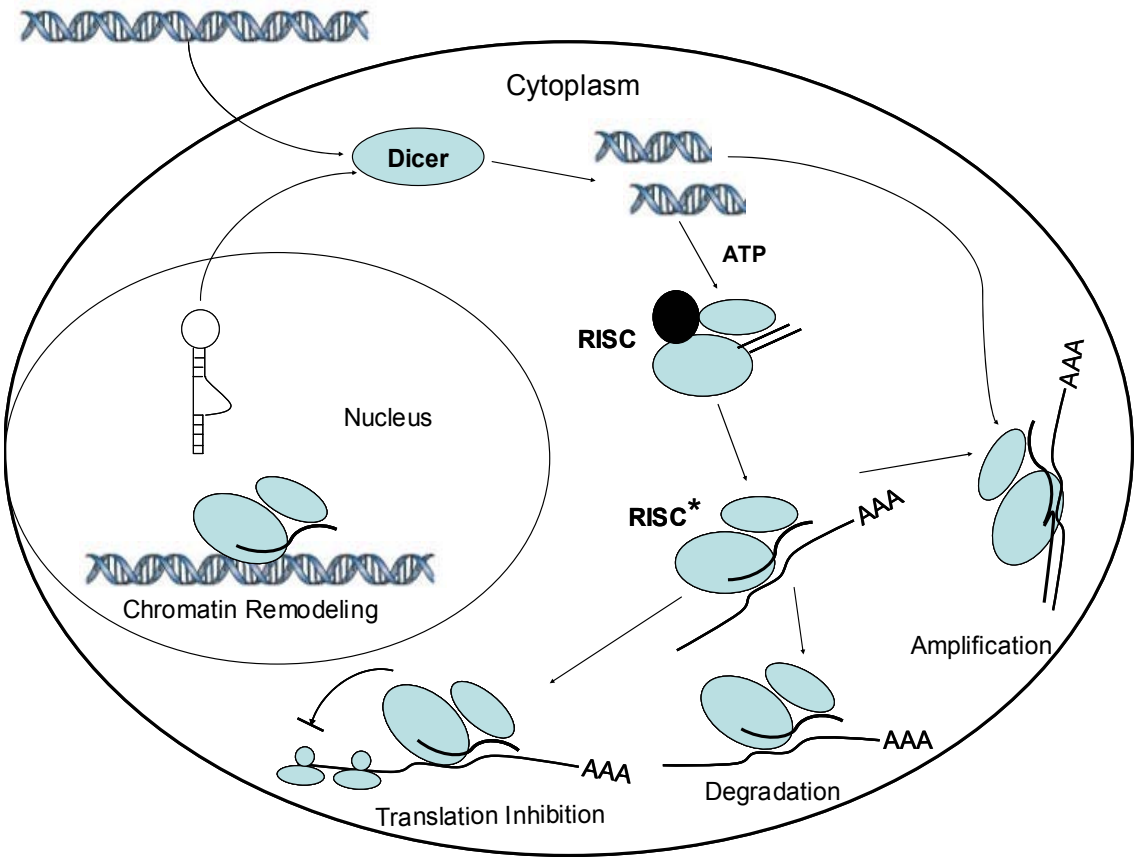


Fig. 1. **A schematic representation of RNAi-related gene silencing mechanisms.** dsRNAs are recognized and processed by Dicer. The douplex siRNAs are passed to RISK which upon activation can regulate gene expression at different levels. Modified from (27).

Chromatin modification - Recent studies have suggested that the RNAi machinery may also affect gene expression at the level of chromatin structure in *Drosophila*, *C. elegans* and fungi (10,18,28,29). Connection between the RNAi machinery and the genome, and mechanistic links between PTGS and TGS has been investigated in many systems. For example, in *C. elegans*, mut-7 and rde-2 mutations de-repress transgenes that are silenced at the level of transcription by a polycomb-dependent mechanism (28). Polycomb-group proteins function by organizing chromatin into 'open' or 'closed' conformations, creating stable and heritable patterns of gene expression. Recently, it has been found that the polycomb proteins MES-3, MES-4 and MES-6 are required for RNAi (29). Mutant worms were deficient in the RNAi response if high levels of dsRNA were injected, but were not deficient in the presence of limiting dsRNA. The effects of these mutants could be indirect, altering the expression of other elements or regulators of the RNAi pathway. However, links between altered chromatin structures and dsRNA-induced gene silencing have also emerged from plant and *Drosophila* systems. In particular, alterations of either methyltransferases (MET1) or chromatin remodelling complexes (for example, DDM1) can affect both the degree and persistence of silencing in Arabidopsis (23,30). Conversely, mutations in genes required for PTGS (for example, AGO1 and SGS2) decrease both co-suppression and transgene methylation (13). Furthermore, mutation of piwi, a relative of the RISC component Argonaute-2, compromises co-suppression of dispersed transgenes in *Drosophila* at both the post-

transcriptional and transcriptional levels (18). One model suggests that a variant, nuclear RISC carries a chromatin remodelling complex rather than a ribonuclease to its cognate target. RNAi machinery may have to form heterochromatic domains in the nucleus that are critical for genome organization and stability (31).

Translation inhibition - In *C. elegans*, endogenously encoded inducers of the RNAi machinery (for example, lin-4) operate at the level of protein synthesis (32). Although translational control by dsRNA has not been established definitively in other systems, the conservation of let-7 and related RNAs (33) suggests that this regulatory mode may be a further common mechanism through which RNAi pathways control the expression of cellular genes.

Post-transcriptional gene silencing It has been shown that injection of dsRNA into *Drosophila* embryos induced sequence-specific silencing at the post-transcriptional level (34). The possibility that *Drosophila* embryo extracts, previously used to study translational regulation, might be competent for RNAi has been tested (35). Incubation of dsRNA in these cell-free lysates reduced their ability to synthesize exogenous luciferase from a synthetic mRNA. This suggests that dsRNA might bring about silencing by triggering the assembly of a nuclease complex that targets homologous RNAs for degradation. These findings support a link between transgene co-suppression in plants and RNAi in animals. A model for RNAi and related silencing phenomenon began to emerge (Fig. 2). According to this model, initiation of silencing occurs upon recognition of

dsRNA by machinery that converts the silencing trigger to ~21–25 nucleotide RNAs. These small interfering RNAs (siRNAs) are a signature of this family of silencing pathways and, by joining an effector complex RISC; they guide that complex to homologous substrates.

In the initiation step, the dsRNA silencing trigger is cleaved to produce siRNAs (Fig. 2). Support for this step emerged first from studies of *Drosophila* embryo extracts, which contained an activity capable of processing long dsRNA substrates into ~22-nucleotide fragments (36). These RNAs were shown to be double-stranded and contained 5'-phosphorylated termini (36,37). The enzyme that initiates RNAi is a member of RNase III ribonuclease family, which displays specificity for dsRNAs and generates such termini. RNase III enzymes can be divided into three classes based upon domain structure: bacterial RNase III contains a single catalytic domain and a dsRNA-binding domain; Drosha family nucleases contain dual catalytic domains (38); and a third family also contains dual catalytic domains and additional helicase and PAZ motifs (39). Members of the third class of RNases were found to process dsRNA into siRNAs and were therefore proposed to initiate RNAi (39). This family, now named the Dicer enzymes, are evolutionarily conserved, and proteins from *Drosophila*, Arabidopsis, the insect *Spodoptera frugiperda*, tobacco, *C. elegans*,

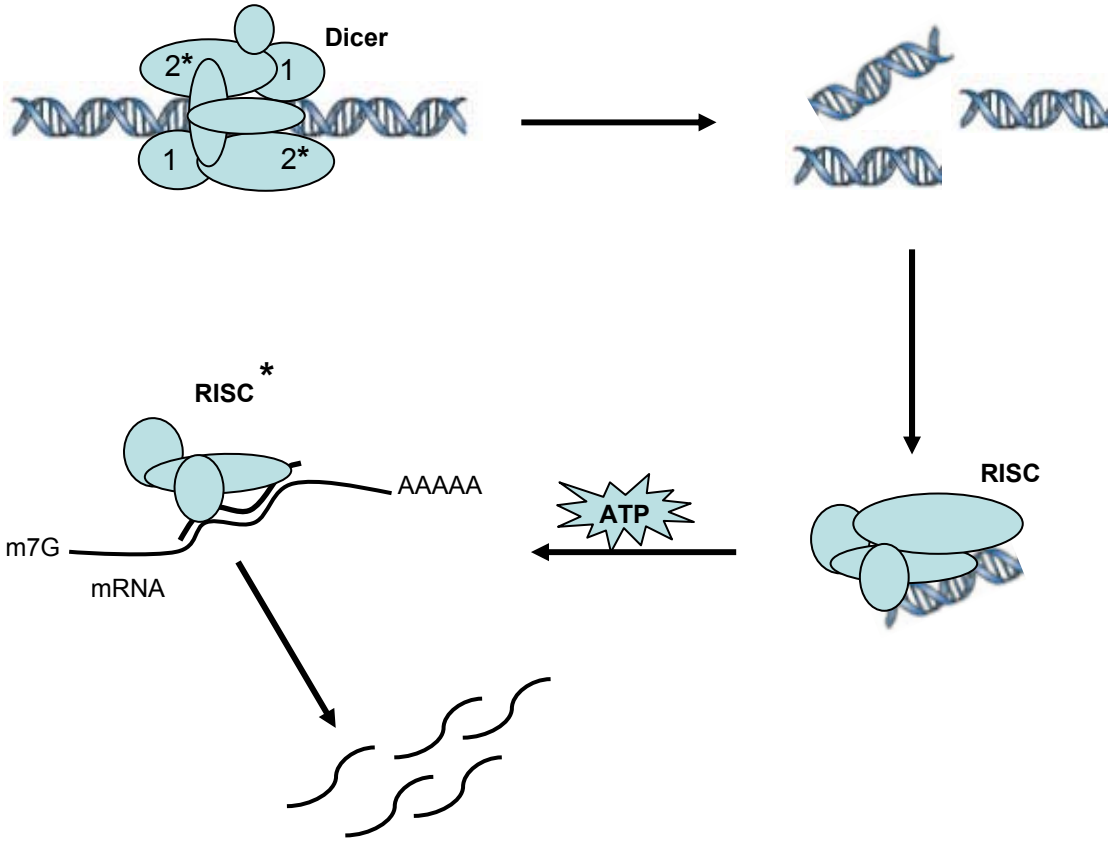


Fig. 2. **Initiation and effector complexes in post-transcriptional gene silencing.** RNAi is initiated by the dimeric enzyme Dicer which cleaves long dsRNA into siRNAs. Activation of RISC produce smaller complex with the antisense strands of unwinded siRNA. Modified from (40).

mammals and *Neurospora* have all been shown to recognize and process dsRNA into siRNAs of a characteristic size for the relevant species (39,41). Recently, the structure of an RNase III catalytic domain has led to a model for the generation of ~22-nucleotide RNAs by Dicer cleavage (42). It is thought that bacterial RNase III functions as a dimeric enzyme and, in the structural model, antiparallel RNase III domains produce two compound catalytic centres, each of which is formed by contributions from both monomers. The sequences of Dicer and Drosha RNase III domains reveal deviations from the consensus in both enzymes. Introduction of these alterations into bacterial RNase III permitted a genetic test for domain function: defects were noted upon introduction of residues that form part of the catalytic centre from the second RNase III domain of Dicer family members. Antiparallel alignment of Dicer's RNase III motifs on a dsRNA substrate could produce four compound active sites, but the central two of these would be inactive. In this way, cleavage would occur at ~22-base intervals (42) (Fig. 2).

In the effector Step, RNAi is enforced by RISC, a protein–RNA effector nuclease complex that recognizes and destroys target mRNAs. The first subunit of RISC to be identified was the siRNA, which presumably identifies substrates through Watson–Crick base-pairing (43,44). One study showed that RISC is formed as a precursor complex of ~250K; which becomes activated by the addition of ATP to form a ~100K complex that can cleave substrate mRNAs (45). Cleavage occurs only in the region homologous to the siRNA. siRNAs

configuration of two-nucleotide 3' overhangs and 5'-phosphate termini (36,37) are functionally important for incorporation into RISC complexes (37,45). However, single-stranded siRNAs should be most effective at seeking homologous targets, and one intriguing correlation with the transition of RISC zymogens to active enzymes is siRNA unwinding (45). Another study showed that RISC purified from *Drosophila* S2 cells was ~500K ribonucleoprotein with slightly different characteristics (43,46). RISC* (the 100K active RISC species) cleaves its substrates endonucleolytically (45).

Intermediate cleavage products are never observed in even the most highly purified RISC preparations from S2 cells, suggesting the presence of an exonuclease in this enzyme complex. RISC from S2 cells co-purifies with AGO2, a member of the Argonaute gene family (46). These proteins are characterized by the presence of two homology regions, the PAZ domain and the Piwi domain, the latter being unique to this group of proteins. The PAZ domain also appears in Dicer proteins, and may be important in the assembly of silencing complexes (39). Argonaute proteins were linked to RNAi by genetic studies in *C. elegans*, whose genome contains >20 related genes. The rde-1 gene was isolated from a mutant worm that was unable to sustain RNAi in germline or soma (28). Genetic studies showed a requirement for RDE-1 and RDE-4(small dsRNA binding protein) for initiation of silencing in a parental animal (47); however, neither function was required for systemic silencing in F_1 progeny. It is believed that RDE-4 initially recognizes dsRNA and delivers it to the Dicer enzyme. This

would be consistent with the observation that siRNA levels are greatly reduced in worms that lack RDE-4 function, but are abundant in worms that lack RDE-1 (48). In *Neurospora*, mutations in the Argonaute family member qde-2 eliminate quelling (transgene co-suppression), but do not alter accumulation of siRNAs (49). Thus RDE-1 and perhaps other Argonaute proteins might shuttle siRNAs to appropriate effector complexes (RISCs).

Biological role of RNAi. Since the discovery of this evolutionarily conserved phenomenon, there has been a major question about the biological function(s) of RNAi. Three distinct roles for this process have emerged. First, RNAi clearly acts as an antiviral defense. Second, genetic studies have considered RNAi as a geno-protective mechanism. Third, recent findings have demonstrated a role for components of the RNAi machinery in the regulation of cellular gene expression and developmental timing.

Antiviral response - In mammals, there exist well-characterized responses to dsRNA that act as an antiviral defense. Therefore, one obvious role for the RNAi/PTGS machinery was as a functional homolog of such systems. Indeed definitive evidence for the use of RNAi as a viral defense comes from genetic studies in plants. Arabidopsis mutants that lose the ability to mount a PTGS response are hyper-susceptible to virus infection (50). Just as plants have evolved a defense against viral invasions, viruses have evolved a counterattack. For example, proteins such as cucumber mosaic virus 2b and p25 of potato virus X inhibit the spread of silencing within the plant (51). As expected if PTGS

is considered as a primary defense mechanism against such viruses, these inhibitors are essential determinants of virulence. Numerous mammalian viruses have evolved the ability to block PKR as an aid to efficient infection. For example, adenoviruses express viral RNAs, which mimic dsRNA with respect to binding but not to activation of PKR (52). Vaccinia virus uses two strategies to evade PKR. First is expression of E3L, which binds and masks dsRNAs (53). The second is expression of K3L, which binds and inhibits PKR via its ability to mimic the natural substrate of this enzyme, eIF2α (53).

Genome defense - In all complex genomes, a significant fraction of sequence is formed by endogenous repetitive elements, including numerous copies of defective and intact transposons. Suppression of these elements contributes to genetic stability in two ways. First, intact transposons are potential mutagens. Second, both defective and intact transposons provide potential sites for nonhomologous crossovers that could occur during DNA repair. Genomic stability requires that they be packaged into heterochromatin (31). In *C. elegans*, some RNAi-deficient strains are “mutators” owing to increased mobility of endogenous transposons (28,54). In many systems, transposons are silenced by their packaging into heterochromatin (31). Therefore, RNAi may stabilize the genome by sequestering repetitive sequences such as mobile genetic elements, preventing transposition and making repetitive elements unavailable for recombination events that lead to chromosomal translocations. However, it remains to be determined whether RNAi regulates transposons through effects

at the genomic level or by post-transcriptionally targeting mRNAs (for example, those encoding transposases) that are required for transposition.

Regulation of endogenous genes and developmental timing - a role for RNAi pathways in the normal regulation of endogenous protein-coding genes was originally suggested through the analysis of plants and animals containing dysfunctional RNAi components. Mutations in the Argonaute-1 gene of Arabidopsis, for example, cause pleiotropic developmental abnormalities that are consistent with alterations in stem-cell fate determination (55). A hypomorphic mutation in Carpel Factory, an Arabidopsis Dicer homologue, causes defects in leaf development and overproliferation of floral meristems (56). Mutations in Argonaute family members in *Drosophila* also impact normal development. In particular, mutations in Argonaute-1 have drastic effects on neuronal development (57), and piwi mutants have defects in both germline stem-cell proliferation and maintenance (58). This should not be interpreted as a demonstration that PTGS pathways regulate endogenous gene expression per se. In fact, separation-of-function ago1 mutants have recently been isolated that preferentially affect PTGS without affecting development. Mutations in Zwille, another Argonaute family member, also alter stem-cell maintenance (59), and this occurs without perceptible impact on dsRNA-mediated silencing. Thus, components of the RNAi machinery, and related gene products, may function in related but separable pathways of gene regulation. A possible mechanism underlying the regulation of endogenous genes by the RNAi machinery emerged

from the study of *C. elegans* containing mutations in their single Dicer gene, DCR-1. Unlike most other RNAi-deficient worm mutants, dcr-1 animals were neither normal nor fertile: the mutation induced a number of phenotypic alterations in addition to its effect on RNAi (41,60-62).

Dicer mutants showed alterations in developmental timing similar to those observed in let-7 and lin-4 mutants. The lin-4 gene was originally identified as a mutant that affects larval transitions (63), and let-7 was subsequently isolated as a similar heterochronic mutant (33). These loci encode small RNAs, which are synthesized as ~70-nucleotide precursors and post-transcriptionally processed to a ~21-nucleotide mature form. Genetic and biochemical studies have indicated that these RNAs are processed by Dicer (41,60-62).

The small temporal RNAs (stRNAs) encoded by let-7 and lin-4 are negative regulators of specific protein-coding genes, as might be expected if stRNAs trigger RNAi. However, stRNAs do not trigger mRNA degradation, but regulate expression at the translational level (64,65). This raised the possibility that stRNAs and RNAi might be linked only by the processing enzyme Dicer. A model in which the effector complexes containing siRNAs and stRNAs are closely related, but regulate expression by distinct mechanisms is illustrated in Figure 3. Neither LIN-4 nor LET-7 forms a perfect duplex with their cognate target (66). Thus, in one possible model an analogous RISC complex is formed containing either siRNAs or stRNAs. In the former case, cleavage is dependent upon perfect complementarity, while in the latter, cleavage does not occur, but

the complex blocks ribosomal elongation. Alternatively, siRNAs and stRNAs may be discriminated and enter related but distinct complexes that target substrates for degradation or translational regulation, respectively. Consistent with this latter model is the observation that siRNAs or exogenously supplied hairpin RNAs that contain single mismatches with their substrates fail to repress, rather than simply shifting their regulatory model to translational inhibition (37,67,68). RISC may be viewed as a flexible platform upon which different regulatory modules may be superimposed. The core complex would be responsible for receiving the small RNA from Dicer and using this as a guide to identify its homologous substrate. Depending upon the signal (for example, its structure and localization), different effector functions could join the core: in RNAi, nucleases would be incorporated into RISC, whereas in stRNA-mediated regulation, translational repressors would join the complex. Transcriptional silencing could be accomplished by the inclusion of chromatin remodelling factors.

Recent findings show that let-7 and lin-4 are archetypes of a large class of endogenously encoded small RNAs (Table I). Over 100 of these microRNAs or miRNAs have now been identified in *Drosophila*, *C. elegans* and mammals, although most of their functions are unknown, their prevalence hints that RNAi-related mechanisms may have pervasive roles in controlling gene expression (69-72).

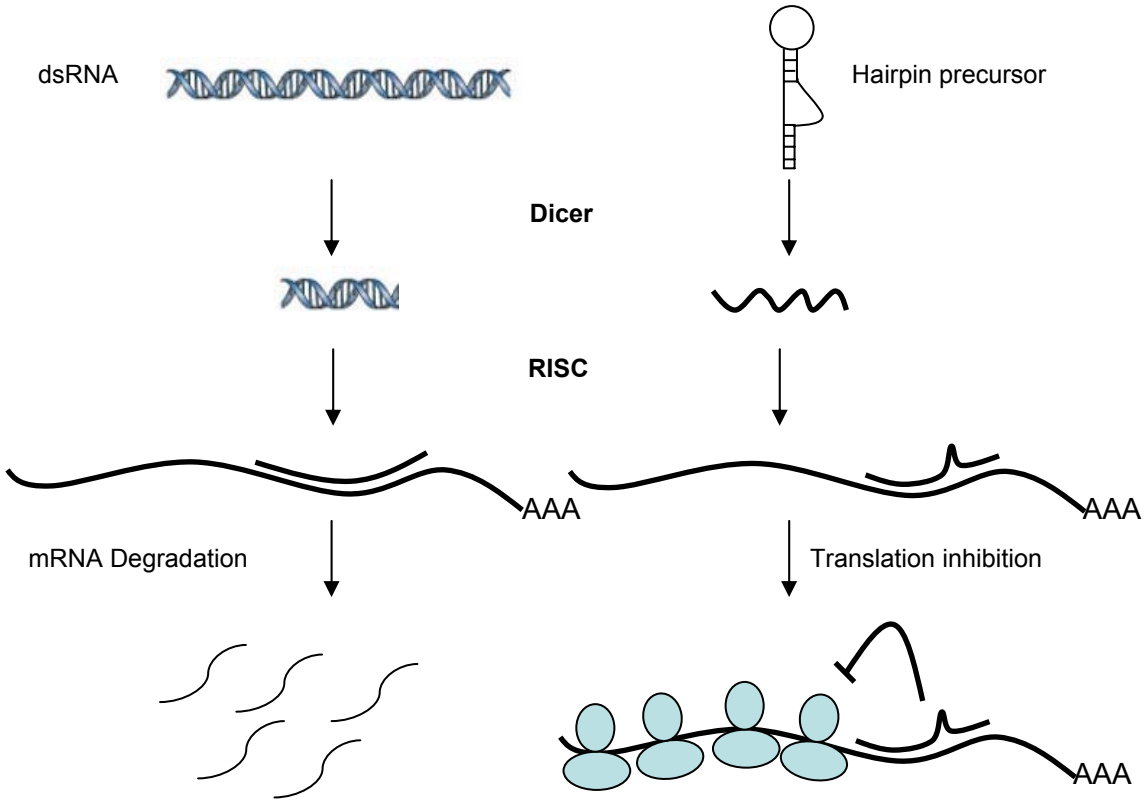


Fig. 3. **Small interfering RNA versus micro RNA gene silencing mechanisms.** siRNAs but not microRNAs have perfect complementarity to their target mRNA which cause different mechanism of gene silencing. Modified from (68).

Table I

Types and functions of microRNAs. Modified from (73).

Process	**Example**	**Function**
Transcription	184-nt *E. coli* 6*S*	Modulates promoter use
	331-nt human 7SK	Inhibits transcription elongation factor P-TEFb
	875-nt human SRA	Steroid receptor coactivator
Gene silencing	16,500-nt human *Xist*	Required for X-chromosome inactivation
	~100,000-nt human *Air*	Required for autosomal gene imprinting
Replication	451-nt human telomerase RNA	Core of telomerase and telomere template
RNA processing	377-nt *E. coli* RNase P	Catalytic core of RNase P
	186-nt human U2 snRNA	Core of spliceosome
RNA modification	102-nt *S. cerevisiae* U18 C/D snoRNA	Directs 2'-*O*-ribose methylation of target rRNA
	189-nt *S. cerevisiae* snR8 H/ACA snoRNA	Directs pseudouridylation of target rRNA
	68-nt *T. brucei* gCYb gRNA	Directs the insertion and excision of uridines
RNA stability	80-nt *E. coli* RyhB sRNA	Targets mRNAs for degradation?
	Eukaryotic miRNA?	Targets mRNAs for degradation?
mRNA translation	109-nt *E. coli* OxyS	Represses translation by occluding ribosome binding
	87-nt *E. coli* DsrA sRNA	Activates translation by preventing formation of an inhibitory mRNA structure
	22-nt *C. elegans lin-4* miRNA 22-nt *C. elegans* let-7 miRNA	Represses translation by pairing with 3' end of target mRNA
Protein stability	363-nt *E. coli* tmRNA	Directs addition of tag to peptides on stalled ribosomes
Protein translocation	114-nt *E. coli* 4.5*S* RNA	Integral component of signal recognition particle central to protein translocation across membranes

Heritable nature of RNAi. The classification of RNAi/PTGS as an epigenetic phenomenon rests largely upon its ability to provoke heritable changes in gene expression. Inheritance of silencing could be derived from either of two sources. The first is the persistence of the signal. The second is persistence of the silenced state. The former case refers to instances such as stable incorporation of transgene arrays into the genome, the presence of endogenous repetitive elements such as transposons, or the enforced expression of hairpin RNAs. Such cases require no additional mechanisms to explain heritable silencing because the trigger is expressed from an endogenous and heritable genetic element. The latter case is more provocative and requires consideration of mechanisms that propagate either the signal or the silenced state independently of the silencing trigger.

The classical example of silencing that is inherited after a transient introduction of the silencing trigger comes from observation with *C. elegans*. Worms that have been injected with dsRNA can impart the silenced state to the next generation, and this has been demonstrated for numerous genes (1). Experiments targeting genes that are expressed in the maternal germline demonstrated interference in the F2 generation; however this effect waned in later generations (74). So far, no genetic mutants have emerged that specifically affect the heritability of silencing without affecting the interference process itself.

Small interfering RNA as a tool for the analysis of genes function. Although RNAi has been used in diverse systems, harnessing RNA to study

gene function in mammals seemed potentially problematic. Indeed, mammals have evolved robust systems for responding to dsRNAs, specifically as an antiviral defense (75,76). In somatic cells, dsRNA activates a variety of responses. Predominant among these is PKR, a kinase that is activated by dimerization in the presence of dsRNA (52). PKR, in turn, phosphorylates EIF2α, causing a nonspecific translational shutdown (75). Double stranded RNA also activates 2'-5' oligoadenylate polymerase, the product of which is an essential cofactor for a nonspecific ribonuclease, RNase L that non-specifically degrade all mRNA (77). In some situations, several-hundred-base-pair long dsRNA represents an alternative to siRNAs. Long dsRNA effectively silences genes expressed in insect cells (43,78-81) and in embryonic mammalian cells that have not yet established the interferon system (82-85). In somatic mammalian cells, the application of long dsRNA is prohibited, because these cells trigger a sequence-nonspecific innate immune response (interferon-mediated defense) when exposed to dsRNA greater than thirty base pairs (86). The use of siRNAs of 21-23 nucleotides bypass the interferon response and produce extraordinary effect in gene silencing in mammalian cells (37).

Characteristics of functional siRNA. siRNA duplexes produced by the action of Dicer contain 5'-phosphates and free 3'-hydroxyl groups. The central base-paired region is flanked by two-to-three nucleotides of single-stranded 3'-overhangs (37). The 5' -phosphate termini of siRNAs is essential for guiding mRNA degradation (45). Nevertheless, for their practical application in gene

targeting experiments, siRNAs may be used without 5'-phosphate termini because a kinase activity in the cell rapidly phosphorylates the 5' ends of synthetic siRNA duplexes (37,45,87). Under certain circumstances (e.g., injection experiments in D. melanogaster), 5'-phosphorylated siRNA duplexes may have slightly enhanced properties as compared to 5'-hydroxyl siRNAs (87). In gene targeting experiments using human HeLa cells, no differences in siRNA-mediated "knockdown" of gene expression were observed, as a function of 5'-phosphorylation (88). The sequencing of the human genome has greatly stimulated research on gene function for validating new targets for drug discovery and development of therapeutic strategies for many common disorders including infectious, cardiovascular, neurological diseases and cancer. siRNAs are excellent tools for target validation in biomedical research , because of their exquisite specificity, efficiency and endurance of gene-specific silencing. siRNAs are probably also suitable for the design of novel gene-specific therapeutics by directly targeting mRNAs of disease-related genes.

Small interfering RNAs have brought reverse genetics to mammalian cultured cells, and have made large-scale functional genomic analysis a possibility (89). For example targeting of essential and non-essential genes resulted in cellular phenotypes that were identical to phenotypes previously observed in cells derived from transgenic knockout mice (89), illustrating the value of siRNA methodology for the analysis of mammalian gene function.

Until recently, the application of siRNAs in somatic cells was restricted to the delivery of chemically or enzymatically synthesized siRNAs (37,84,90), however, methods for intracellular expression of small RNA molecules have now been developed. The use of RNA polymerase III (Pol III) promoters to direct *in vivo* synthesis of functional siRNAs has been reported (84,91-97). There are several reasons for using Pol III. Unlike RNA Pol II, Pol III normally transcribes small, noncoding transcripts that are not capped or polyadenylated at the 5' and 3' ends, respectively. Pol III initiates transcription at defined nucleotides, and terminates transcription when it encounters a stretch of four or five thymidines (98). Consequently, it is possible to design small RNAs synthesized by Pol III that carry 3' overhangs of one to four uridines, a structural feature resembling that defined for siRNAs to be effective in vitro (37).

Two approaches using Pol III promoters have yielded robust gene-specific inhibition (Fig. 4). In the first case, the design is modeled after the naturally occurring microRNAs (miRNA) that are ~22-nt hairpins and can modulate gene expression *in vivo* (99). Pol III promoter, U6 or H1, is used to direct transcription of small inverted repeats separated by a spacer region of varying lengths. The U6 or the H1 promoter initiates transcription at guanine or adenine, respectively. The resulting RNAs are predicted to form hairpins containing 19- to 29-nts stems that match target sequences precisely, three- to nine-nt loops and 3' overhangs of four or fewer uridines). It is believed that these hairpin RNAs are processed by Dicer into active siRNAs *in vivo* (84). In the

second case, two U6 promoters are placed in tandem(94,96) or on two separate vectors (93)to direct transcription of a sense and an antisense strand of a small RNA with 19 nt matching the target gene sequence precisely and four or fewer Us as 3' overhangs. The sense and antisense strands are believed to form a duplex *in vivo* similar to the chemically synthesized siRNAs described by Elbashir and coworkers (67). However, the hairpin siRNA strategy appears to inhibit gene expression more efficiently than the duplex siRNAs expressed from two separate plasmids (93). Another way of generating small RNAs that can function as siRNAs from DNA templates is through the generation of modified miRNAs. Naturally occurring miRNAs are noncoding RNAs that have been identified in a range of organisms from *C. elegans* to humans (98). The best characterized miRNAs (also known as stRNAs for small temporal RNAs) are *C. elegans* lin-4 and let-7, both of which are crucial in the control of developmental timing (63,100). Artificial miRNAs whose sequences are completely complementary to the target RNAs have been shown to function as siRNAs that inhibit gene expression by reducing RNA transcript levels (97,101).

Unlike the small hairpin RNAs directed by U6 or H1 promoters, the siRNA-acting miRNAs are generated from ~70-nt miRNA precursors. The artificial miRNA precursor contains a substitution of the stem sequence with a sequence entirely complementary to the intended target gene, enabling the resulting miRNA to function as a siRNA to induce target RNA degradation (101).

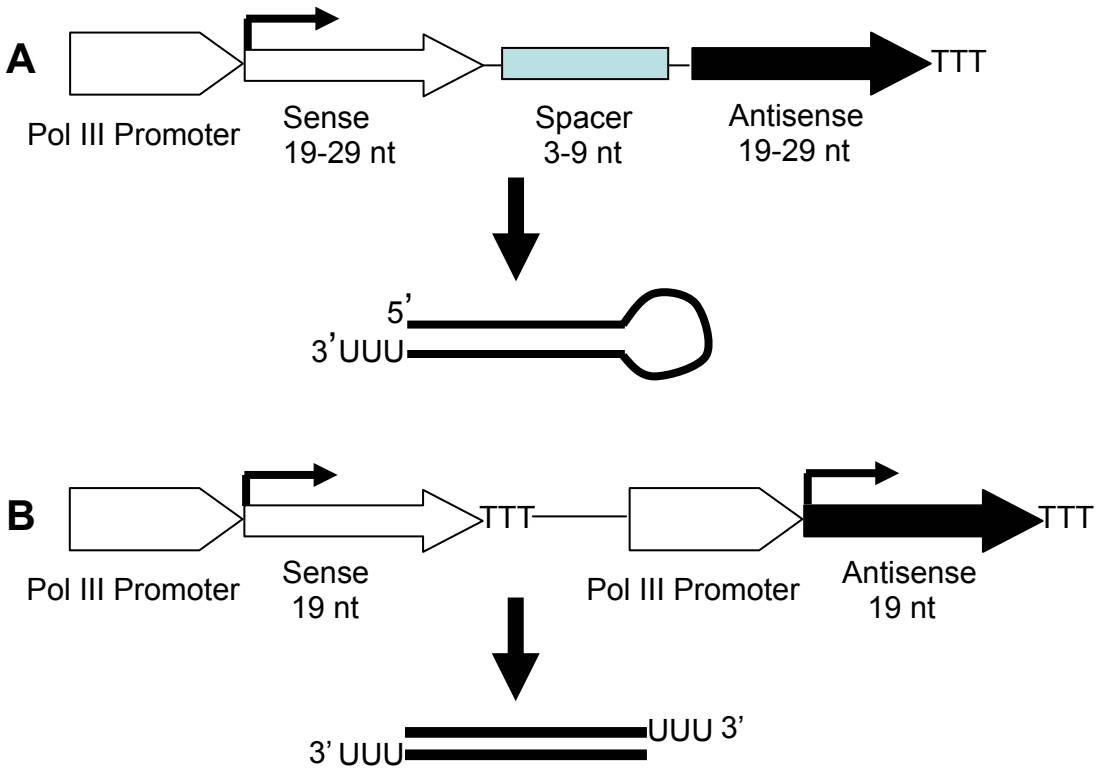


Fig. 4. **Approaches for *in vivo* synthesis of functional siRNA.** [A] Generation of hairpin siRNA from inverted repeat. [B] Generation of siRNA from two complementary strands. Modified from (68).

An important advantage of the DNA vector-based RNA approach is that it can be used to express siRNAs stably in cells and thus provide long-term gene inhibition. This principle was demonstrated recently by Brummelkamp and coworkers (92) who reported sustained inhibition of p53 by stably integrated siRNA-expressing DNA templates. Another advantage of the DNA vector system is that it can be useful for inducible knockdown of gene expression. One study has shown that doxycycline-regulated form of the H1 promoter of RNA polymerase III allows the inducible knockdown of gene expression by siRNA. β-Catenin in colorectal cancer was used as target in this study as a proof-of-principle (102). The resistance of important cell types to transfection using the previous approaches, both *in vivo* and *in vitro*, has limited the use of siRNA (103). Recently, several viral vectors have been developed for efficient delivery of siRNA into mammalian cells (104,105). Retroviral vectors were designed to produce siRNA driven by either U6 or H1-RNA promoter for efficient, uniform delivery and immediate selection of stable knock-down cells (104,106). Adenovirus vectors using RNA polymerase II CMV promoter (105) and the well defined pol III promoter (91) were also developed and shown to mediate gene silencing both *in vitro* and *in vivo*. Recently, a lentiviral system has been used for delivery of siRNA into cycling and non-cycling mammalian cells, stem cells, zygotes and their differentiated progeny (107). Lentiviruses have two key advantages over other gene delivery system. Firstly, they can infect non-cycling and post-miotic cells (108,109). Secondly, transgenes expressed from

lentiviruses are not silenced during development and can be used to generate transgenic animals through infection of embryonic stem cells or embryos (110,111).

Small interfering RNAs as therapeutic agents. siRNAs are highly sequence-specific reagents and discriminate between single mismatched targeted RNA sequences (67,92), and may represent a new avenue for gene therapy for several diseases.

Infectious disease - Viral inhibitors of the mammalian RNAi machinery have not yet been described and it is feasible that the application of siRNAs could extend our understanding of viral protein function and viral life cycle and could be used as antiviral therapy. One study reported suppression of HIV-1 infection and replication in permanent cell lines and primary activated CD4 T cells by siRNA specific for different regions of HIV-1(112). Cells harboring proviral HIV, such as reservoir or acutely infected cells that have progressed past proviral integration, can also be targeted by RNAi-mediated inhibition of viral replication by targeting viral RNA transcripts produced from the provirus (112). Other studies have reported efficient inhibition of hepatitis C (113) and B (114) viruss replication as well as protein synthesis by using both synthetic and vector derived siRNAs. Although viral replication was inhibited and mRNAs transcribed from the viral genome were effectively silenced, it was not possible to cleave the viral genomic or antigenomic RNA because of its chromatin-like condensed structure. Recently one study reported effective siRNA-mediated

degradation of HIV-1 rev transcripts in a cell assay by co-transfection of proviral DNA and siRNA expression vectors, thus raising the possibility that siRNAs can be developed for treating HIV infection (94).

Genetic disorders and neoplastic disease - The expression of mRNAs coding for mutated proteins, which give rise to dominant genetic disorders and neoplastic growth, might be decreased or blocked completely by specific siRNAs (Table II). In leukemias and lymphomas (the most frequent cancers in childhood), oncogene activation frequently occurs through reciprocal chromosomal translocations. These translocations lead to juxtaposition of gene segments normally found on different chromosomes, and the creation of a composite gene (115). Translocation of the BCR gene from chromosome 22 and ABL gene from chromosome 9 creates an oncogenic BCR-ABL hybrid gene (116). The BCR-ABL fusion protein has dramatically increased tyrosine kinase activity compared to that of the normal ABL protein, leading to aberrant phosphorylation of several downstream molecules. RNAi was used to target the BCR-ABL mRNA, and this approach was effective in reducing the expression of BCR-ABL mRNA, followed by a reduction of BCR-ABL oncoprotein, leading to apoptosis in leukemic cells (117). Silencing of these tumor-specific, chimeric mRNAs by siRNAs might become an effective fusion gene-specific tumor therapy. The extraordinary sequence specificity of the RNAi mechanism may also allow for the targeting of individual polymorphic alleles expressed

Table II

Suggested targets in human malignancy for siRNA-mediated therapy.

Genes or Fusion Genes	**Aberration**	**Tumors**
RAS	Point mutations	Pancreatic carcinoma, chronic leukemia, colon carcinoma, lung cancers
c-MYC, N-MYC	Overexpression, translocation, point mutation, amplification	Burkitt's lymphoma, neuroblastoma
ERBB1	Overexpression	Breast cancer
ERBB2	Overexpression	Breast cancer
MLL fusion genes	Translocation	Acute leukemias
BCR-ABL	Translocation	Acute and chronic leukemia
TEL-AML1	Translocation	Acute and chronic leukemia
EWS-FLI1	Translocation	Childhood acute leukemia
TLS-FUS	Translocation	Ewing sarcoma
PAX3-FKHR	Translocation	Myxoid liposarcoma
BCL-2	Overexpression, translocation	Alvelolar rhabodomyosarcoma
AML1-ETO	Overexpression, translocation	Lung cancers, Non-Hodgkin lymphoma, prostate cancer

in loss-of-heterozygosity tumor cells, as well as targeting point-mutated transcripts of transforming oncogenes such as Ras and tumor suppressor genes such as P53. P53 which is called the guardian of the genomes inactivated by point mutation in 50% of human cancers. One study has demonstrated that a single base difference in siRNAs discriminates between wild type and mutant P53 in cells expressing both forms, resulting in restoration of wild type protein function (118). RNAi also can be useful in decreasing overexpressed apoptosis inhibitors such as Bcl-2 and c-Myc which may be beneficial in cancer therapy. siRNA-mediated therapy can be used to silence many gene targets that contribute to human malignancy (Table 2).

Neurological disease - Another potential application of siRNA is in the treatment of neurodegenerative disorders, siRNAs were recently directed against a mutated mRNA associated with the spinobulbular muscular atrophy in tissue culture (119). Spinobulbular muscular atrophy, together with Huntington Disease, belongs to a growing group of neurodegenerative disorders caused by the expansion of trinucleotide repeats (120). Targeting the CAG-expanded mRNA transcript with dsRNA may be an alternative to commonly used therapeutic strategies (119).

Use of siRNA as a tool is advancing in almost every field of biomedical research as mentioned before, but some of the most dynamic and exciting applications of siRNA are in cancer research in particular functional validation of tumorigenic genes in cell culture and animal tumor models. Research in this

laboratory has focused on different types of cancers including hormone-dependent cancers such as breast, endometrial and prostate cancers as well as hormone-independents cancers such as pancreatic and colon cancers. siRNAs will be used in this research to knock down selected genes in mammalian cancer cells as an approach for determining their role in cancer cells growth and progression. In addition, this approach will be used to understand the mechanism of action for some of the compounds that are being synthesized in this laboratory such as PPARγ agonists.

WHAT IS CANCER?

Cancer is the second leading cause of death in the United State and one half of all men and one- third of all women in the United States will develop cancer during their lifetime (121). The term cancer describes a subset of lesions of a disease termed neoplasia. Neoplasia literally means “new matter” and refers to any abnormal growth of cells. Neoplasms can be classified as benign or malignant. Benign tumors do not metastasize to other tissues and they usually grow very slowly, their cells are often well differentiated and tend to stay together because they are surrounded by a capsule of dense tissue. Benign tumors are usually not life threatening unless they disrupt the function of a vital organ. Unlike benign tumors, malignant tumors or cancers have undifferentiated, rapid growing cells that are not encapsulated and tend to metastasized to other regions of the body through blood and lymphatic vessels. For example cells from

malignant breast cancer usually form new (secondary) tumors in bone, brain, and lung tissues (122). Benign tumors that arise from epithelial tissues include papilomas, adenomas, and nevus while those that arise from connective tissues include lipomas, osteomas and chondromas. Malignant tumors from epithelial cells are generally called carcinomas while those that arise from connective tissues are called sarcomas (122). There are three types of changes that occur when a cell becomes tumorigenic. First, immortalization, where the cells have the property of indefinite growth without any other changes in the phenotype. Second, transformation, which describes the failure to observe the normal constrains of growth, where the transformed cells become independent of factors usually needed for cell growth. Finally, metastasis, where cancer cells gain the ability to invade normal tissues and form new colony elsewhere in the body away from the tissue of origin (123) (Fig.5).

The etiology of various forms of cancer is not well defined; it is known that cancer involves hyperplasia (too many cells) and/or anaplasia (abnormal, undifferentiated cells) but it is unclear what causes these phenotypes. There are several factors known to play a role in cancer development. These include genetic factors (123), exposure to carcinogens (123), physical agents such as ionizing radiation (124) and ultra violet light (125). In addition, infection with oncogenic viruses (such as human immunodeficiency and hepatitis C viruses) is associated with a number of human cancers (126). Lifestyle plays a major role for increasing risk of cancer. For example tobacco smoke is the principle cause

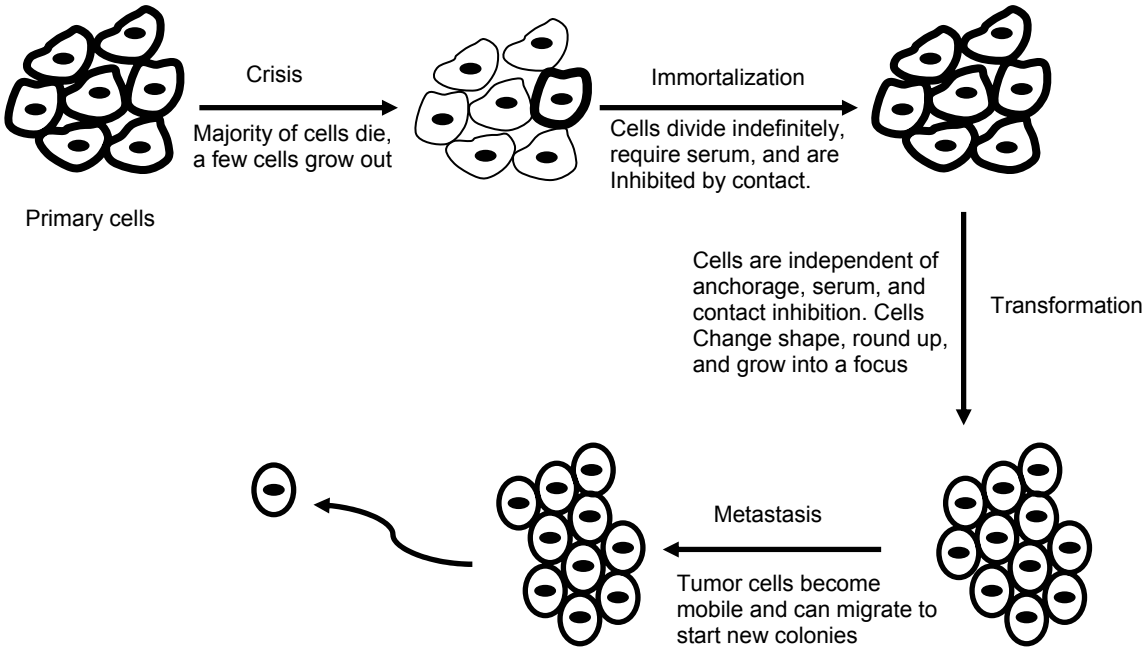


Fig. 5. **Sequential changes that distinguish a cancer cell from a normal cell.** Modified from (123).

of lung cancer (127). On the other hand, consumption of certain phytochemicals found in a complex human diet, such as carotenoids (green, yellow-red, and yellow-orange vegetables), phytoestrogens (soy and some soy products), organosulfides (garlic), phenolic acids (green tea, citrus) has exhibit anti-mutagenic and anti-carcinogenic effects (128).

BREAST CANCER

Breast cancer is an endocrine-responsive tumor that accounts for one in four of all female cancers, making it by far the most common cancer in women in the Western world. One in eight or nine women in the United Kingdom will develop breast cancer at some time in their lives (129). About 211,300 women in the United States will have invasive breast cancer in 2003 and about 39,800 women will die from the disease (121).

The breasts lie over the pectoral muscles and are attached to them by a layer of connective tissue, each breast consist of several lobes separated by septa of connective tissue. Each lobe consists of several lobules which are composed of connective tissues in which are embedded the secreting cells (aloveoli) of the gland, arranged in grapelike clusters around the minute ducts. Ducts from various lobules unite form a single luciferous duct for each lobe. These main ducts converge toward the nipple (Fig. 6). There is large amount of adipose tissue deposited around the surface of the glands; the breast size is determined by amount of this fat around the glandular tissue. There is an

extensive network of lymphatic vessels and nodes that receive lymph from the breast. The breast is drained by two sets of lymphatic vessel, one originate in and drain the skin over the breast with the exception of areola and nipple and the other drains the substance of the breast itself, as well as the skin of the areola and nipple (122). Knowledge of the lymphatic drainage of the breast is important in clinical medicine because cancerous cells from malignant breast tumors often spread to other areas of the body through the lymphatics.

Normal breast development and breast cancer. Mammary development begins during embryogenesis; in humans, males and females have a similar rudimentary mammary gland at birth. Subsequent mammary development is initiated with the onset of female puberty and is dependent on the high levels of estrogen. The most abundant circulating form of estrogen is 17β-estradiol (E2) which primarily produced along with progesterone in the ovary (Fig. 7). After puberty, the mammary gland undergoes cycles of growth and involution, which are regulated with the menstrual cycle, and with cycles of pregnancy and lactation. Post-pubertal development results in cyclical increases in ductal branching, resulting in a ductal tree that fills the mammary fat pad. During pregnancy, further branching and end-bud development lead to an appearance that is like bunches of grapes. After weaning, mammary-gland regression to a near pre-pregnancy state occurs through massive programmed cell death or apoptosis (130,131).

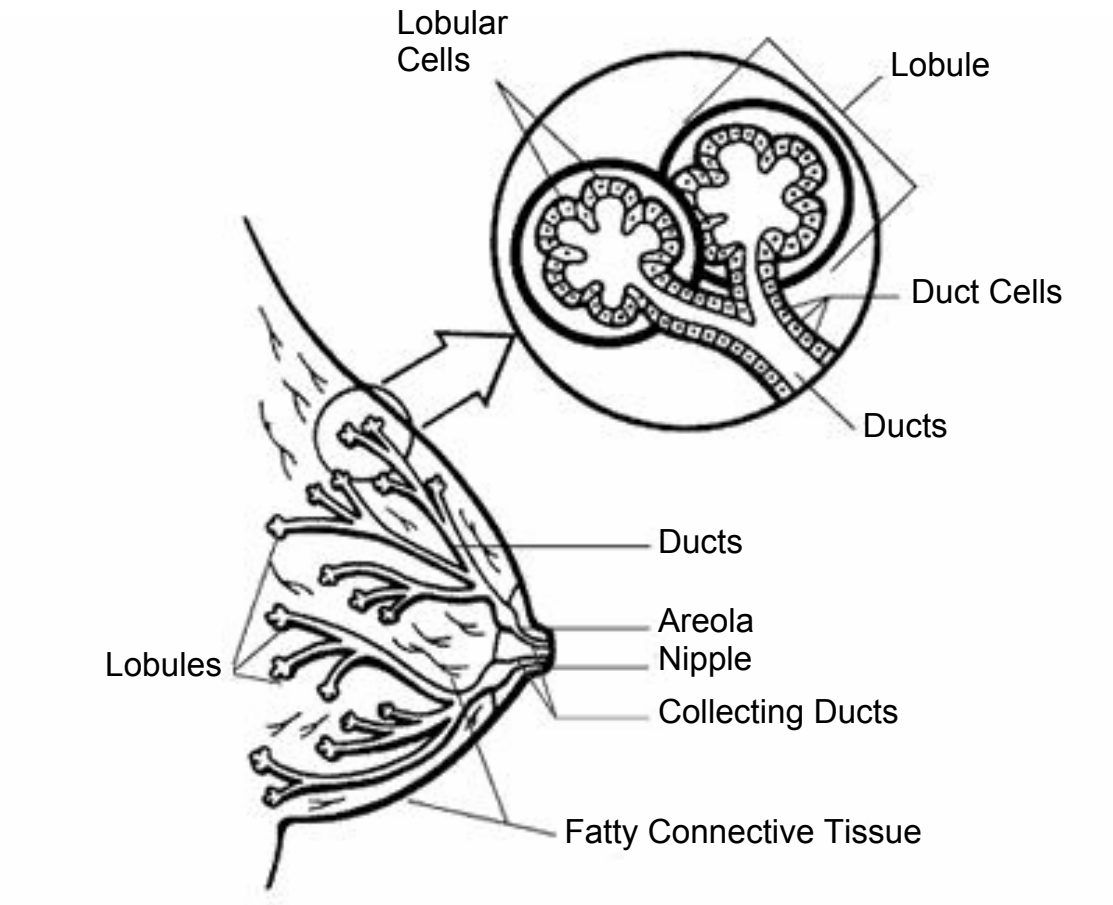
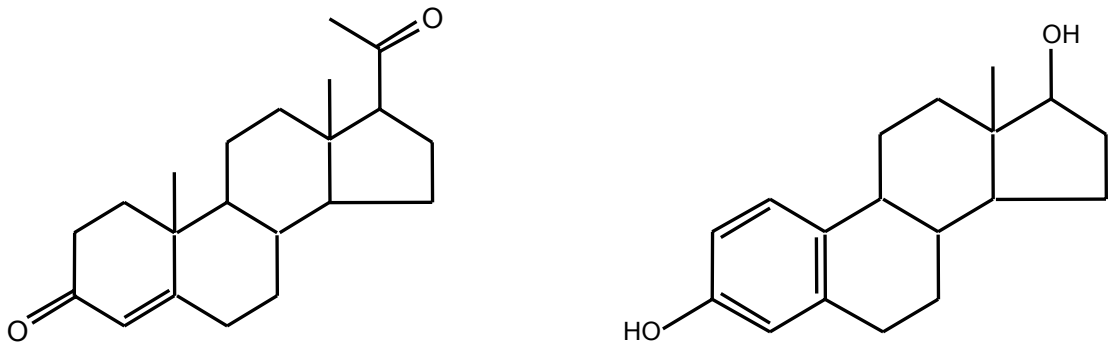


Fig. 6. **Anatomy of female breast.** Adapted from (122).



Progesterone Estrogen

Fig. 7. **Chemical structures of progesterone and estrogen.**

Shedding of placenta after delivery of the baby cuts off a major source of estrogen. The resulting rapid drop in the blood concentration of estrogen stimulates anterior pituitary secretion of prolactin which stimulates alveoli of the mammary glands to secrete milk. Also, the sucking movement of a nursing baby stimulates anterior pituitary secretion of prolactin and posterior pituitary secretion of oxytocin which stimulates alveoli of the braest to eject milk into the ducts which is accessible to the infant by sucking (122). Epithelial cells seem to be the main site of estrogen action in the breast. Immunohistochemical analysis reveals that the epithelial cells contain the receptor that mediates the action of estrogen (132). Histologically, it is the luminal epithelial cells that are responsible for most breast tumors, and this is also supported by biochemical comparisons (133).

Risk factors for breast cancer. The cause of breast cancer remains unknown in the majority of patients despite identification of numerous risk factors in epidemiological studies. A family history of breast cancer is one of the strongest risk factors, especially in families with multiple first-degree relatives. About 5%-10% of breast cancer cases are due to inheritance of highly penetrant mutations in breast cancer susceptibility genes. These include BRCA-1 and BRCA-2 genes, p53 gene mutations in Li-Fraumeni syndrome, PTEN mutations in Cawden's disease, and the AT gene in ataxia teleangiectasia (134). BRAC1 is one of the most common breast cancer susceptibility genes which was first identified in 1994 as an autosomal dominant mutation for breast cancer and it was later shown to increase risk for ovarian cancer (135,136). Women that

carry the mutation in BRCA1 have a 60-80% lifetime risk for developing breast cancer and a 20-40% lifetime risk of developing ovarian cancer (137-139). BRCA1 is believed to be a tumor suppressor gene which functions to regulate transcription, cell cycle control, and DNA repair. BRCA1 can interact with RNA polymerase II, as well as to enhance p53 transactivation (133,140). Like BRCA1, mutations in the BRCA2 gene increase lifetime risk of breast and ovarian cancer in carriers of this mutation (141,142). Unlike BRCA1 gene, the function of BRCA2 is not well defined, but may be involved in some of the same processes as BRCA1 (143).

There is considerable evidence that associates increased breast cancer risk with lifetime exposure to estrogens. Risk for breast cancer in women is associated with early menarche, late first full-term pregnancy and late menopause. Oral contraceptives and estrogen-replacement therapy have also increase the risk for breast cancer. In addition, dietary and environmental agents that can act as estrogens have been linked to breast cancer risk (144,145). Moderate alcohol consumption (146) and smoking in women with genetic defects in aromatic amine metabolism modestly increase breast cancer risk (147). Women with a history of prior invasive breast cancer or a history of noninvasive breast lesions such as atypical hyperplasia and lobular carcinoma *in situ* (LCIS), carry an increased risk for developing invasive breast cancer (148).

Breast cancer treatment. There are many different classes of drugs that have been approved for the treatment of breast cancer both in the presence or absence of surgery and radiotherapy.

Chemotherapeutic drugs - This type of therapy is based on the fact that cancer cells are rapidly dividing cells, and these drugs interfere or inhibit cancer cell proliferation. There are three major classes of chemotherapeutic drugs for treating different types of cancer including breast cancer and these include anthracyclins such as doxorubicin and epirubicin, taxanes such as paclitaxil and docetaxil and alkylating agents such as cyclophosphamide (149). The disadvantage of using such drugs is the lack of specificity, since these also kill normal cells in our bodies that have rapid dividing nature such as blood cells and epithelial cells of intestine and skin.

Endocrine therapy - Two-thirds of breast cancers are ER-positive and most of these respond to endocrine therapy (150). Several drugs have been used to block the effects of estrogen in ER-positive breast cancer (Fig. 8). The anti-estrogen tamoxifen was first used in the treatment of metastatic breast cancer, and led to disease regression in approximately 30% of these cancers (151). In ER-positive breast cancer, tamoxifen is now the principal form of adjuvant treatment in pre- and post-menopausal women. In addition, medical ovariectomy with luteinizing-hormone-releasing hormone (LHRH) agonists such as goserelin is also a commonly used treatment in pre-menopausal women. LHRH agonists decrease luteinizing-hormone secretion by the pituitary, leading

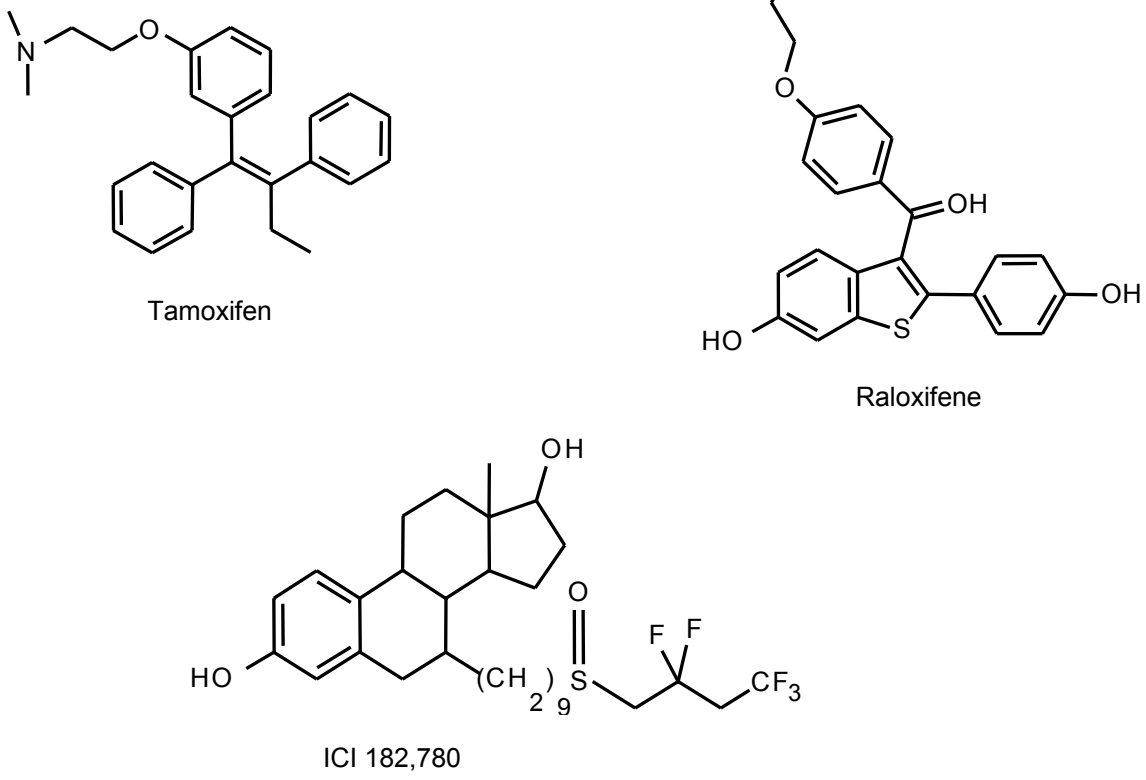


Fig. 8. **Chemical structure of tamoxifen, raloxifene, and ICI 182,780.**

to a block in follicular activity and consequent reduction in estrogen production by the ovaries (152). Tamoxifen was originally developed as an oral contraceptive, but the potential of its anti-estrogenic action in breast cancer was recognized (153) and this drug is now become the most widely used endocrine agent for the treatment of breast cancer. Tamoxifen treatment for one or two years provides some reduction in recurrence and death for women with operable breast cancer. Treatment for five years provides a maximal benefit with a 51% reduction in recurrence and about 28% reduction in deaths during years 0–4. Reduction in recurrence and mortality is sustained in year 5 and beyond. The benefits of adjuvant tamoxifen treatment are independent of age, but are restricted to women with ER-positive breast cancer (150,151). Because of this success with tamoxifen, several other anti-estrogens have been developed and clinically tested. Raloxifene and faslodex (ICI 182, 780) (Fig.8) (154) are examples of the new generation of anti-estrogen. Raloxifene, like tamoxifen, is mixed ER agonists/antagonists, with antagonistic activity in the breast. Unlike tamoxifen which has estrogenic activity in endometrial tissue and results in increased risk for endometrial cancer, raloxifene had reduced estrogenic activity in the endometrium and is now being tested as chempreventive agent for breast cancer (155). On the other hand, Faslodex is a 'pure' ER antagonist, and is active in patients with metastatic breast cancer who had relapsed on tamoxifen therapy. Several Phase III studies with ICI show higher efficacy compared with other endocrine therapies (156). The use of raloxifene in breast cancer

prevention is also being investigated. Aromatase is responsible for local estrogen synthesis in post-menopausal women. Aromatase inhibitors are now being used to reduce peripheral estrogen synthesis as second- and third-line agents for treatment of hormone-sensitive disease, once resistance to tamoxifen has developed. Recent study indicates that third-generation aromatase inhibitors might be superior to tamoxifen in causing regression of breast cancers, in terms of both response rates and duration of response (157).

Retinoids - Many studies have shown that retinoids such as trans-retinoic acid or 9-cis retinoic acid can be used to inhibit breast tumor promotion and/or progression (158,159). This effect is mediated by transcribtional activation of nuclear retinoic acid receptor (RAR) and retionoid X receptor (RXR). Many genes that are important for growth and cell cycle progression are regulated by the active RAR/RXR complex. Examples of these genes include p27 (158), (160), cyclin D1 and cdk-2 (161). It is worth noting that ER (-) breast cancer cells are more resistant to retinoid treatment than ER (+) cells and this maybe due to different expression patterens of RARα (162).

PPAR γ agonist - Many PPAR γ ligands have been used for the treatment of different types of cancer including breast cancer. PPAR γ is expressed in many cancer cell lines (163) and in both primary and metastatic carcinomas (164). Elstner and coworkers had shown that PPAR γ agonist troglitazone can inhibit cellular proliferation by 50 % (165). These agonists produce their effect by binding to PPAR γ followed by heterodimerization with RXR (166). 15-deoxy-

$\Delta^{12,14}$-prostagladin J_2 (15dPGJ2) is another PPAR γ ligand that also has a potential use in breast cancer treatment. Like troglitazone, 15dPGJ2 inhibits cell proliferation and promotes apoptosis (167). On going research in this laboratory has investigated a new class of PPARγ agonists as a potential treatment for breast cancer. These compounds include 1,1-bis(3'-indolyl)-1-(ρ trifluoromethylphenyl)methane (DIM-C- ρPhCF3) and several ρ-substituted phenyl analogs. Compounds containing *para t*-butyl, cyano, dimethylamino and phenyl substitutents were the most active PPARγ agonists.

Vitamin D analogs - Vitamin D or its analogues has been investigated as drugs for treatment of breast cancer. 1alpha, 25-Dihydroxyvitamin D-3 (1α, $25(OH)_2D_3$), the active metabolite of vitamin D, inhibits growth of many cancer cell lines including those from the breast cancer (168,169). 1γ,$25(OH)_2D_3$ produces its effect by activating the vitamin D receptor (VDR) which is another member of nuclear receptor superfamily (170).The active VDR heterodimerizes with RXR and binds the vitamin D-responsive element on vitamin D responsive gene promoters.

Treatment with antibodies - Another potential treatment for breast cancer is the use of humanized monoclonal antibodies against growth factor and/or growth factor receptors. One of the important characteristic of ER(-) and SERM resistant breast cancers is the upregulation of receptor tyrosine kinases on the surface of these cells and this mimics the action of the growth factors (171-175).

The EGF family of structurally related receptor tyrosine kinases known as the ErbB receptors mediate proliferation of breast cancer cells/tumors primarily through activation of the MAP kinase and PI3-K signal transduction pathways (174,176). Four ErbB receptors have been identified: ErbB1 (HER1), ErbB2 (also known as Her2neu), ErbB3 (HER3), and ErbB4 (HER4) (177). ErbB2 (HER-2/neu) is a potential target for drugs since this receptor is overexpressed in 20-30 % of mammary tumor (178). Herceptin is one of the humanized monoclonal antibody, which binds to and downregulates the Her2/neu receptors on the cell surface by causing them to be endocytosed into the cell and thereby limiting tumor growth regulated by these signaling pathways (179,180).

Gene therapy - One of the most promising treatments for several types of cancer including breast cancer is the use of gene therapy. Gene therapy can be used to replace and/or knockout defective genes or alleles in tumor cells with the prospect of causing cancer remission. Target genes for this therapy include tumor suppresser genes, cell cycle kinase inhibitors, or genes that inhibit growth factor receptors. P53 is one of these targets since it is mutated or deleted in almost 50% of all cancers (181,182). P53 mutations are also present in many breast cancer cells and tumors (182). Gurnani and cowrkers have used adenovirus constructs to introduce a wild type copy of p53 into a breast cancer cell line (MDA-MB-231) which express mutated p53 and *in vivo* into athymic nude mice injected with MDA-MB-468 cells. Results of this study showed that the growth of MDA-MB-231 cells expressing Adp53 was inhibited compared to

cells treated with empty vector Moreover, adenoviral p53 expression prevented tumor growth in the nude mice (183). Recently, siRNAs have been used as a new avenue for gene therapy. These siRNAs are highly sequence-specific that are high discriminatory in targeting specific mRNA. siRNA has been used to target mutant p53 allele in cancer cells and this results in restoration of wild type protein function (118) .

TRANSCRIPTION

The sequential changes that occur during development of cancer are related in changes in levels of gene transcription. The remarkable diversity between cancer and normal cells is achieved through deregulated expression of genes involved in cell cycle regulation, DNA synthesis and tumor suppression and transformation. The fact that more than 5% of our genes are predicted to encode transcription factors underscores the importance of this protein family in normal and cancer cell biology (184). When activated, transcription factors bind to gene regulatory elements and, through interactions with other components of the transcription machinery, promote access to DNA and facilitate recruitment of the RNA polymerase enzymes to the transcriptional start site. RNA transcription is multi-step process that involves several factors. Briefly, soon after RNAP II initiates transcription, the nascent RNA is modified by the addition of a “cap” structure at its 5′-end. This cap serves initially to protect the new transcript from attack by nucleases and later serves as a binding site for proteins involved in

export of the mature mRNA into the cytoplasm and its translation into protein (185).The capping process appears to coordinate early transcriptional events by regulating the transition between transcription “initiation,” during which RNAP II begins RNA synthesis, and transcription “elongation,” in which the polymerase moves 5′ to 3′ along the gene sequence to extend the transcript. A family of “elongation factors” is responsible for regulation of the elongation phase of transcription (186). Coding sequences in the gene (exons) are often interrupted by long noncoding sequences (introns), which are removed by pre-mRNA splicing. Once a gene has been transcribed, RNAP II stops transcription (“termination”), the newly synthesized RNA is cleaved (“cleavage”) and a polyadenosine poly (A) tail is added to the 3′ end of the transcript (“polyadenylation”) (185).

Cellular DNA is not naked, but packaged into a highly organized and compact nucleoprotein structures known as chromatin. Nucleosome, the basic organizational unit of chromatin, is consists of 146 bp of DNA wrapped almost twice around a protein core containing two copies each of four histone proteins: H2A, H2B, H3, and H4 (187). These small, positively charged proteins are highly conserved among eukaryotes and are the protein building blocks of chromosomes. Further compaction of genes is achieved via poorly defined levels of higher-order nucleosome folding. It is clear now that chromatin plays a crucial role in regulating gene transcription by marshalling access of the transcriptional apparatus to genes (188). All chromatin is not equal; for example

untranscribed regions of the genome are packaged into highly condensed “heterochromatin,” while transcribed genes are present in more accessible “euchromatin” (189). To activate gene expression, transcriptional activator proteins must, therefore, contend with inaccessible and repressive chromatin structures. It is clear now that many transcriptional coregulators are enzymes that modulate chromatin structure and this underlines the importance of DNA packaging in gene expression. Coregulators that act on chromatin can be divided into two general classes: ATP-dependent nucleosome remodeling complexes and activities that catalyze posttranslational modification of histones. ATP-dependent chromatin remodeling complexes facilitate access of DNA binding proteins to DNA by repositioning nucleosomes at the promoter or by inducing conformational changes in nucleosomes (188). There are four classes of histone modifiers that have been implicated in transcriptional regulation. These are the histone acetyltransferases (HATs), the histone deacetylases (HDACs), the histone methyltransferases (HMTs), and the histone kinases (188).

Histone acetylation was the first modification shown to correlate with transcriptional competence and this process initiates the breakdown of chromatin structure (190). After being recruited to promoters by different activator, HATs and HMTs will cause the acetylation and methylation, respectively, of residues located in the N-terminal tails of histones and this is crucial for activation of many classes of gene (190-194). Conversely, recruitment

of HDACs by transcriptional repressors leads to deacetylation of histone tails and this is required for gene repression. However, gene expression and histone tail modifications exhibit a complex relationship.

Decompaction of chromatin at the promoter is not sufficient for efficient transcription. RNAP II often needs to travel thousands of base pairs of compacted chromatin downstream of the promoter. Two protein factors have been involved in this process. The first is the chromatin-specific transcription elongation factor, FACT, which facilitates RNAP II elongation through nucleosomes and plays a role in elongation *in vivo* (195,196). The second complex implicated in disrupting chromatin downstream of the promoter is the elongator, originally isolated as a component of elongating RNAP II (197) and recently shown to promote transcription through chromatin (198). Unlike the prokaryotic enzymes, eukaryotic RNA polymerases cannot recognize the promoters of their target genes and instead rely on a series general transcription factors (GTFs) (199-201).These protein factors recognize the conserved “TATA” box and “initiator” sequences present in most protein-coding genes and recruit RNAP II to the start site of transcription. Different biochemical assays have been used for purification of transcription factors from mammalian cells. This revealed the existence of large families of sequence-specific activators (Sp1, AP-1, C/EBP, NF-B, GR, etc.) as well as a host of accessory factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH) necessary to program a functional RNA Pol II complex (202-207). Most genes are regulated by mixing and matching different

types of activators and repressors in a coordinated fashion. Studying the mechanisms by which co-activators and co-repressors interface with gene regulators and the transcription machinery has become essential for understanding transcriptional regulation in eukaryotes. Generally, transcriptional co-factors are divided into five classes (Table III) that differ in both structure and number.

Mechanism of transcriptional machinery assembly. The process of how the transcriptional machinery may be assembled and targeted to specific promoters is still not clear. However, there are two models suggested for this process.

A stepwise assembly model - This model proposes an ordered assembly of the transcription pre-initiation complex and this is based on the formation of active transcription complexes *in vitro* (208). It was observed that a stepwise addition of purified basal factors was required for promoter binding and transcription initiation from naked DNA templates (Fig.9). Steps leading to Pol II-dependent transcription include: first, formation of a metastable complex between TFIID, TFIIA, and TFIIB (DAB) capable of recognizing and binding to the TATA promoter element; second, a more stable closed complex containing DAB, hypophosphorylated RNA Pol II and TFIIF; third, an activated open complex formed by the further addition of TFIIE and TFIIH, which stimulate ATP-

Table III

Classes and properities of transcription co-factors. Modified from (209).

Class	General properties	Examples
I	activator and repressor targets inherent to the core machinery, promoter recognition, and enzymatic functions	TAFs, TFIIA, NC2, PC4
II	activator and repressor adapters, modulate DNA binding, target other co-regulators and the core machinery	OCA-B/OBF-1, Groucho, Notch, CtBP, HCF, E1A, VP16
III	multifunctional structurally related but highly divergent co-regulators: some interact with RNA Pol II and/or multiple types of activators, some also appear to have inherent enzymatic functions or chromatin-selective properties	yeast: Mediator, SRBs human a: CRSP, PC2 human b: ARC/DRIP/TRAP human c: NAT, SMCC, Srb/Mediator
IV	chromatin-modifying activator and repressor adapters, acetyltransferase or deacetylase activities with multiple substrates: histones, histone-related proteins, activators, other co-regulators and the core machinery	CBP/p300, GCN5, P/CAF, p160s (SRC1, TIF2, p/CIP, etc.), HDAC-1 and HDAC-2 (rpd3), Sir2
V	ATP-dependent chromatin remodeling activities	SNF2-ATPase (SWI/SNF, RSC) and ISWI-ATPase (NURF, ACF, ChrAC, RSF, etc.)

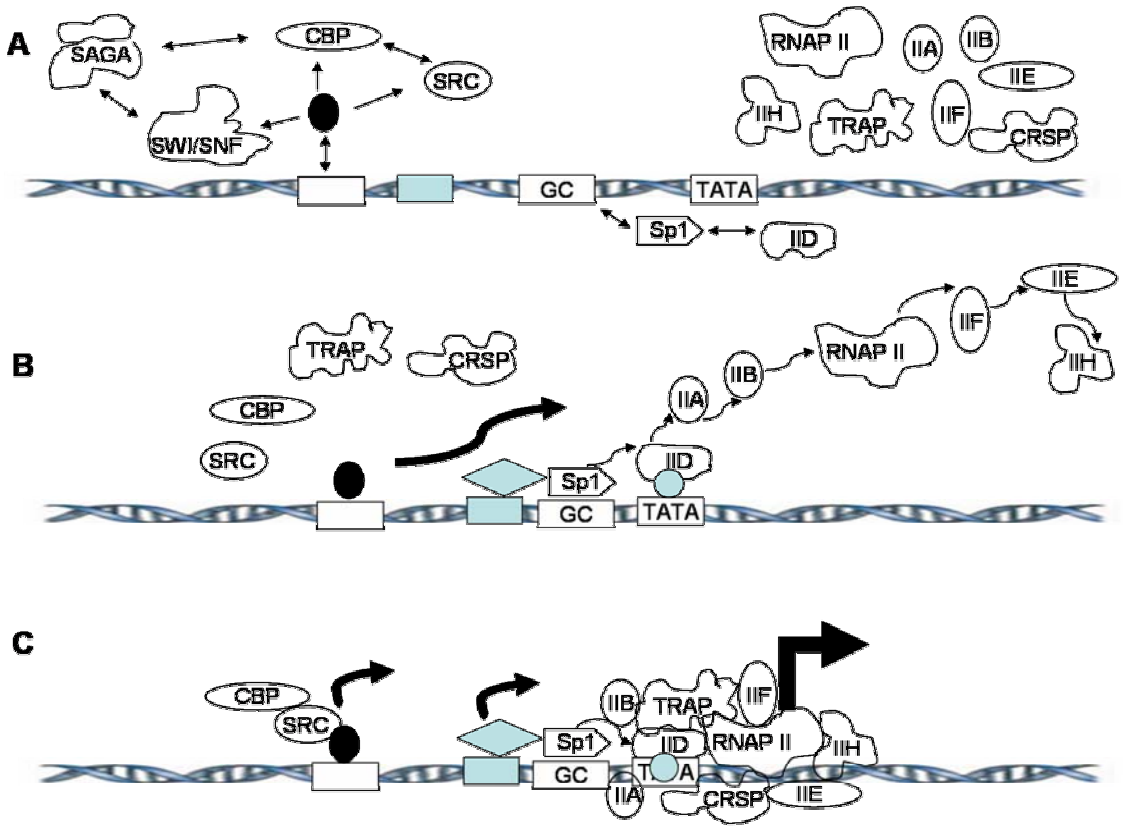


Fig. 9. **Stepwise assembly model for gene transcription.** [A] Chromatin remodeling and template access. [B] Stepwise recruitment of core machinery. [C] Activated initiation complex and transcription. Modified from (209).

dependent isomerization and promoter-melting. Finally, promoter clearance and nascent RNA synthesis occurs upon hyperphosphorylation of the RNA Pol II C-terminal domains (CTD) (199,210-212). Many studies have shown that direct or indirect interaction of activators with constituents of the general machinery affect rates of complex formation and transcription (213-215). This stepwise model for assembly of the core initiation machinery is consistent with the observed biochemically defined steps and could satisfy a biological requirement for dynamic regulation. However, it is now understood that the RNA Pol II core initiation machinery is more elaborate than previously anticipated and contains up to 40 polypeptides comprising separable activities that govern the distinct steps leading to transcription described above.

Pre-assembly complex model - This model proposes recruitment of a completely pre-assembled RNA Pol II holoenzyme for transcription initiation (Fig.10). This model was first proposed when certain preparations of RNA Pol II were observed to co-purify with subsets of the basal machinery along with some co-regulators, including chromatin remodeling factors such as SWI/SNF and CBP, and even proteins involved in DNA replication and repair (216-219). Despite considerable heterogeneity of these RNA Pol II preparations, one invariant property has been the absence of TFIID in these holoenzyme conglomerates. Consequently, at least two targeted steps are required to form an active pre-initiation complex with the holoenzyme model since recruitment of TFIID (or a functional equivalent) is a prerequisite for transcription.

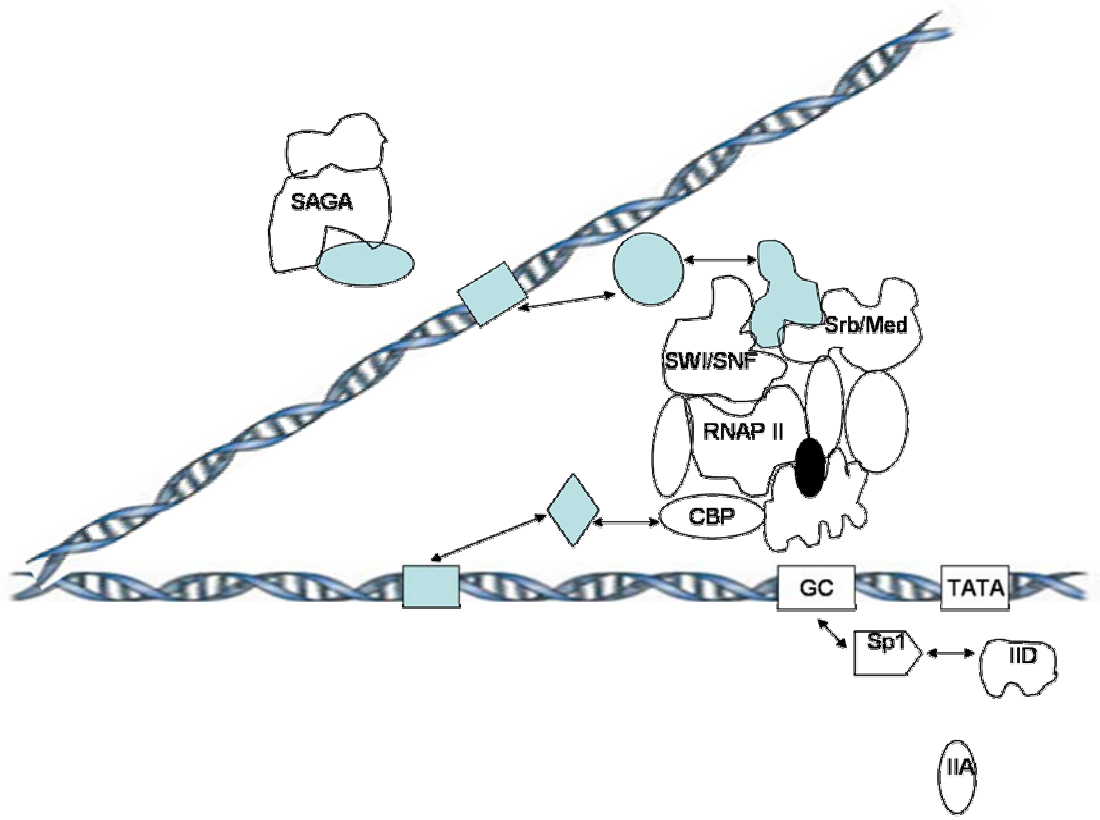


Fig. 10. **Pre-assembly complex model for gene transcription.** Holoenzyme recruitment. Modefied from (209).

One possible advantage of a holo-complex is the ability to obviate the limited cellular concentrations of individual transcription factors. A pre-assembled RNA polymerase complex could, in principle, facilitate rapid responsiveness to arrayed regulators that might cooperatively recruit the transcriptional machinery via targeting of multiple interfaces. On the other hand, the recruitment of a monolithic universal holoenzyme does not fit well with the observed need for the vast diversity of co-regulators in animal cells. It would be more favorable to employ multiple regulators that act at different stages of the transcription reaction. Such a multi-faceted mechanism could impose controls at different barriers to the transcription process and thereby provide greater flexibility for modulating rates of transcription. Since the basal machinery and co-regulator activities are separable and can be reconstituted biochemically with distinct rate-limiting steps, it is likely that there are multiple stages employed by sequence-specific DNA-binding factors to exercise regulation *in vivo* that cannot be explained by the simple binary recruitment of an RNA Pol II holoenzyme. This suggests that eukaryotes have evolved adaptable and interchangeable transcriptional complexes along with attendant co-regulators that incorporate subsets of multifunctional polypeptides.

Sp1 TRANSCRIPTION FACTOR

Sp1 was one of the first transcription factors to be purified and was cloned from mammalian cells in the early 1980s (202,220). Sp1 was shown to bind DNA via three Cys_2His_2 zinc-finger motifs. A similar DNA-binding domain had been found in many developmental regulators, including the *Drosophila* embryonic pattern regulator Krüppel (220). Krüppel-like factors have been named after the *Drosophila* segmentation gene Krüppel that shows a similar arrangement of zinc fingers (221). Sp1-like/KLF members recognize the same GC-(GGGGCGGGG) and GT-(GGTGTGGGG) boxes albeit with different affinities due to the substitutions of amino acids in the zinc fingers. GC and GT boxes are important for the expression of many different ubiquitous as well as tissue-specific cellular and viral genes (222). In addition, these motifs are involved in the maintenance of the methylation-free status of the CpG islands in several genes (223,224). Many members of the Sp1-like/KLF family have acquired multiple names over time and because of this the nomenclature for these proteins is currently being revised and standardized. In this Dissertation, we follow the current nomenclature of Sp1-Sp6.

At least 21 Sp1-like/KLF genes have been identified in humans by a variety of cloning approaches (Table IV). So far, homologs of 17 of the 21 human Sp1-like/KLF proteins have been identified in mouse, and 11 in rat. Several subgroups have been defined within the Sp1-like/KLF family and this is based on sequence and functional similarities (Fig.11). One subgroup contains

the factors that are highly related to Sp1, namely Sp1-Sp6 (the 'Sp' proteins or subgroup I). The other Sp1-like/KLF proteins make up additional subgroup. According to the rules of nomenclature, these proteins are numbered as KLF

Table IV

Characteristics of Sp/KLF family members. Modified from (222).

Protein	**KLF number**	**Species**	**Chromos-omal localizati-on**	**Transcriptional activity (and functional domains)**	**Expressio-n pattern**	**Cellular functions**
Sp1	-------	Human, mouse, rat and *Drosophil a*	12q13	Transcriptional activity (and functional domains)	Ubiquitous	Embryogen esis
Sp2	--------	Human, mouse and rat	17q21	Unknown (Q-rich domain)	unknown	Unknown
Sp3	--------	Human, mouse and rat	2q31	Activator and/or repressor (Q-rich domains)	Ubiquitous	Unknown
Sp4	--------	Human, mouse and rat	7p15	Activator (Q-rich domains	Brain-enriched	Post-natal survival and male fertility
mSp5	-------	Mouse		Unknown	Ubiquitous	Unknown
Sp6	KLF14	Human and mouse	17q21	Activator	Ubiquitous	Unknown
EKLF	KLF1	Human and mouse	19p13	Activator (acidic domain)	Erythroid and mast cells	Erythropoie sis
LKLF	KLF2	Human and mouse	19p13	Activator (acidic domain)	Lung, blood vessels, lymphocyt es	Blood vessel, lung developmen t, T-cell survival
BKLF			4p14	Activator/repress or (PVDLS/T motif)	Erythroid and brain-enriched	Unknown

Table IV. Continued.

GKLF	KLF4	Human, mouse, rat and zebrafish	9q31	Activator and/or repressor (acidic domain)	Gut-enriched	Anti-proliferation, survival
IKLF	KLF5	Human, mouse and rat	13q21	Activator	Gut and epithelial tissues	Cell growth
CPBP	KLF6	Human and mouse	10p15	Activator	Ubiquitous	Putative tumor suppressor
UKLF	KLF7	Human and mouse	2q32	Activator (acidic domain)	Ubiquitous	Cell-cycle arrest
BKLF3	KLF8	Human	Xp11	Repressor (PVDLS/T motif)	Ubiquitous	Unknown
BTEB1	KLF9	Human, mouse and rat	9q13	Activator/repressor (SID)	Ubiquitous	Neurite outgrowth and carcinogen metabolism
TIEG1	KLF10	Human	Xp11	Repressor (PVDLS/T motif)	Ubiquitous	Apoptosis, anti-proliferation
TIEG2/ FKLF	KLF11	Human	2p25	Activator and/or repressor (SID, R2, R3)	Ubiquitous	Anti-proliferation
AP-2rep	KLF12	Human, mouse, rat and zebrafish	13q21	Repressor (PVDLS/T motif)	Brain, kidney, liver and lung	Unknown
BTEB3 /RFLAT-1/FKLF-2	KLF13	Human, mouse and rat	15q12	Activator/repressor (SID, R2 and R3)	Ubiquitous	Anti-proliferation and carcinogen metabolism
KKLF	KLF15	Human, mouse and rat	3q13	Repressor	Ubiquitous	Unknown
BTEB4 / mDRRF	KLF16	Human and mouse	19q13	Repressor (SID)	Ubiquitous	Carcinogen metabolism
BTEB5	-------	Human	7	Unknown	Unknown	Unknown

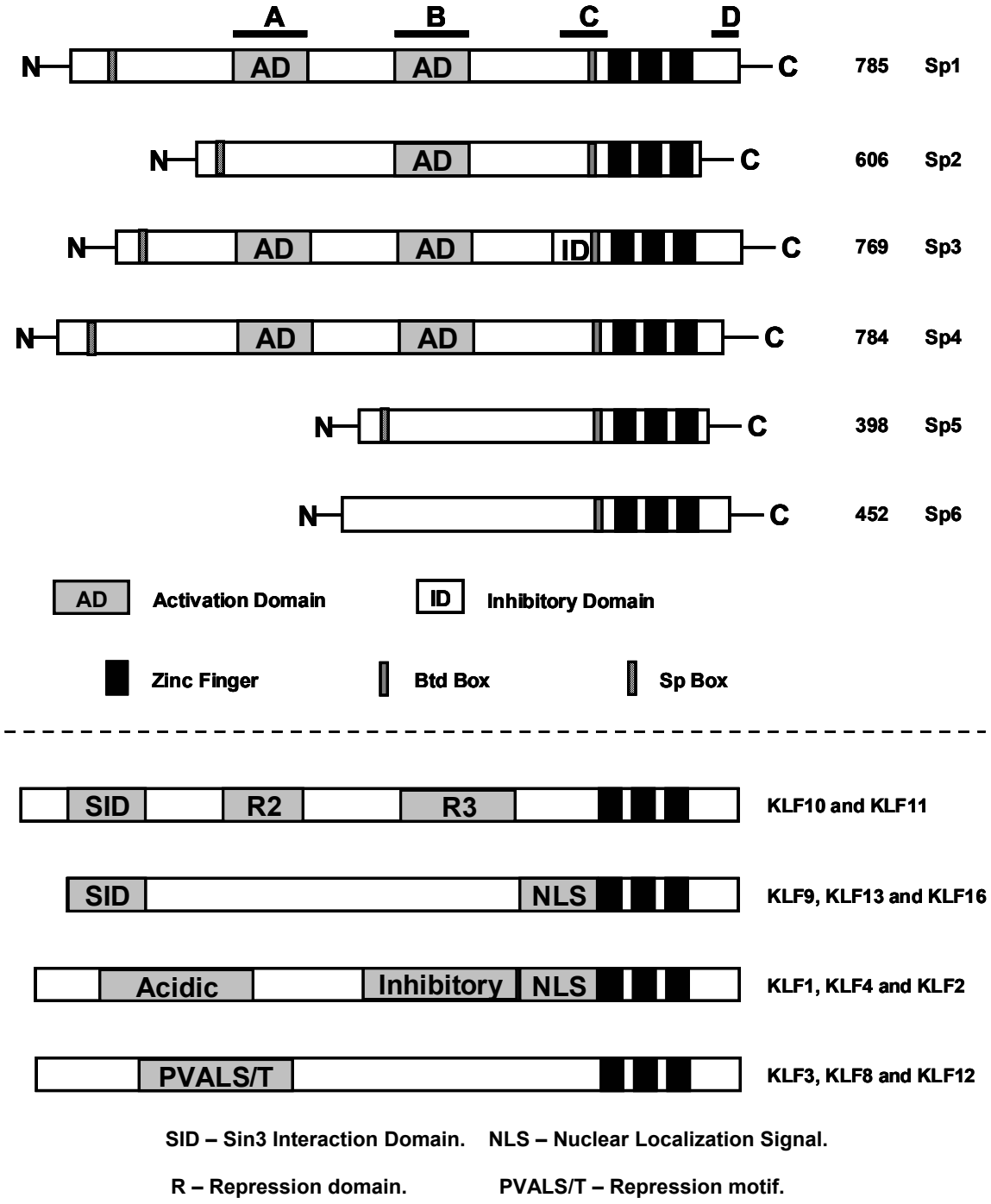


Fig. 11. **Structural features of Sp/KLF family members.** Modefied from (222,225).

factors, corresponding to the approximate order in which the genes were described (KLF1-KLF16).

There are three domains required for a functional site-specific transcription factor: a DNA-binding domain, a nuclear localization signal, and a transcriptional regulatory domain. The defining feature of Sp1-like/KLF proteins is a highly conserved DNA-binding domain (more than 65% sequence identity among family members) at the carboxyl terminus that has three tandem Cys_2His_2 zinc-finger motifs. The zinc-finger motifs may also function in protein-protein interactions that modulate DNA-binding specificity (226,227). The amino-terminal regions of the Sp1-like/KLF proteins are much more variable and contain transcriptional activation or repression domains. Nuclear localization sequences have been found in Sp1-like/KLF proteins, which can occur immediately adjacent to, or within, the zinc-finger motifs (228,229).
Many DNA binding studies have shown that most Sp1-like/KLF proteins have similar affinities for different GC-rich sites (230-232). Importantly, several Sp/KLF proteins have identical amino acids sequence that interact with DNA and competition for DNA binding has been shown for some of these members.For example, Sp1 and Sp3 compete for the same sites in many promoters, as do Sp1 and KLF9 (BTEB1), Sp1 and KLF13 (BTEB3), Sp1 and KLF4 (GKLF), and KLF1 and KLF3 (BKLF) (231,233-236). There are some differences in DNA binding among Sp proteins: For example, Sp2, which has a leucine residue within the first zinc-finger motif in place of the histidine found in the

corresponding region of Sp1, preferentially recognizes GT box (5'-GGTGTGGGG-3'), found in many different promoters, rather than GC box (233,237).

Based on their similar modular structures, Sp1, Sp2, Sp3, and Sp4 form a subgroup. Sp1, Sp3, and Sp4 contain two major glutamine-rich transactivation domains A and B that are essential for transcriptional activation. Serine/threonine-rich sequences that may be targets for post-translational modification are located adjacent to these A and B domains. While Sp2 has only one glutamine-rich domain, it does share a highly charged domain C and a serine/threonine-rich region with the other factors (237). The so-called Buttonhead box is located N-terminal to the zinc finger domain in all Sp proteins (238). This conserved stretch of 11 amino acid residues was originally identified in the *Drosophila* Sp1 homologue Buttonhead (Btd) (239). It is believed that this box may contribute to the transactivation potential of these factors, since a deletion of an overlapping region results in reduced activity of Sp1 *in vitro* (240). One study has shown that Btd element within domain C is involved in synergistic activation by Sp1 or Sp3 with sterol-regulatory element-binding proteins (SREBP) (241). Another stretch of conserved amino acids consisting of the sequence SPLALLAATCSR/KI (Sp box) has been identified at the N-terminus of the proteins (238). This element contains an endoproteolytic cleavage site and is situated close to a region at the N-terminus of Sp1 that targets proteasome-dependent degradation *in vitro* (242). The fact that the Sp box is highly

conserved indicates that it may have a function in regulation of Sp protein proteolysis.

Sp factors- physiological function and transactivation properties. Although Sp1-like/KLF proteins have high degree of similarity in their DNA-binding activities, however, transcriptional regulation among different family members can be highly variable.

Sp1 - Sp1 stimulates transcription from both proximal and distal enhancers (243). Sp1 tetramers may be involves in the synergistic activation via distant sites (244), looping out the intervening DNA (244-246). For multimerization, activation domain B appeared to be of critical importance (247). Together with domain A, domain B also mediates superactivation of Sp1-dependent transcription and this can be achieved by non-DNA-binding mutants in case of multiple binding sites (243,248). For synergistic activation via binding to multiple sites, domain D on both transactivation domains are required (247). Heterotypic interactions of Sp1 with different classes of nuclear proteins have been reported. These include general transcription machinery factors, such as the TATA-box binding protein TBP (249) and the TBP-associated factors dTAFII110/hTAFII130 (250,251), and hTAFII55 (252). Sp1 can bind to its target sequence in assembled nucleosomes (253), and so it is interesting to note the interaction with a large coactivator complex called CRSP (cofactor required for Sp1 activation) which stimulates Sp1-mediated transcription *in vitro* (254). Sp1 is involved in the activation of a very large number of genes, such as

housekeeping, tissue-specific and cell cycle-regulated genes, and is required to prevent methylation of CpG islands (223,255).

Mouse model with genetically engineered disruption or “knockout” of Sp1 protein has shown that Sp1-deficient embryonic stem cells (ES cells) are viable, have normal growth characteristics and can be induced to differentiate and form embryoid bodies as efficiently as wild type ES cells (256). Nevertheless, Sp1 is essential for normal mouse embryogenesis. The Sp1-knockout embryos are severely retarded in development and died around day 11 of gestation. They displayed a marked heterogeneity in phenotype indicating that Sp1 has multiple functions in many cell types. Sp1 appears to be a transcription factor whose function is essential for differentiated cells after day 10 of development. The only genes which were found to be expressed at a lower level in Sp1-/- mice are the thymidine kinase and the methyl-CpG binding protein 2 (MeCP2) genes (256). It was suggested that the MeCP2 gene might be a key target of Sp1 (257). However, whether Sp1 acts as a direct regulator of MeCP2 expression by binding to the promoter, enhancer or local control region elements in the MeCP2 gene, or whether additional proteins mediate downregulation of MeCP2 remains to be established.

Sp2 - unlike Sp1, Sp2 is unable to activate promoters containing GC boxes because the binding site specificity of Sp2 differs from that of the other Sp proteins (237,258,259). It has been shown in one study that Sp2 represses Sp1- and Sp3-driven activation of a construct containing the murine

CTP:phosphocholine cytidylyltransferase γpromoter in *Drosophila* cells but activates the same construct in C3H10T1/2 mammalian cells (260). It is likely that Sp2 has different characteristics than Sp1, 3 and 4 since it has only one glutamine-rich transactivation domain, whereas two domains are required for superactivation and synergistic activation by Sp1 (247).

Sp3 - Unraveling the transcriptional role of Sp3 is complicated by the fact that three Sp3 isoforms exist, a 110-115 kDa Sp3 protein and two approximately 60-70 kDa Sp3 species. The two smaller Sp3 species arise from the first two internal AUG codons (261). Several reports have shown that Sp3 act as a transcriptional activator similar to Sp1 (262-264). In other studies, Sp3 remained inactive or acted only as a very weak activator (265-267). Most of these reports are based on co-transfection experiments into the insect cell line SL2. Usually, a distinct promoter fragment containing appropriate Sp-binding sites fused to a reporter gene has been co-transfected along with expression plasmid for Sp1, Sp3 or both in combination. If Sp3 is expressed to the same extent as Sp1 but does not act as a strong activator, it will compete for the same binding site and thus lower Sp1-mediated activation. Decreased endogenous Sp3 expression in the myelomonocytic cell line HL60 using antisense oligonucleotides showed that Sp3 participates in activation of the CD11c and CD11b promoters (268). The experimental conditions required for Sp3 to act as a strong activator or a transcriptional inactive molecule which represses Sp1-mediated activation are not completely understood. The structure and arrangement of the recognition

sites appear to determine whether Sp3 is transcriptionally inactive and can repress Sp1-mediated activation or whether it acts as a strong activator. Promoter analysis studies have shown that promoters containing a single binding site are activated, whereas promoters containing multiple binding sites often are not activated or respond weakly to Sp3 (269,270). Whether Sp3 acts as an activator or as a repressor of Sp1-mediated activation may also depend on cell context. Transfected Sp3 stimulated transcription from the HERV-H long-terminal repeat in the teratocarcinoma cell line NTera2-D1 but acted as a repressor in HeLa and insect cells (270).

In insect and in mammalian cells, it is clear that both N-terminal glutamine-rich regions of Sp3 can act as strong activation domains (270,271). The molecular basis for the inactivity of Sp3 under certain conditions has been linked to an inhibitory domain located between the second glutamine-rich activation domain and the first zinc finger. To have repressor function, the amino acid triplet KEE within this domain is absolutely essential (270). Mutation of these amino acids to alanine residues converted almost inactive Sp3 to a strong activator. The inhibitory domain of Sp3 can act independently and after transfer to other activation domains there is a loss of transactivation properties (270). It is not clear how this domain functions mechanistically, however, it is possible that additional proteins such as SIF-1 (Sp3-interacting protein 1) which acts as co-repressors are involved in the inhibitory function of Sp3 (272).

Sp4 - Sp4 is a tissue restricted member of the Sp-family. It is predominantly expressed in the brain but also detectable in epithelial tissues, testis and developing teeth (232,273). Despite obvious structural similarities, the functional properties of Sp4 are different from those of Sp1. Like Sp1, Sp4 shows similar transactivation potential through its glutamine-rich activation domains. In addition, Sp4 can be superactivated by fingerless Sp1 and repressed by Sp3 (248). Although Sp1 can synergistically activate promoters containing multiple binding sites, transactivation by Sp4 only occurs in an additive manner (248). Unlike Sp1 and Sp3, the transactivation potential of Sp4 has not been intensively investigated with respect to different promoters and cell types. Several promoters are activated by Sp4 in mammalian cell lines as well as in *Drosophila* cells (248,266,274), but others only appeared to respond to different Sp family members (275,276). Disruption of the mouse Sp4 gene revealed that it is important for early post-natal survival (273). Approximately two thirds of the Sp4-/- mice die within a few days of birth. The cause of the early death remains unknown and survivors of these mice are significantly smaller than their wild type littermates. It is believed that the reduced body weight results from an unknown growth hormone-independent mechanism (273). In addition, surviving mice exhibit a striking sex-specific abnormality. Fertility of the female mutants appears normal. In contrast, although male reproductive organs are fully developed and apparently normal, they do not breed and it is possible that male Sp4-/- mice have lost their ability to copulate. The most likely cause of this

abnormal behavior is a neurological defect. The hypothalamus and the vomeronasal organ are known to play important roles in reproductive physiology and behavior. However, both structures are histologically normal in Sp4-/- mice and further studies are required to understand the role of Sp4 and to identify its target genes.

Co-operative interactions of Sp1 with other proteins. Regulation of gene expression by transcription factors depends on the communication with the basal transcription machinery. Sp1 can directly interact with TBP (249) and dTAF(II)110/hTAF(II)130 via the glutamine-rich activation domains A and B (250,251,277) and with hTAF(II)55 through the C-terminal domain (252). It has been found that TAF (II) 250 plays an important role in stimulation of Sp1 transcriptional activity by Rb (278). Furthermore, the multi-subunit complex CRSP (cofactor required for Sp1) promotes efficient activation of transcription by Sp1 (254). CRSP functions in conjunction with the TBP-associated factors. CRSP contains unique subunits and polypeptides that are shared with other cofactor complexes (254). There are several reports that describe functional interactions between Sp factors and proteins and these include sequence-specific transcription factors such as Oct-1 (279), NF-κB (280-282), and E2F-1 (283,284) and also tissue-specific regulators like MEF-2 (285) and GATA proteins (286). These interactions with Sp1 can synergistically activate transcription of various target genes.

In addition to the zinc finger domains, non-conserved domains can also play a role, as has been shown for Sp1 and NF-κB in case of the HIV-1 promoter (287). The interaction of Smad3 with Sp1 but not with Sp3 demonstrates that distinct Sp proteins can specifically co-operate with other transcription factors (288).

Sp1 site-dependent and growth regulation. Early studies identified "Sp1 sites" in the promoters of multiple growth-regulated genes, arguing that these sites may be important for cell growth regulation. Several studies have established the ability of "Sp1 GC-rich sites" to mediate growth induction of a variety of promoters, including those of the genes encoding insulin-like growth factor (IGF)-binding protein 2 (289), vascular/endothelial growth factor (VEGF) (290), thymidine kinase (291), and serum response factor (292). Growth regulation by Sp1-sites has been shown by studying the effects of expressing truncated (C-terminal) Sp1 which contains the zinc finger DNA binding domain but not the major transactivating domains of Sp1 (293). This "dominant negative" Sp1 inhibited HeLa cell growth. This effect was associated with an increase in the duration of S-phase, arguing that "Sp1 site"-dependent transcription is particularly important for this phase of the cell cycle.

In another study, transfection of cells with "Sp1 site" decoy oligonucleotides inhibited the expression of a range of genes and decreased invasiveness and proliferation of A549 lung adenocarcinoma and U251 glioblastoma cells (294). However, it worth noting that "dominant negative" Sp1 protein and the decoy oligonucleotides would act generally for all proteins that

bind the "Sp1 site". Therefore these studies represent the role of "Sp1 site"-dependent transcription and not necessary Sp1 protein in mediating these effects. Interestingly, it has been shown that, upregulation of "Sp1 site"-dependent transcription can be related to positive or negative changes in cell growth and this is promoter context-dependent. For example, "Sp1 sites" in the rep3a and DHFR promoters support upregulation of transcription following growth stimulation of quiescent cells. The opposite is true for "Sp1 sites" in the $p21^{waf1/cip1}$ where transcriptional upregulation is related to growth inhibition (295). Sp1 expression and activity is increased in epithelial carcinomas compared with benign tumors, such as papillomas, suggesting that Sp1 may be involved in tumor progression (296). Another study in several pancreatic cancer cell lines has shown that expression of VEGF is correlated with the expression of Sp1 and both proteins are coordinately over expressed in pancreatic cancers compared to normal pancreatic tissue (297).

MECHANISM OF ESTROGEN-MEDIATED TRANSACTIVATION THROUGH Sp1 and GC-RICH SITES

Estrogen (E2) mediates its effects through interaction with intracellular steroid hormone receptor know as the estrogen receptor (ER). Once in the nucleus, estrogen binds ER causing conformational changes that lead to dissociation of heat shock proteins and formation of transcriptionaly active ER, however, steroid hormone receptors are members of the large nuclear receptor

(NR) family of transcriptional modulators. Transcription is regulated by NRs through intreraction with DNA regulatory sequences that bind discriminately to particular classes of NR as well as with co-activator and co-repressor molecules to regulate the activity of the RNA polymerase complex (298-300). The nuclear receptor family also includes additional steroid hormone receptors such as the progesterone and androgen receptors, receptors for vitamins or metabolites such as the vitamin D or retinoic acid receptors, or receptors with no identified ligand, termed 'orphan' receptors (Table V). A new form of ER (ERβ) has been cloned and characterized and has been shown to share common structural features with ERα, and also some variability (Fig. 12) (301,302).

The structure of ER is shared by all members of the steroid hormone receptor family (Figure 12) and contain six functional domains designated A–F (303). The A/B domain is located in the amino-terminal and contains the hormone-independent activation function 1 (AF-1) which shows the highest variability among all the steroid hormone receptors (304). Domain C contains the DNA binding domain (DBD) which consists of two zinc finger motifs. In combination with the so-called P-box, zinc finger motifs are responsible for ER binding to estrogen response elements (EREs) and, in combination with a D-box, they are essential for dimerization of ER on EREs (305). The D domain, also called the hinge region, is important for co-regulatory protein binding (306). E and F domains makeup the carboxy-terminal region of ER which contains the ligand binding domain (LBD) and a region implicated in modulating the agonist

activity of non-steroidal antiestrogens and binding of coregulatory proteins (307). The LBD itself comprises the ligand-dependent transcription activation function AF-2 (308), an HSP 90 binding region (309), a nuclear localization signal (310),

Table V

Nuclear receptor families of transcription modulators. Modified from (311).

Class	**Receptor**	**Sub-Type**	**Denomination**	**Ligand**	**Response Element**
Class I	TR	α, β	Thyroid hormone receptor	Thyroid hormone (T_3)	Pal, DR-4, IP
	RAR	α, β, γ	Retinoic acid receptor	Retinoic acid	DR-2, DR-5 Pal, IP
	VDR		Vitamin D receptor	1-25$(OH)_2$ vitamin D_3	DR-3, IP-9
	PPAR	α, β, γ	Peroxisome proliferator activated receptor	Benzotriene B4; Wy 14.643Eicosanoids; thiazolidinediones (TZD_S); 15-deoxy-12,41-prostaglandin J_2; polyunsaturated fatty acids	DR-1
	PXR		Pregnane X receptor	Pregnanes; C21 steroids	DR-3
	CAR/MB 67	α, β	Constitutive androstane receptor	Androstanes; 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene	DR-5
	LXR	α, β	Liver X receptor	Oxysterols	DR-4
	FXR		Farnesoid X receptor	Bile acids	DR-4, IR-1
	RevErb	α, β	Reverse ErbA	Unknown	DR-2, Hemisite
	RZR/ROR	α, β, γ	Retinoid Z receptor/retinoic acid-related orphan receptor	Unknown	Hemisite
	UR		Ubiquitous receptor	Unknown	DR-4
Class II	RXR	α, β, γ	Retinoid X receptor	9-*Cis*-retinoic acid	Pal, DR-1

Table V continued.

	COUP-TF	α, β, γ	Chicken ovalbumin upstream promoter transcription factor	Unknown	Pal, DR-5
	HNF-4	α, β, γ	Hepatocyte nuclear factor 4	Fatty acyl-CoA thioesters	DR-1, DR-2
	TLX		Tailles-related receptor	Unknown	DR-1, Hemisite
	PNR		Photoreceptor-specific nuclear receptor	Unknown	DR-1, Hemisite
	TR2	α, β	Testis receptor	Unknown	DR-1 to DR5
Class III	GR		Glucocorticoid receptor	Glucocorticoids	Pal
	AR		Androgen receptor	Androgens	Pal
	PR		Progesterone receptor	Progestins	Pal
	ER	α, β	Estrogen receptor	Estradiol	Pal
	ERR	α, β, γ	Estrogen-related receptor	Unknown	Pal, Hemisite
Class IV	NGFI-B	α, β, γ	NGF-induced clone B	Unknown	Pal, DR-5
Class V	SF-1/FTZ-F1	α, β	Steroidogenic factor 1Fushi Tarazu factor 1	Oxysterols	Hemisite
Class VI	GCNF		Germ cell nuclear factor	Unknown	DR-0
Class 0	SHP		Small heterodimeric partner	Unknown	
	DAX-1		Dosage-sensitive sex reversal	Unknown	

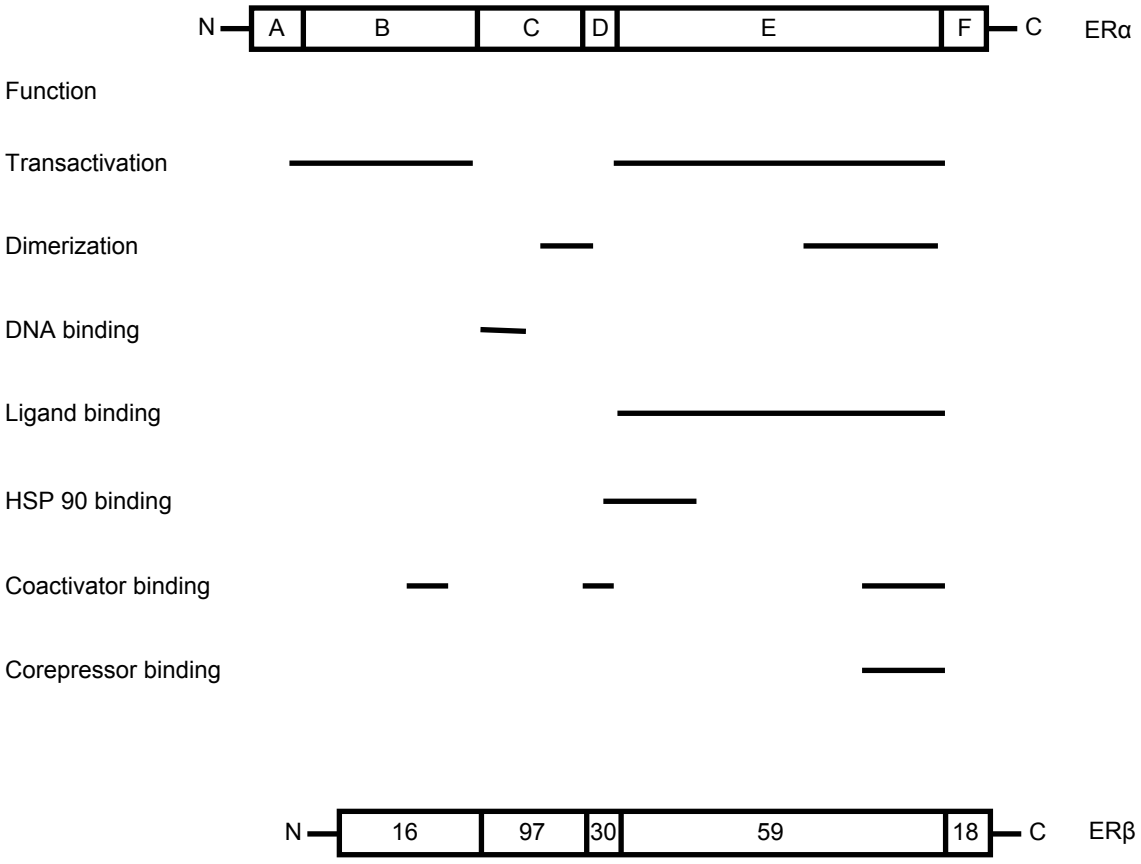


Fig. 12. **Structural domains of human ERα and ERβ.** Modified from (312).

and another dimerization domain (313). AF-1 and AF-2 are responsible for transcriptional activation of ER-regulated genes. These domains can function either independently or synergistically, depending on the cellular context. Both of these domains interact with distinct components of the basal transcription machinery (314), mediate cell context-specific agonist and antagonist activities of selective ER modulators (SERMs) (315) and bind steroid receptor co-regulatory proteins such as SRC-1, TIF1a, and RIP140 (316-319).

Results of previous studies suggest that cell-specific activity of AF-1 and AF-2 depend in part on the relative availability of co-regulatory proteins which could either facilitate or disrupt the interactions of ER AF-1 and AF-2 with the basal transcription machinery. Crystal structures of ERs have shown that LBD consist of 12α-helices. Helix 12 is of special importance since it undergoes extensive repositioning upon ligand binding, the extent of which depends on the type of ligand (320). Interestingly, hydrophobic residues on the surface of helix 12 have been identified as mediators of receptor–coactivator interaction (321). These data in combination with functional studies of ER imply that ligand-induced conformational changes in steroid receptors affect the recruitment of co-factors and receptor-mediated transactivation. It has been suggested that recruitment of cofactors is affected by ligand-induced conformational changes in the ER.

Mouse models in which ERs were genetically disrupted or 'knocked out' exhibit defects in reproductive function as well as alterations in physiological and genomic responses (322). Estrogen receptor α knockout (ERKO) females are infertile because they are anovulatory; their LH regulation is disrupted and the ERKO mouse uterus is insensitive to estrogen. On the other hand, ERβ knock out (βERKO) females are sub-fertile and primarily lack efficient ovulatory function. Deletion of both ERα and ERβ (both ERKO) produces effects similar to these seen in ERKO mice and they also exhibit a unique ovarian pathology. Table VI summarizes different phenotypes observed in reproductive tissues of ER knockout mice.

Several mechanisms have been proposed for estrogen-mediated transcription. In the classical model, ER homo- or heterodimers bind palindromic E_2- responsive elements (ERE) (GGTCANNNTGACC) in E_2- responsive gene promoters (323-325) (Fig. 13). In addition to DNA- binding, ligand-activated ER recruits a series of transcriptional-mediating proteins. These proteins include TAFs, coactivators, corepressors, cointegrators and other proteins with histone acetyltransferase (HAT) and histone deacetylase (HDAC) activetie (300,326,327). However, many estrogen responsive genes such as collagenase and IGF-1 do not have consensus and nonconsensus ERE in their promoters. These genes contain AP1 sites that bind Jun homodimers or Jun-Fos heterodimer.

Table VI

Different phenotypes in ERs knockout mice. Adapted from (322).

Component	Function	Phenotypes
Pituitary gland	Production and secretion of gonadotrophins	ERKO and both ERKO: high LH βERKO: none
Ovary	Production of progesterone and estradiol, ovulation	ERKO: haemorrhagic cystic, high oestrogen and testosterone due to high LH, anovulatory βERKO: reduced ovulations both ERKO: lack of ovulation, sex-reversed follicles
Uterus	Proliferation, secretion	ERKO and both ERKO: estrogen insensitive – no growth or induction of oestrogen target genes βERKO: normal growth and responses to estrogen
Embryo/uterus	Implantation/decidualization	ERKO: estrogen independent decidualization, no implantation βERKO: normal
Mammary gland	Pubertal development, lactation	ERKO and both ERKO: no pubertal development βERKO: normal development and lactation

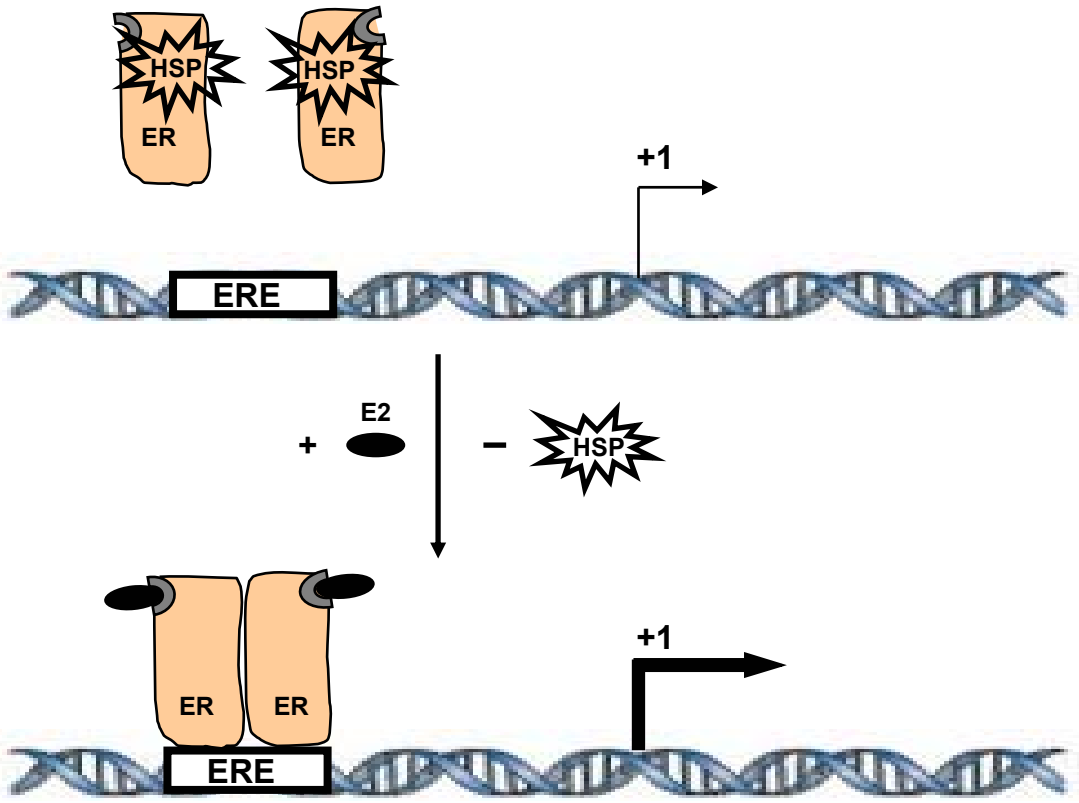


Fig. 13. **Classical mechanism of ER-mediated transcription.** Modified from (328).

Promoter analysis studies have shown that ER can modulate gene expression in a cell context dependent manner (329-333). This regulation involves ER interaction with AP-1 sites through protein-protein binding (Fig.14). Promoter analysis studies of another set of estrogen responsive genes have identified another cis-element that is required for estogenic-inducibility of these genes. This GC-rich site (Sp1-site) has been shown to bind the specificity transcription factor Sp1 (334-335). Studies in this laboratory have identified two types of promoters in which GC-sites are important for estrogen action. First type contains Sp1-site in addition to ERE-1/2 site. In this type of promoter both sites are essential for estrogen inducibility. The mechanism of transactivation requires DNA-binding of ER and direct or indirect interaction of ER with Sp1. Cathepsin D, heat shock protein 27 (HSP27), and transforming growth factor α (TGFα) are E_2-responsive genes which contains GC-rich/ERE1/2 motif (336-338).

The second type of Sp1-site containing promoter has only GC-rich sites that are essential for estrogen inducibility. The mechanism of estrogen action in these promoters involves ER interaction with Sp1 protein but not with DNA and ERα/Sp1 mediated transactivation dose not require the DBD of ERα (339).

Research in this laboratory has provided many examples of E_2-responsive genes in which only Sp1-sites but not EREs are required for estrogen inducibility and these include retinoic acid receptor α1 (RAR α1), C-fos, DNA polymerase alpha (DNA pol α), bcl-2, CAD, and VEGF (340-345).

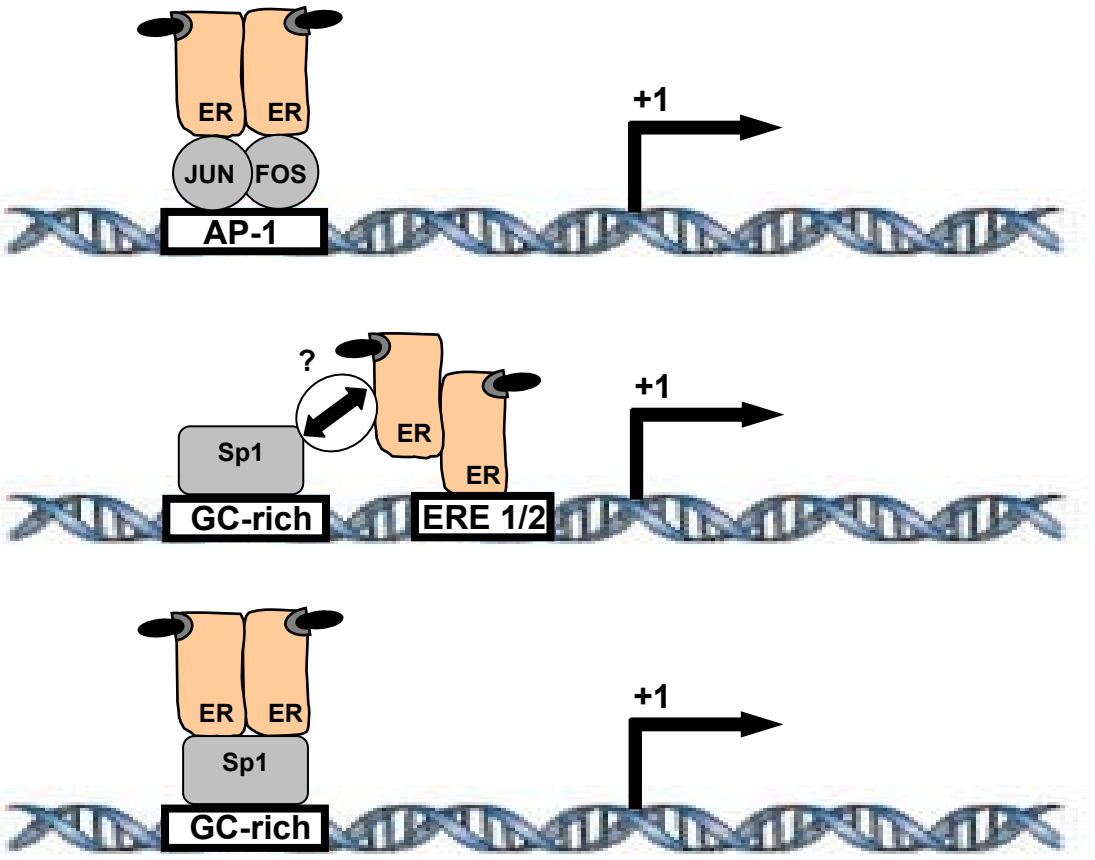


Fig. 14. **Non-classical mechanisms of ER-mediated transcription.** Modified from (329,335,339).

INHIBITORY AhR-ERα CROSSTALK

The aryl hydrocarbon receptor (AhR) is a member of basic-helix-loop-helix –PAS (bHLH-PAS) family of transcription factors. It was first identified in mouse liver by showing high affinity and specific binding to radiolabeled 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (346). Subsequent studies with [^{3}H]TCDD have demonstrated that AhR is widely expressed in mammalian tissues, and it was suggested that the broad spectrum of biochemical and toxic responses of TCDD is due to initial binding to the AhR (347). The hepatic AhR is a cytosolic protein which exists in a complex with HSP90, co-chaperone p23 and immunophilin-like protein XAP2 (also AIP or ARA9) (348-350). XAP2 and p23 are thought to be required for maintaining the stability of the HSP90 complex. After ligand binding the AhR complex undergoes a conformation change exposing a nuclear localization sequence (NLS). The complex then translocates into the nucleus (351,352), dissociates from the protein complex and binds to a closely related nuclear bHLH–PAS protein called Arnt (AhR nuclear translocator).

The AhR:Arnt heterodimeric complex binds to xenobiotic or dioxin responsive elements (XREs OR DREs) in the promotor region of several TCDD-inducible genes (302) and confers TCDD- and AhR-responsiveness upon these genes (Fig. 15). The core sequence (GCGTG) is necessary for AhR/Arnt binding, and the flanking sequences are essential for transcriptional activation (353-356). The presence of the AhR and AhR signal transduction pathway in a

diverse range of species, tissues and cell types (357-360) and its ability to act as a ligand-dependent transcription factor suggests that many of the toxic and biological effects of AhR ligands result from differential alteration of gene expression in target tissues/cells.

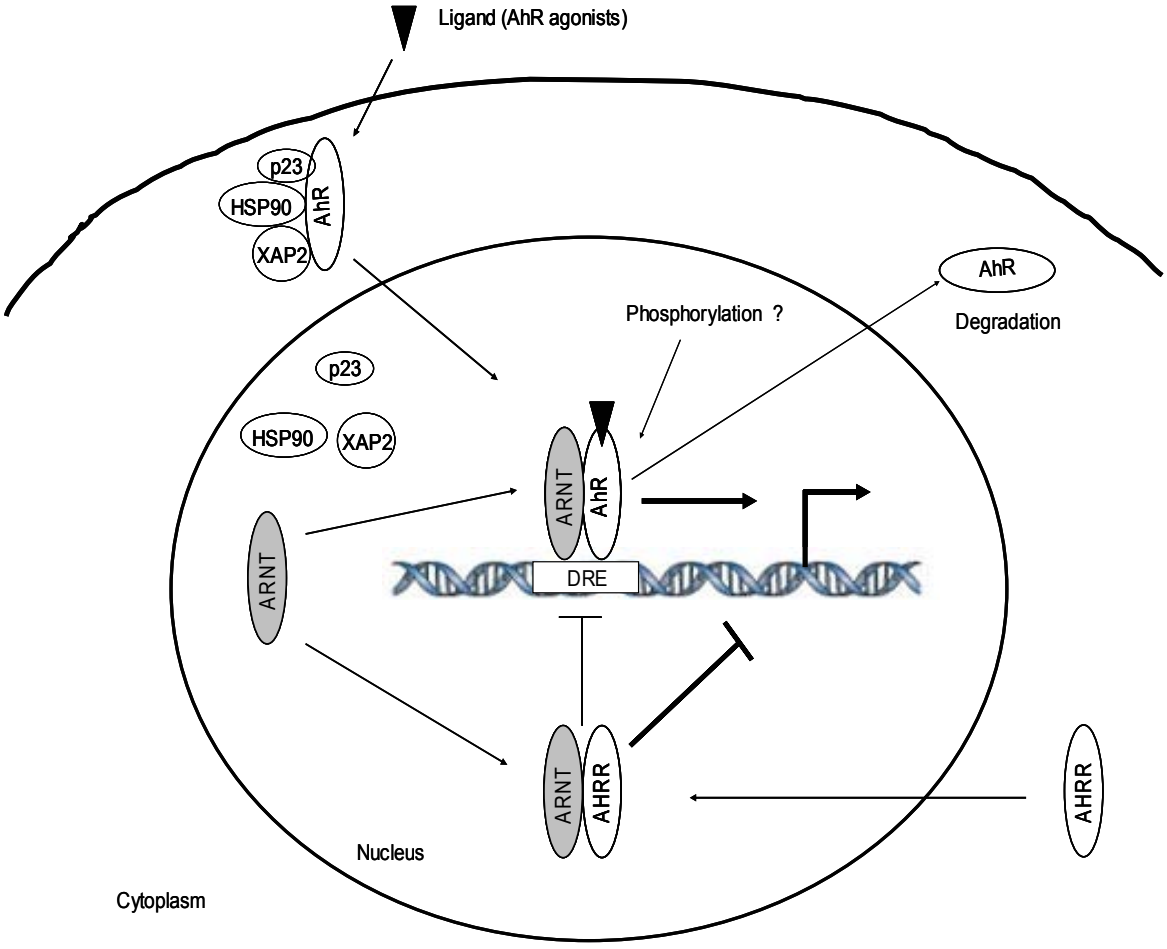


Fig.15. **Mechanism of transcriptional activation by AhR.** Modified from (361).

Phosphatase treatment decreases the binding of the AhR/Arnt heterodimer to XRE suggesting that the phosphorylation state of AhR/ARNT complex is important for transactivation (362). In 1996, an AhR-related factor which is termed as the AhR repressor (AhRR) was cloned (363). The AhRR localizes in the nucleus and forms a heterodimer with Arnt. Like the AhR/ARNT complex, AhRR/ARNT heterodimer recognizes the DRE, but functions as a transcriptional repressor. Therefore, AhRR is considered a negative regulator of AhR by competing with AhR for Arnt. Three copies of functional DREs have been found in the promoter region of mouse AhRR gene which suggests that AhRR is inducible in an AhR-dependent manner (363). These results suggest that the AhR and AhRR form a regulatory feedback loop.

AhR structural features and physiological function. AhR and its partner molecule, Arnt, are members of a structurally related gene family which exhibit characteristic structural motifs designated as bHLH (basic helix–loop–helix) and PAS (Per, Arnt/AhR, Sim homology) (364,365) (Fig. 16). These proteins contain a bHLH motif in their N-terminal region which is involved in DNA binding and in hetero- or homodimerization. The sequence next to C-terminal region of the bHLH region constitutes the PAS domain, which was initially identified as a conserved sequence among *Drosophila* PER, human ARNT and *Drosophila* SIM proteins. The PAS domain contains two imperfect repeats of 50 amino acids, PAS A and PAS B, and is considered to function as an interactive surface for hetero- or homodimer formation. The ligand binding domain of AhR

has been shown to overlap in part with the PAS B region, and also with the binding site for HSP90 which keeps AhR structurally competent to bind a ligand (366). In addition to the PAS B region, HSP90 interacts with the bHLH region to mask the nuclear localization signal (NLS) of AhR, resulting in the cytoplasmic maintenance of the hepatic AhR. Binding of ligands to AhR protein results in conformational changes of the HSP90/AhR complex to expose the NLS of AhR, leading to nuclear translocation of the complex (367). In addition, AhR contains a nuclear export signal (NES) in its second helix of the bHLH domain (368,369). This NES is necessary for the nuclear export of the AhR protein followed by proteasome degradation.

In mouse models where the AhR is genetically disrupted there was no embryonic lethality and AhR-null mice were born in normal Mendelian genetics. (370-372). Nevertheless, the growth rate of the AhR-null mice was significantly retarded as compared with wild type mice for the first 3 weeks of life, and the mutant mice were defective in liver and immune system development (372). Several studies have reported that adult AhR-null mice have many defects such as retinoid accumulation in liver and abnormal hepatic and kidney vascular structures (373,374). It has also been reported that female AhR-null mice have difficulty in maintaining conception, lactation, and rearing pups to weaning (375). The AhR knock out mice are resistant to the multiple tissue-specific toxin and biochemical responses induced by TCDD this includes the induction of CYP1A1, CYP1A2 and CYP1B1.

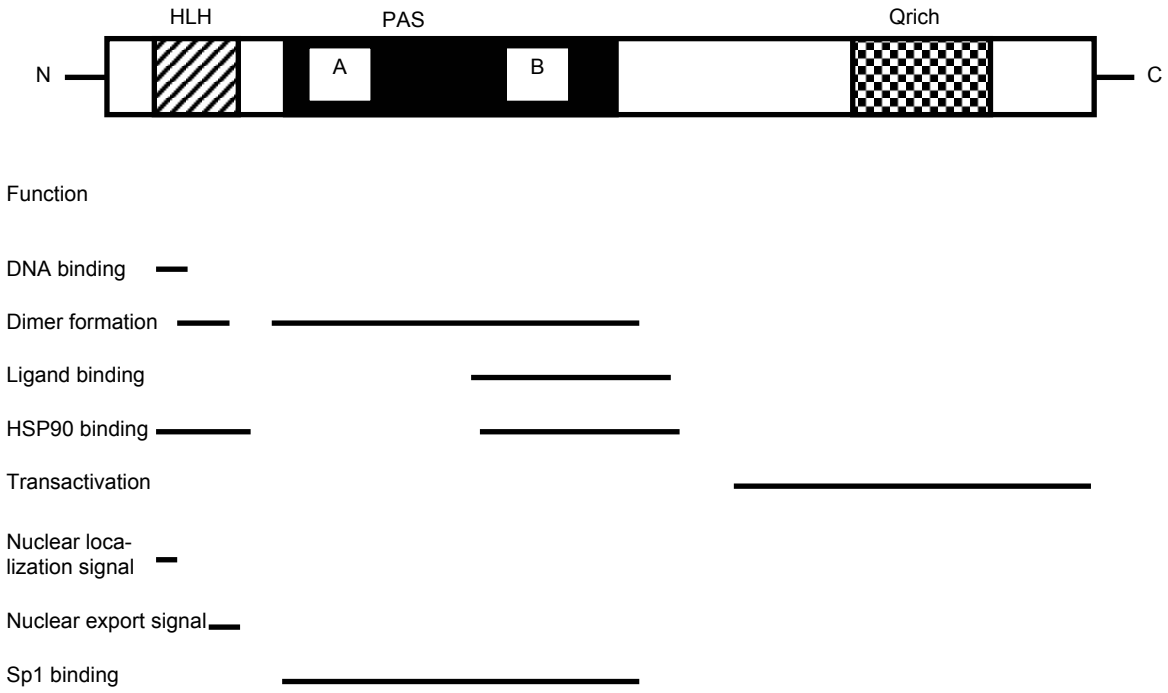


Fig. 16. **Structural features of AhR.** Modified from (361).

AhR-null mice are also resistant to procarcinogens that are metaboloically activated by CYP1A1/1A2 (370,376)

AhR ligans are structurally diverse compounds from both synthetic and naturally sources. Halogenated aromatic hydrocarbon (HAHs) (such as the polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans and biphenyls and related chemicals) and the polycyclic aromatic hydrocarbon (PAHs) (such as benzo(*a*)pyrene, 3-methylcholanthrene, benzoflavones, rutacarpine alkaloids, aromatic amines and related chemicals) are the most extensively studied classes of AhR ligands (302,377-381). HAHs have a relatively high binding affinity for the AhR (in the pM to nM range) whereas the PAHs have a significantly lower affinity (in the high nM to μM range). Structure–activity relationship studies with different AhR ligands have shown that the AhR binding pocket can accept planar ligands with maximal dimensions of 14×12×5 A^{o}. Thermodynamic and electronic properties of the ligands are also important factors that determined binding affinity (302,377-381).

TCDD is a high affinity AhR-ligand that has been used as a mechanistic probe for investing AhR-mediated mechanisms. TCDD is a highly toxic compound that induces various drug-metabolizing enzymes and causes hepatocarcinogenic responses in rodent models (382). The effects of TCDD and related compounds vary widely according to species, sex, age and strain of the animal studied. TCDD toxicity in humans is sometimes seen as acne-like problems on the skin or a wasting syndrome (383). TCDD is also a prototypical

endocrine disruptor that directly or indirectly modulates multiple endocrine signaling pathways. For example TCDD induce toxicities resemble those observed for thyroid dysfunction. Several studies have shown that animals treated with TCDD have decreased circulating thyroid hormone (T_4) level. This decrease is linked to induction of glucuronyl transferase activity by TCDD and subsequent increased formation and excretion of T_4 glucuronides (384,385). TCDD also modulates tissue and serum distribution of retinoids in laboratory animals. For example, TCDD significantly decreases retinoid levels in the rodent liver and this could affect retinoid signaling pathways (386). TCDD also affects steroidgenesis *in vitro* and in laboratory animals' species (386-390) in a manner that may contribute to the profound demasculinization and feminization of rats exposed in utero to TCDD (391).

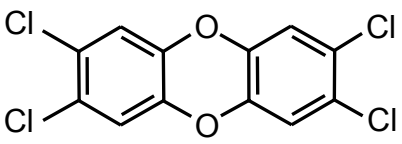
Antiestrogenic activity of AhR agonists. Several epidemiological studies have shown that AhR ligands exhibit antiestrogenic /antitumogenic activity. For example women accidentally exposed to TCDD in Seveso, Italy exhibit decreased incidence of two estrogen-dependent tumors, namely breast and endometrial cancers (392). Another study has shown that cigarette smoking protects against uterine cancers (393,394) and this is possibly because cigarette smoke condensate contains PAHs and other AhR active compounds. Research in this laboratory has extensively investigated and confirmed this antiestrogenic/antitumorgenic activity of TCDD and other AhR ligands in rodent and in breast cancer cell line models, where TCDD not only inhibits cell

proliferation, but also antagonizes the E2-induced expression of various genes including cathepsin D, pS2, c-fos, HSP27, TGFα and PR (336,395-399). Although TCDD is useful as a mechanistic probe for investigating the mechanisms of AhR-mediated antiestogenicity, the potential toxicity of TCDD, especially in liver, precludes the use of this compound for treatment of breast cancer. A new class of compounds called selective AhR modulators (SAhRMs) have been developed and tested in this laboratory as potential treatment option for E2-dependent breast and endometrial cancers in women. Two such classes of SAhRMs include 6-methyl-1,3,8,-trichlorodibenzofuran (6-MCDF) and its related alkyl-polychloro dibenzofurans, and diindolylmethane (DIM) and its related ring substituted DIMs. DIMs are condensation products of indole-3-carbinol (I3C) a major chemoprotective phytochemical in cruciferous vegetables (400,401) (Fig. 17).

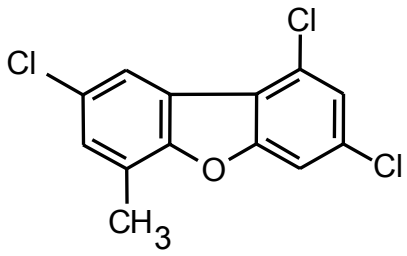
There are four possible mechanisms that have been proposed for inhibitory AhR- ER crosstalk (Fig. 18). Several studies showed that treatment of breast cancer cells with AhR agonists cause a depletion of estrogen (402-404) through induction of CYP 1A1 and CYP 1B1 and subsequent oxidative metabolism of estrogen (mechanism A). However, several SAhRs have been shown to inhibit cells and tumor growth without CYP1A1 induction (336,405-408). Another *in vivo* study has shown that treatment with TCDD did not affect the level of circulating estrogens (409) suggesting that E_2 depletion may not be necessary for inhibitory AhR/ER crosstalk. Competition for the transcription

factors has also been proposed as a possible mechanism for inhibitory AhR/ER crosstalk (mechanism B). This mechanism was supported by a study in which estrogen treatment inhibited induction of CYP1A1 by TCDD and this was linked to competition for limiting level of coregulatory proteins (410). Another study has shown that both ER and AhR interact with common coactivator and corepressor proteins and these may be preferentially sequestered by the liganded AhR complex to inhibit efficient estrogen transactivation (411).

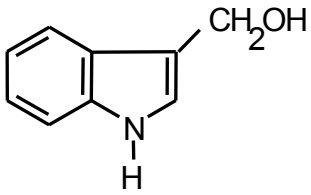
The AhR/ARNT complex may bind directly to the inhibitory DRE (iDRE) sequences in selected estrogen-responsive gene promoters (mechanism B). Promoter analysis of the cathepsin D gene promoter has identified iDRE within upstream estrogen responsive GC $(N)_{19}$ ERE1/2 motif. The importance of this iDRE in mediating inhibitory AhR/ER crosstalk has been confirmed in transactivation studies and in gel mobility assays (336). Functional iDREs have been identified in several estrogen responsive gene promoters including pS2, HSP27, and c-fos where the mechanism of action in these genes is promoter specific (396,398,399). The ligand-activated AhR also induces proteasome-dependent degradation of ER (mechanism D) and in combination with E_2; ER may be below the levels required for hormone-induced transactivation. A recent study in this laboratory has shown that decreased protein levels of AhR and ER were observed in different breast cancer cells after treatment with TCDD



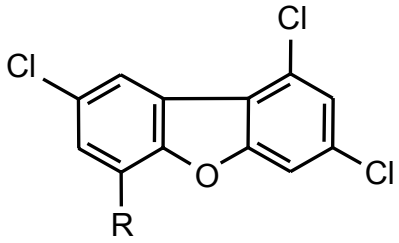
2,3,7,8-TCDD



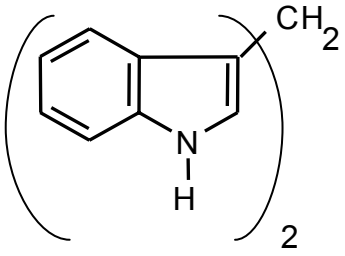
6-MCDF



Indole-3-carbinol (I3C)



6-alkyl-1,3,8-triCDFs



DIM

Fig. 17**. Chemical structure of TCDD and selected SAhRMs.**

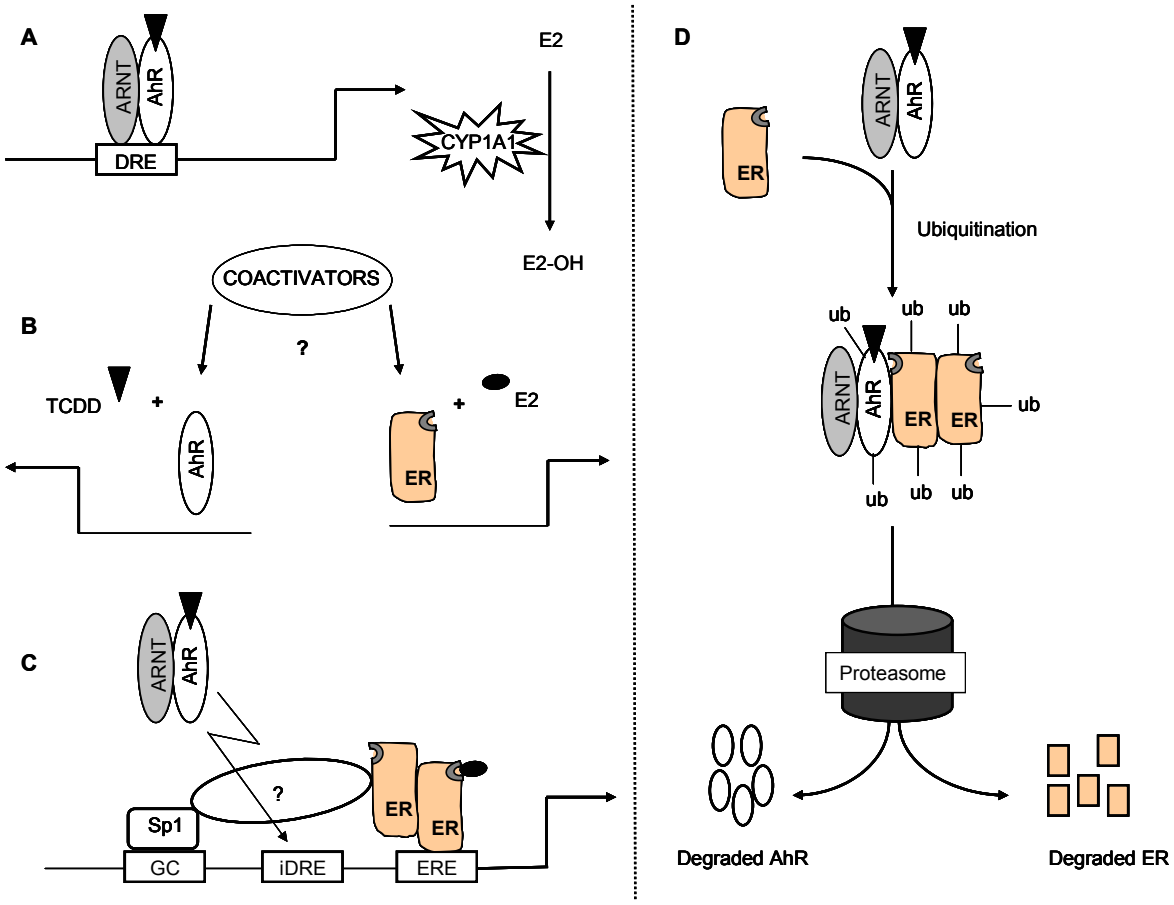


Fig. 18. **Suggested mechanisms of inhibitory AhR/ER crosstalk.** (A) Estrogen depletion. (B) Competition for common coactivators and transcription factors. (C) Presence of functional iDRE in the promoter region. (D) Proteosome-dependent degradation of ER. Modified from (412).

and this protein degradation was blocked by proteasome but not protease inhibitors (413). It is possible that inhibitory AhR/ER crosstalk is a multipathway mechanism, in which two or more pathways (Fig. 18) are involved in block hormone-dependent induction of specific genes.

PANCREATIC CANCER

An estimated 29,200 new cases of pancreatic cancer will be diagnosed in year 2001 in the United States of America (414) and over the past few decades there has not been a significant change in the incidence of this disease. The five-year survival for pancreatic cancer patient is only 4–5%, making pancreatic cancer the fourth leading cause of cancer mortality in the United States (415). Due to a lack of specific symptoms and limitations in diagnostic methods, the disease often eludes detection during its formative stages. A recent National Cancer Institute group (known as a Progress Review Group) articulated the crucial questions and challenges facing the field and provided recommendations to address key unmet needs in the clinical and basic research arenas (416). The pancreas is a grayish pink-colored gland about 12 to 15 cm long, weighing about 60 g. It resembles a fish with its head and nieck in the C-shaped curve of the duodenum (Fig. 19). The pancreas is composed of two different types of glandular tissue, one exocrine and one endocrine. Most of the tissue exocrine, with a compound acinar arrangement where the cells are in a grapelike formation and they release their secretions (pancreatic juice) into a microscopic

duct within each unit. Pancreatic juice contains different digestive enzymes. All of them are secreted as zymogens (inactive enzyme). Trypsin (protein digesting enzyme) is secreted as trypsinogen which is converted to active trypsin by enterokinas of the intestinal lumen. Trypsin can then activate other enzymes such as chymotrypsin (another protein digesting enzyme), lipase (lipid digesting enzyme), nuclease (RNA and DNA digesting enzyme) and amylase (a starch digesting enzyme) (122). Cells along the exocrine ducts of the pancreas also have a secretory function; they produce sodium bicarbonate which keeps the pH of the body balanced and avoids homeostasis stability loss. Between the exocrine units of the pancreas lie clusters of endocrine cells called pancreatic islets (Fig. 19). They are about 2% of the total mass of the pancreas and each islet contains a combination of four primary types of endocrine cells. One type of these cells is the alpha cell, which secrets the hormone glucagon. Beta cells secret the hormone insulin, delta cells secret the hormone somatostatin and pancreatic polypeptide cells (PP) secret pancreatic polypeptides (122).

Risk factors for pancreatic cancer. The etiology of pancreatic adenocarcinoma remains poorly defined, although important clues of disease pathogenesis have emerged from epidemiological and genetic studies. Pancreatic adenocarcinoma is a disease that is associated with advancing age (rare before the age of 40) (417), it culminates in a 40-fold increased risk by the age of 80.

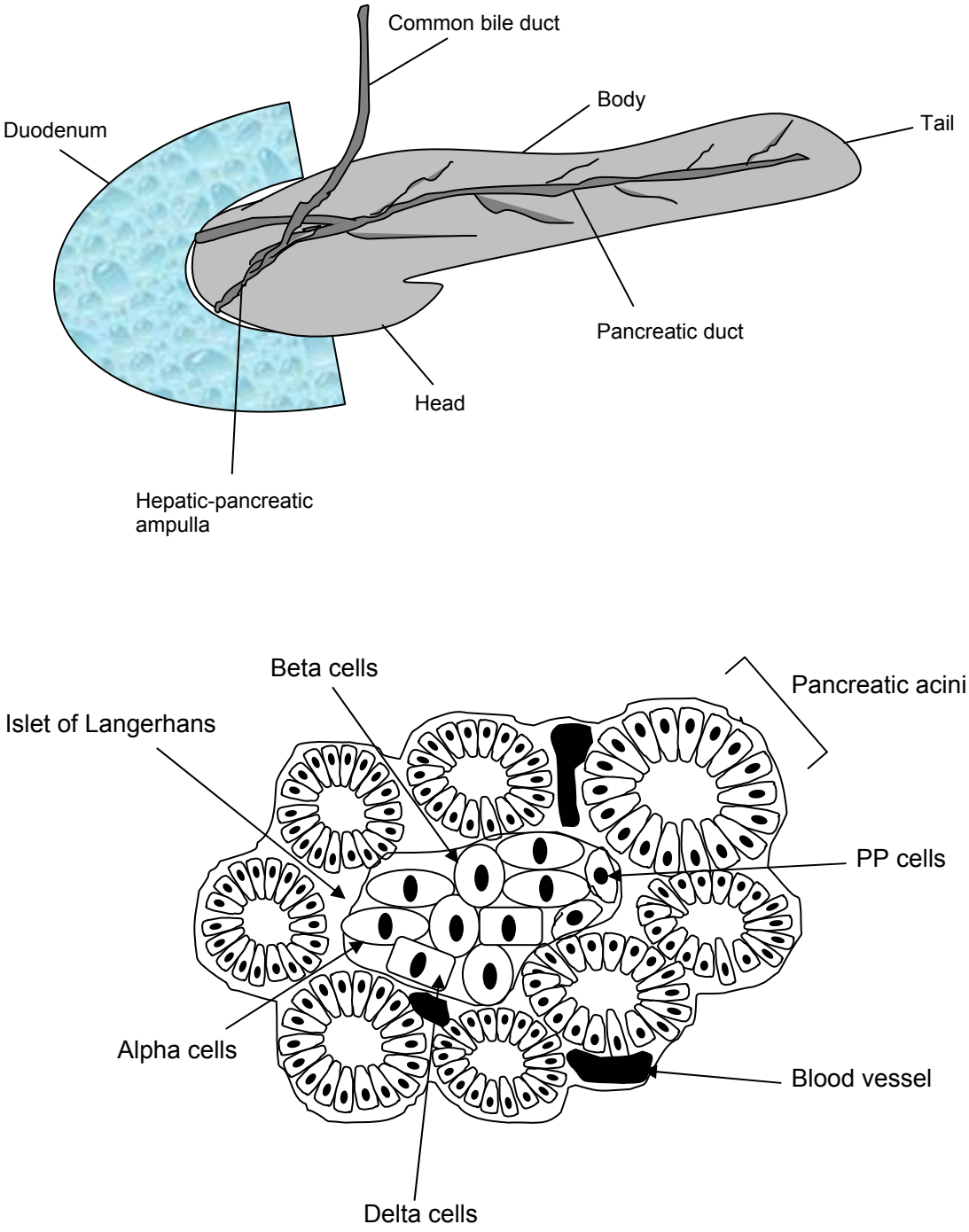


Fig. 19. **Structure of human pancreas.** Modified from (122).

Cigarette smoking is the most prominent and consistent risk factor in pancreatic cancer (417-421). The risk increases with the increasing of cigarette smoking. The highest risk ratio, 10-fold, has been seen in males who smoke more than 40 cigarettes daily (419). The second important risk factor associated with pancreatic cancer is diet, although the data for dietary effects are limited (417-419). Generally, increased risks have been associated with animal protein and fat consumption, and decreased risks, with intake of vegetables and fruits. Methods of food preparation evaluated in several studies showed an association of increased pancreatic cancer risk with high consumption of salt, smoked meat, dehydrated food, fried food, and refined sugar (422-425). An inverse association was found with the consumption of food containing no preservatives and additives, raw food, food prepared by high-pressure cooking, and food prepared in an electric or microwave oven (422-425).

Occupational exposure to some carcinogens is considered the third suspected risk factor for pancreatic cancer. High rates of pancreatic cancer have been reported in workers in certain occupations, such as chemists, coal and gas exploration workers, those in metal industries, leather tanning, textiles, aluminum milling, and transportation (426-427). Suggestive findings exist in relation to the products of incomplete combustion, (428,429) to certain pesticides (430,431) and to other chemicals and chemical processes. Other suggested risk factors for pancreatic cancer include some medical conditions, chronic pancreatitis, and inherited susceptibility is another suggested risk factor

for pancreatic cancer (417-419). On the genetic level, there is an increased risk in relatives of pancreatic adenocarcinoma patients (approximately three fold) and it is estimated that 10% of pancreatic cancers are due to an inherited predisposition (432). However, unlike familial cancer syndromes for breast, colon and melanoma, pancreatic adenocarcinoma linked to a familial setting has a lower penetrance (<10%) and maintains a comparable age of onset to sporadic cases in the general population. Among the genetic lesions that are linked to familial pancreatic adenocarcinoma are germline mutations in CDKN2A (which encodes two tumor suppressors (INK4A and ARF), BRCA2, LKB1 and MLH1 (433). Additional genetic defects seem to be operative in rare families in which pancreatic cancer is inherited as an autosomal-dominant trait with very high penetrance (432). A pancreatic cancer syndrome (so far identified in a single family) has been linked to chromosome 4q32-34 (434) and is associated with diabetes, pancreatic exocrine insufficiency and pancreatic adenocarcinoma, with a penetrance approaching 100%. Patients with hereditary pancreatitis, which is associated with germline mutations in the cationic trypsinogen gene PRSS1, experience a 53-fold increased incidence of pancreatic adenocarcinoma (435,436).

Molecular genetics of pancreatic adenocarcinoma. The identification of signature gene mutations in pancreatic adenocarcinoma was recognized as a valuable starting point, for analyzing the genesis and development of this disease. Molecular and pathological analysis of evolving pancreatic

adenocarcinoma has revealed a characteristic pattern of genetic lesions. Understanding how these signature genetic lesions (mutations of KRAS, CDKN2A, p53, BRCA2 and SMAD4/DPC4) contribute to the biological characteristics and evolution of this disease is of great interest. The progression model for colorectal cancer has served as a template for relating sequential, defined mutations to increasingly atypical growth states (437). It is believed that the pancreatic-duct cell is the progenitor of pancreatic adenocarcinoma. The increased incidence of abnormal ductal structures (now designated pancreatic intraepithelial neoplasia, PanIN) (417,438) in patients with pancreatic adenocarcinoma, and the similar spatial distribution of such lesions to malignant tumors, are consistent with the hypothesis that such lesions might represent incipient pancreatic adenocarcinoma (439).

Histologically, PanINs show a spectrum of divergent morphological alterations relative to normal ducts that seem to represent graded stages of increasingly dysplastic growth (440). A growing number of studies have identified common mutational profiles in simultaneous lesions, providing supportive evidence of the relationship between PanINs and the pathogenesis of pancreatic adenocarcinoma. Common mutation patterns in PanIN and associated adenocarcinomas have been reported for KRAS and for CDKN2A (441). In addition, similar patterns of loss of heterozygosity (LOH) at chromosomes 9q, 17p and 18q (harbouring CDKN2A, p53 and SMAD4, respectively) have been detected in coincident lesions and studies have

consistently shown an increasing number of gene alterations in higher-grade PanINs (442-445).

KRAS - Activating KRAS mutations are the first genetic changes that are detected in the progression series, occurring occasionally in histologically normal pancreas and in about 30% of lesions that show the earliest stages of histological disturbance (446). KRAS mutations are found in nearly 100% of pancreatic adenocarcinomas; they seem to be a virtual rite of passage for this malignancy (447). WAF1 (also known as p21 and CIP1) seems to be coordinately induced with the onset of KRAS mutations, perhaps due to activation of the mitogen-activated protein kinase (MAPK) pathway (448). Activating mutations of RAS-family oncogenes lead to induction of proliferation, survival and invasion through the stimulation of several effector pathways (449).

CDKN2A - The inheritance of mutant CDKN2A tumor-suppressor gene confers a 13-fold increased risk of pancreatic cancer (450,451). CDKN2A loss is generally seen in moderately advanced lesions that show features of dysplasia. Loss of CDKN2A function (caused by mutation, deletion or promoter hypermethylation) occurs in 80–95% of sporadic pancreatic adenocarcinomas (447). The role of CDKN2A has attracted the attention of many researchers as this tumor-suppressor locus, at 9q21, encodes two tumor suppressors (INK4A and ARF) via distinct first exons and alternative reading frames in shared downstream exons (452). Given this physical juxtaposition and frequent homozygous deletion of 9p21 (in ~40% of tumors), many pancreatic cancers

sustain loss of both the INK4A and ARF transcripts, thereby disrupting both the retinoblastoma (Rb) and p53 tumor-suppression pathways. INK4A inhibits CDK4/CDK6-mediated phosphorylation of RB, thereby blocking entry into the S (DNA synthesis) phase of the cell cycle; ARF stabilizes p53 by inhibiting its MDM2-dependent proteolysis. INK4A seems to be the more important pancreatic cancer suppressor at this locus, as germline and sporadic mutations have been identified that target INK4A, but spare ARF (447,453,454). Loss of INK4A usually occurs only in later stages of pancreatic neoplasia.

p53 - The p53 tumor-suppressor gene is mutated, generally by missense alterations of the DNA- binding domain in more than 50% of pancreatic adenocarcinomas (447). p53 mutations arise in later-stage PanINs that have acquired significant features of dysplasia, reflecting the function of p53 in preventing malignant progression. p53 loss probably facilitates the genetic instability that characterizes this malignancy. These tumors have profound aneuploidy and complex cytogenetic rearrangements, as well as intratumoral heterogeneity, which is consistent with ongoing genomic rearrangements (455,456). Cytogenetic studies indicate that telomere dynamics might contribute to this genomic instability (457,458).

BRCA2 - Although inherited BRCA2 mutations are typically associated with familial breast and ovarian cancer syndrome, it is also a significant risk for development of pancreatic cancer. Goggins and coworkers (459) has found that approximately 17% of pancreatic cancers that occur in a familial setting have

mutations in this gene. Loss of wild-type BRCA2 is a late event in those individuals who inherit germline heterozygous mutations of BRCA2, which is restricted to severely dysplastic PanINs and adenocarcinomas (459). BRCA2 is necessary for the maintenance of genomic stability by regulating the homologous-recombination-based DNA-repair processes; consequently, BRCA2 deficiency in normal cells results in the accumulation of lethal chromosomal aberrations (460). The loss of p53 and BRCA2, and the detection of abnormal mitosis and severe nuclear abnormalities in PanIN-3 lesions, indicate that genomic instability is initiated at this stage of tumor progression.

SMAD4/DPC4 - Loss of SMAD4/DPC4 is a frequent alteration in pancreatic adenocarcinoma (461); SMAD4/DPC encodes a transcriptional regulator that is a keystone component in the transforming growth factor-β (TGF-β) - family signaling cascade (462). The pathogenic role of SMAD4 inactivation is strongly supported by the identification of inactivating intragenic lesions of SMAD4 in a subset of tumours. SMAD4 seems to be a progression allele for pancreatic adenocarcinoma and its loss occurs only in later-stage PanINs (443,444). Loss of SMAD4 is considered a predictor of decreased survival for pancreatic adenocarcinoma patients (445), and this is consistent with its role in disease progression. The mechanism by which SMAD4 loss contributes to tumorigenesis is likely to involve its role in TFG-β-mediated growth inhibition. One study showed that TGF-β inhibits the growth of most normal epithelial cells by either blocking the G1–S cell-cycle transition or by

promoting apoptosis (462). The cellular responses to TGF-β are partially, but not exclusively, SMAD4-dependent (463) and, correspondingly, pancreatic adenocarcinomas show a degree of TGF-β resistance.

Vascular Endothelial Growth Factor and pancreatic cancer. In addition to all previous genetic lesions, the progressive growth of pancreatic cancer depends on vascularization from the surrounding stromal tissue into the tumor tissue. Vascular endothelial growth factor (VEGF) and its receptors (VEGFRs) play a critical role in tumor angiogenesis, and VEGF and/or VEGFRs are over expressed in multiple tumors including pancreatic cancer (464,465). Both physiological and pathological angiogenesis are regulated by vascular endothelial growth factors (VEGFs) and their receptors. There are six members in the VEGF family which includes VEGF-A, placenta growth factor PlGF, VEGF-B, VEGF-C, VEGF-D and orf virus VEGF also called VEGF-E. These factors are secreted as dimeric glycoproteins, all of which contain the characteristic regularly-spaced eight cysteine residues, the so-called cystine knot motif. VEGF forms an antiparallel homodimer, which is covalently linked by two disulphide bridges. The receptor-binding face of this dimer consists of residues presented from both sub-units and are located at each pole(466).Like most growth factors, VEGF utilizes predominantly hydrophobic interactions for binding to both of its receptors (466,467).

VEGF-A is the original VEGF and the major regulator of both physiological and pathological neovascularization (468). It has been shown that

VEGF has a pronounced mitogenic and angiogenic activities in a variety of *in vivo* models. VEGF stimulates endothelial cells to degrade extracellular matrix ECM, migrate and form tubes *in vitro* (469). Genetic engenering studies have shown that embryos lacking a single VEGF allele are growth retarded, exhibit developmental anomalies in both the central nervous system and the cardiovascular system, and die between E11 and E12. (470,471). Although VEGF mRNA is still expressed in heterozygous embryos, angiogenesis and blood-island formation are impaired which suggests that embryonic vessel formation is VEGF dose-dependent. Gene knock out studies had shown that other members of the VEGF gene family are apparently not equally important for vascular development and survival. For example, PlGF knockout mice have impaired wound healing processes, but they are viable and fertile (472). Many isoforms of VEGF have been shown to be produced by alternative exon splicing of the VEGF gene. Human VEGF is expressed as a combination of 121, 145, 165, 189 or 206 amino acid isoforms.

The action of VEGF is mediated by VEGF receptors (VEGFRs). These receptors transducer signals for mediating endothelial cell proliferation, migration, and organization into functional vessels. At least three different VEGFR genes have been identified: VEGFR-1 (Flt-1), VEGFR-2 (KDRrFlk-1) and VEGFR-3 (FLT-4). These receptors form a subfamily within the platelet-derived growth factor PDGF receptor class. All three consist of seven immunoglobulin-homology Ig domains, a transmembrane sequence and an

intracellular portion containing a split kinase domain (473). During embryogenesis, the VEGFRs are expressed in vascular endothelial cells from the stage of blood island formation. In adult tissues VEGFR-1 and VEGFR-2 localize to vascular endothelial cells, whereas VEGFR-3 is expressed mainly in the lymphatic endothelium (474). The ligand specificities of the receptors are different. For example VEGFR-1 binds VEGF, VEGF-B and PlGF, VEGFR-2 binds VEGF, VEGF-C, VEGF-D and the orf virus VEGF, whereas VEGFR-3 binds VEGF-C and VEGF-D (464). Ligand binding induces receptor dimerization and subsequent auto/transphosphorylation and the second Ig domain of VEGFR-1 is critical for specific binding of VEGF (475,476). In 1998 another VEGFR was identified, namely neuropilin-1 NP-1 which functions as a cell surface glycoprotein that mediates axonal repulsion during development. It has been identified as an isoform-specific $VEGF_{165}$ and PlGF-2-receptor (477,478). NP-1 enhances the mitogenic effects of VEGFR-2 and may have a signaling function of its own. Studies in which VEGFR genes have been genetically disrupted have shown indispensable, but distinct roles of VEGFRs in vasculogenesis and angiogenesis during embryonic development. In mice homozygous for a disrupted VEGFR-1 allele, the endothelial cells hyperproliferate and fail to organize into normal vascular channels, leading to embryonic death (479). Unlike VEGFR1 knockout mice, the VEGFR-2 knockout mice display defects in yolk sac blood-island formation and vasculogenesis and hematopoietic cells fail to develop, resulting in death *in utero* (480). This

suggests that VEGFR-2 is necessary for the differentiation and proliferation of endothelial and hematopoietic cells. VEGFR-3 knockout mice exhibit a failure in remodeling the primary vascular network, abnormal endothelial organization and embryonic death (481). Thus, VEGFR-3 seems to be necessary for the maturation of the vascular plexus into a hierarchy of large and small vessels. At the same time there were no major defects occurred in the differentiation of endothelial cells, formation of primary vascular networks or vascular sprouting. All these differences distinguish VEGFR-3 functions from those of the other VEGF receptors (481).

Mechanism of VEGF gene regulation - Transcription of VEGF mRNA is induced by different condition and by multiple factors (Table VII). Sp/KLF family members play a role in angiogenesis and members of this family are involved in regulating of VEGF expression (482). Notably, "Sp1 GC-rich sites" are important elements in VEGF promoters (483). The relevance of Sp factors to cancer-related angiogenesis is highlighted by the following findings: First, "Sp1 site" decoy oligonucleotides inhibit expression of VEGF in lung cancer and glioblastoma cells (484). Secondly, TNFα and basic FGF upregulate Sp1 levels in glioma cells and this upregulation correlates temporally with VEGF synthesis (485), Thirdly, mithramycin, an inhibitor of Sp1 DNA-binding, blocks the induction of VEGF and its receptors by basic FGF and/or TNFα (485,486), Fourthly, downregulation of VEGF in von Hippel-Lindau disease-associated tumors is related to the ability of the VHL tumor suppressor to bind to and

inactivate Sp1 (487). Fifth, Sp1 antisense oligonucleotides block TNFα induction of VEGF and tubular morphogenesis in vascular endothelial cells (488). Finally, growth stimulation of vascular endothelial cells leads to a Ras-dependent upregulation in the binding of Sp1 and other "Sp1 site"-binding proteins to the cyclin D1 promoter without changes in Sp1 levels (489).

Table VII

Regulatory factors of VEGF gene expression.

Condition or Factor	**Reference**
Hypoxia	(490-493)
Hypoglycemia	(494,495)
Growth factors	(496-498)
Hormones	(493,499,500)
Suppressors p53	(501)
p73	(502)
V-src oncogene	(503)
VHL tumor suppressor gene product	(487)
Ultraviolet radiation	(504,505)
Mechanical stress	(506,507)
Acidic growth conditions	(508-510)
Cytokines	(511,512)

In pancreatic cancer cells, analysis of the VEGF gene promoter showed that basal expression of VEGF was dependent on proximal GC-rich motifs that bind Sp1 protein, thus linking VEGF and Sp factors as potential key factors in the growth and metastasis of this cancer. In addition, the expression of VEGF is correlated with the expression of Sp1 and both proteins are overexpressed in the same cancer cell line and tumors (297).

Research in this laboratory has demonstrated that constitutive and hormonal regulation of several genes, including those involved in purine/pyrimidine synthesis and cell cycle progression, are dependent on GC-rich promoter elements that bind Sp1 or ERα/Sp1 (339,340-342,513,514). In breast and endometrial cancer cells basal and hormone-induced VEGF expression requires the GC-rich proximal promoter region. However, activation of VEGF is dependent on both cell context and Sp factors protein expression. For example, estrogen-dependent down regulation of VEGF in HEC1A endometrial cancer cell is dependent on ERα/Sp3 interaction with proximal GC-rich sites, whereas induction of VEGF by estrogen in ZR75 breast cancer cells requires ERα/Sp1 and ERα/Sp3 interaction with same GC-rich motifs (345,515).

Pancreatic cancer treatment. At present radical surgery is the only curative therapy for pancreatic cancer. However, only 5–25% of the patients present with potentially resectable disease (415). Approximately 50% of patients with pancreatic cancer have locally advanced disease, which is nonresectable because of involvement of the celiac axis, superior mesenteric artery, or vein.

Thirteen to twenty months is the median survival time after pancreatic resection with less than 10–20% of patients being long-term survivors. (415,516).

Chemotherapy - Although cytotoxic chemotherapy has a very low objective response rate, it is still considered the conventional treatment for advanced pancreatic cancer. The most widely used drugs included 5-fluorouracil (5-FU), mitomycin-C, streptozocin, doxorubicin, and nitrosoureas (Table VIII). 5-FU was the most active and best tolerated of these agents. The response rates to 5-FU from studies in the precomputer tomography era, were approximately 15 to 25% but with negligible impact on survival or disease-related symptoms. (517,518). Studies of chemotherapy in pancreatic cancer have demonstrated that single agents have limited anti-tumor activity with a minimal impact on overall survival. Therefore, there has been interest in studying combination therapies in pancreatic cancer. Table IX shows fluoropyrimidine-based combinations and Gemcitabine-based doublet that has been used in pancreatic cancer treatments.

Chemotherapy plus radiation therapy - In patients with locally advanced pancreatic cancer, therapy is directed to achieve both local control of disease as well as the treatment of systemic disease. A similar consideration is made in patients undergoing therapy after resection in the adjuvant setting. Chemotherapeutic agents have been used as radiosensitizers in pancreatic cancer since it is a relatively radio-resistant tumor.

Table VIII

Single chemotherapy used to treat pancreatic cancer.

Treatment	**Example**	**Refeference**
Nucleoside analogue	Gemcitabine	519
Oral Fluoropyrimidines	Capecitabine	520
Topoisomerase inhibitors	9-nitrocamptothecin (9NC) Exatecan	521,522

Table IX

Combination therapy used to treat pancreatic cancer.

Type	**Combination**	**Reference**
Fluoropyrimidine-based Combination	FAM (5FU, doxorubicin and mitomycin) SMF (streptozocin, mitomycin and 5FU)	(523,524)
Gemcitabine-based doublet	Gemcitabine/cisplatin Gemcitabine/oxaliplatin Gemcitabine/docetaxel Gemcitabine/5-FU (CIVI) Gemcitabine/5-FU (bolus) Gemcitabine/epirubicin Gemcitabine/irinotecan Gemcitabine/cisplatin/epirubicin / 5-FU	(525,526) (527) (528,529) (530,531) (532) (533) (534,535) (536)

Several randomized studies have suggested an improvement in survival for patients treated with modality therapy combining 5-FU-based chemotherapy and radiation (537-539). The role of radiation therapy in this setting remains controversial. Moreover, the use of ineffective chemotherapy as a radiosensitizer resulted in the lack of control of disease outside the pancreatic bed (540) Newer chemotherapeutic regimens with better systemic activities are in clinical trials to improve local and systemic tumor control. Gemcitabine has exhibit potent radio-sensitizing properties in preclinical studies (541). Radiation enhancement may be achieved by doses lower than those resulting in cell kill.

Novel systemic therapies - The benefit of chemotherapy, radiation, and surgery for patients with pancreatic cancer has been very modest. In an attempt to develop more effective systemic therapies for this disease, many researchers are focusing on new therapeutic strategies. A better understanding of the molecular mechanisms of cancer has identified dysregulated fundamental processes in malignant cells. These include molecular aberrations involving cell cycle control, signal transduction, apoptosis, angiogenesis, and extracellular matrix invasion. A number of drugs have been designed to target these molecules with high specificity. In general, two overlapping strategies have been adopted for development of new drug for pancreatic cancer: Drugs have been developed to target molecules that are critical for cell proliferation, differentiation, and induction of apoptosis. Other strategies to sensitize pancreatic tumor cells to conventional cytotoxic drugs by circumventing drug resistance and reducing the

apoptotic threshold of the pancreatic cancer cells are being persued. Table X summarizes some suggested targets for novel therapies in pancreatic cancer. Some of the drugs are already in clinical trials as single agents or in combination with chemotherapy.

Table X

Suggested targets for novel therapies in pancreatic cancer.

Molecular Target	**Examples of Therapeutic Agent**	**Reference**
Ras	Farnesyl transferase inhibitors (e.g. R115777)	(542)
Growth factor receptors	Monoclonal antibodies (e.g., C225, trastuzamab)	(543,544)
Angiogenesis	VEGF inhibitors (e.g. SU5416, TNP 470)	(545)
Cell signaling molecules	PI_3-K/AKT inhibitor (eg. LY294002)	(546)
COX-2 enzyme	COX-2 inhibitors (e.g., celecoxib, rofecoxib)	(547)
Matrix metalloproteinase	MMP inhibitors (e.g.marmastat)	(548)
Tumor suppressor genes	Gene replacement therapy (e.g., p53, p16)	(549,550)

RESEARCH OBJECTIVES

Several genetic approaches have been used to inhibit gene expression. For example gene targeting by homologous recombination is commonly used to determine gene function in mammals, but this is a costly and time-consuming process. In addition, the function of targeted genes might not be determined by this approach due to lethal and redundant phenotypes. Alternatively, the function of many genes can be determined by ribozyme and antisense technologies. Although successful in some situations, these techniques have been difficult to apply universally.

siRNA-directed silencing has created a revolution in somatic cell genetics, allowing inexpensive and rapid analysis of gene function in mammals. RNAi technology can be applied to many cell types and because the genome sequence for human is available, RNAi technology has the potential to determine the function of each gene that is expressed in a cell-type or pathway specific manner. siRNA is now being used as a tool in almost every field of biomedical research and one of the most dynamic and exciting applications is in cancer research for identifying and validating gene function or oncogenic properties or exploring the therapeutic potential for siRNA . This research will focus on applications of the siRNA technique for gene silencing to investigate several pathways associated with the growth of breast, liver and pancreatic cancer cell lines.

Objective 1. Sp1 interacts with GC-rich binding sites in multiple gene promoters to regulate gene expression. There are an increasing number of studies showing that Sp1 also interacts with other nuclear proteins including promoter-bound transcription factors and nuclear receptors such as ERα (eg. Sp1/ERα). Research in this laboratory has focused on the molecular mechanism of the ligand-dependent activation of ERα/Sp1 in breast and endometrial cancer cells lines. The first objective is to investigate the role of Sp1 protein in mediating hormone-responsivness in MCF-7 and ZR 75 cells using sequence-specific siRNA targeted to Sp1 mRNA.

Objective 2. TCDD and related AhR agonist inhibit expression of estrogen-induced genes and proliferation of ER-positive breast cancer cells. In AhR-deficient rodent liver cancer cells, AhR expression was associated with enhanced cell proliferation. The transcriptionally active AhR:ARNT complex interacts with inhibitory DREs in some gene promoter however other E_2-responsive genes that do not contain functional inhibitory DREs are inhibited by AhR agonist. The molecular mechanisms of inhibitory AhR-ERα crosstalk maybe complex and dependent on multiple factors including cell context. The second objective is to investigate AhR-ERα crosstalk and other AhR-mediated pathways in breast and hepatic cancer cells using RNAi technology.

Objective 3. Pancreatic adenocarcinoma is a highly aggressive neoplasm that is frequently not detected in patients until the tumor is advanced or metastatic. Prevention of pancreatic cancer is difficult because little is known about etiology. VEGF and VEGFRs play a major role in tumor angiogenesis in addition, VEGF and/or its receptors VEGFRs are over expressed in pancreatic cancer. Sp1 protein is expressed in pancreatic tumors and in pancreatic cells in culture, and it has been suggesting that Sp1 plays an important role in regulation of vascular endothelial growth factor (*VEGF*) expression in Panc-1 and other pancreatic cancer cells. Sp family proteins play a complex role in regulation of cancer cell growth and expression of genes required not only for growth but also apoptosis and angiogenesis. The third objective is to use RNA interference to investigate the role of Sp proteins in *VEGF* expression and cell cycle progression of selected pancreatic cancer cells.

SMALL INHIBITORY RNA DUPLEXES FOR Sp1 mRNA BLOCK BASAL AND ESTROGEN-INDUCED GENE EXPRESSION AND CELL CYCLE PROGRESSION IN MCF-7 BREAST CANCER CELLS *

Sp1 is a member of the Sp and Krüppel-like family of transcription factors that bind GC and CACCC boxes to regulate gene expression (222,551,552). Sp1 is widely expressed in multiple tissues (553) and targeted disruption of Sp1 in mice results in retarded development and embryolethality (256). Sp1 interacts with GC-rich "Sp1 binding sites" in multiple promoters to regulate gene expression, and there are an increasing number of studies showing that Sp1 interacts with other nuclear proteins including promoter-bound transcription factors to attenuate tissue-specific expression of selected genes (222,551,552). For example, Sp1 and NF-Y cooperatively interact to regulate multiple genes through NFY-GC-rich motifs and both proteins also physically interact (554-558). Sp1 also binds estrogen receptor α (ERα) and other members of the nuclear receptor family of transcription factors (339,559-566).

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* Reprinted with permission from “Small inhibitory RNA duplexes for Sp1 mRNA block basal and estrogen-induced gene expression and cell cycle progression in MCF-7 breast cancer cells” by Abdelrahim, M., Samudio, I., Smith, R. 3rd, Burghardt, R., and Safe, S. (2002) *J. Biol. Chem.* 277, 28815-28822.

Research in our laboratory has focused on the molecular mechanisms of the ligand-dependent activation of ERα/Sp1 in breast and endometrial cancer cell lines (340-343,383,513,514,567-571). Promoter analysis studies in breast cancer cells have identified GC-rich sites required for hormone activation of several genes including E2F1, DNA polymerase α, cyclin D1, insulin-like growth factor, growth factor binding protein 4, retinoic acid receptor α1, cathepsin D, vascular endothelial growth factor, c-fos, heat shock protein 27, bcl-2 , thymidylate synthase, and adenosine deaminase (340-343,383,513,514,567-570). Studies in other cell lines have also demonstrated a role for ERα/Sp1 activation of the progesterone receptor, epidermal growth factor receptor, telomerase, and receptor for advanced glycation end products (512,572-574). Activation of ERα/Sp1 does not require the DNA binding domain of ERα (promoter DNA-independent) and is primarily dependent on activation function-1 (AF1) of ERα (569), whereas DNA-dependent activation through ER binding to estrogen response elements (EREs) is primarily dependent on AF2 of ERα.

Recent studies have demonstrated that RNA interference through small inhibitory RNAs (iRNAs) targeted to endogenous or heterologous genes can be used to suppress intracellular expression of these genes in mammalian cells, and this technique is well suited for mechanistic studies on gene/protein function (46,67,89,575-578). This study investigates the role of Sp1 protein in mediating hormone-responsiveness in MCF-7 cells using sequence-specific duplexes of 21 nucleotides targeted to Sp1 mRNA as well as Lamin B1 and the heterologous

firefly luciferase gene (GL2) mRNAs. Transfection of iRNA for Sp1 (iSp1) decreases (40-60%) expression of nuclear Sp1 protein in ER-positive MCF-7 and ZR-75 human breast cancer cell extracts. In transfected cells, Sp1 protein is barely detectable by immunofluorescence, and both basal and estrogen-inducible transactivation is decreased in cells transfected with iSp1 and a GC-rich construct. These data, combined with results showing that iSp1 inhibits hormone-induced MCF-7 cell cycle progression from G_0/G_1 to S phase, demonstrate that ERα/Sp1-mediated transactivation plays a major role in ER-positive breast cancer cell growth.

MATERIALS AND METHODS

Cell lines, chemicals and biochemicals. MCF-7 and ZR-75 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). DME/F12 with and without phenol red, 100X antibiotic/antimycotic solution, propidium idodide, and E2 were purchased from Sigma (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Intergen (Purchase, NY) [γ-^{32}P] ATP (300ci/mmol) was obtained from NEN Life Science Products (Boston, MA). Poly [d(I-C)] and T4-polynucleotide kinase were purchased from Roche Molecular Biochemicals (Indianapolis, IN). Antibodies for proteins were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The $pSp1_3$ construct contains three consensus Sp1 binding sites and the $pERE_3$ construct contains three EREs. The oligonucleotides were linked to the bacterial luciferase gene and cloned into XP-2 plasmid obtained from ATCC. Lysis buffer, luciferase reagent,

and RNase were obtained from Promega Corp. (Madision, WI). iRNAs were prepared by IDT (Coralville, IA) and targeted the coding region 153-173, 672-694, and 1811-1833 relative to the start codon of GL2, Lamin B1, and Sp1 genes, respectively. Single stranded RNAs were annealed by incubating 20 μM of each strand in annealing buffer (100 mM potassium acetate, 30 mM HEPES buffer at pH 7.4, 2 mM magnesium acetate) for 1 min at 90°C followed by 1 h at 37°C. The iRNA duplexes used in this study are indicated below.

GL2 5' – CGUACGCGGAAUACUUCGATT
TTGCAUGCGCCUUAUGAAGCU – 5'

LMN 5' – AACGCGCUUGGUAGAGGUGGATT
TTUUGCGCGAACCAUCUCCACCU – 5'

Sp1 5' – AUCACUCCAUGGAUGAAAUGATT
TTUAGUGAGGUACCUACUUUACU – 5'

Transfection of MCF-7 and ZR-75 cells and preparation of nuclear extracts. Cells were cultured in 6-well plates in 2 ml DME/F12 medium supplemented with 5% FBS. After 16-20 h when cells were 50-60% confluent, iRNA duplexes and/or reporter gene constructs were transfected using Lipofectamin Plus Reagent (Life Technology, Carlsbad, CA). The effects of iSp1 on hormone-induced transactivation was investigated in MCF-7 cells treated with 10 nM E2 and cotransfected with $pSp1_3$ (500 ng) or $pERE_3$ (500 ng) and ERα expression plasmid (200 ng). Based on results of preliminary studies 0.75 μg iRNA duplex was transfected in each well to give a final concentration of 50 nM.

Cells were harvested 36-44 h after transfection by manual scraping in 1X lysis buffer (Promega). For whole cell extracts, cells were frozen in liquid nitrogen for 30 s, vortexed for 30 s, and centrifuged at 12,000 x *g* for 1 min. Lysates were assayed for luciferase activity using luciferase assay reagent (Promega); β-galactosidase activity was measured using Tropix Galacto – Light Plus assay system (Tropix, Bedford, MA) in a Lumicount Micro-well plate reader (Packard Instrument Co.). For nuclear extracts, cells were washed in PBS (2X), scraped in 1ml 1X lysis buffer, incubated at 4°C for 15 min and centrifuged at 14,000 x *g* for 1 min at 20°C. Cell pellets were initially washed in 1 ml of lysis buffer (3X), lysis buffer supplemented with 500 mM KCl was then added to the cell pellet and incubated for 45 min at 4°C with frequent vortexing. Nuclei were pelleted by centrifugation at 14,000 x *g* for 1 min at 4°C, and aliquots of supernatant were stored at -80°C and used for Western blot analysis and gel shift assays.

Western immunoblot. An aliquot of nuclear protein (30 μg) was diluted with loading buffer, boiled, and loaded on 7.5% SDS-polyacrylamide gel. Samples were electrophoresed at 150-180 V for 3-4 h, and separated proteins were transferred to PVDF membrane (Bio-Rad, Hercules, CA) in buffer containing 48 mM Tris-HCl, 29 mM glycine, and 0.025% SDS. Proteins were detected by incubation with polyclonal primary antibodies Sp1-PEP2, Lamin B1-C20, and ERα-G20 (all 1:1000 dilution) against Sp1, Lamin B1, and ERα proteins, respectively, followed by blotting with horseradish peroxidase-conjugated anti-rabbit (for Sp1 and ERα) or anti-goat (for Lamin B) secondary

antibody (1:5000 dilution). Blots were then exposed to chemiluminescent substrate (NEN Life Science Products) and placed in Kodak X-Omat AR autoradiography film. Band intensities were determined by a scanning laser densitometer (Sharp Electronics Corp., Mahwah, NJ) using Zero-D Scanalytics software (Scanalytics Corp., Billerica, MA).

FACS analysis. Cells were transfected with iRNAs for Sp1 or GL2 and, after 20-24 h, treated with Me_2SO or 20 nM E2 for 18-20 h in serum free medium. Cells were then trypsinized and approximately 2 x 10^6 cells were centrifuged, resuspended after removal of trypsin in 1 ml of staining solution containing 50 μg/ml propidium iodide, 4 mM sodium citrate, 30 units/ml RNase and 0.1% TX-100, pH 7.8). Cells were incubated at 37°C for 10 min, then prior to FACS analysis, sodium chloride was added to give final concentration of 0.15 M. Cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA), using CellQuest (Becton Dickinson) acquisition software. Propidium iodide fluorescence was collected through a 585/42-nm bandpass filter, and list mode data were acquired on a minimum of 12,000 single cells defined by a dot plot of PI-width versus PI-area. Data analysis was performed in ModFit LT (Verity Software House, Topsham, ME) using PI-width versus PI-area to exclude cell aggregates. FlowJo (Treestar,Inc., Palo Alto, CA) was used to generate plots shown in the Figures.

Electrophoretic Mobility Shift Assay (EMSA). Consensus Sp1 oligonucleotide (569,571) was synthesized and annealed, and 5 pmol aliquots

were 5'-end-labeled using T4 Kinase and [γ-^{32}P]ATP. A 30 μl EMSA reaction mixture contained approximately 100 mM KCl, 3 μg of crude nuclear protein, 1 μg poly (dI-dC), with or without unlabeled competitor oligonucleotide, and 10 fmol radiolabeled probe. After incubation for 20 min on ice, antibodies against Sp1 protein were added and incubated another 20 min on ice. Protein/DNA complexes were resolved by 5% polyacrylamide gel electrophoresis in 1X TBE (0.09 M Tris–base, 0.09 M boric acid, 2 mM EDTA, pH 8.3) at 120 V at 4°C for 2-3 h. Specific DNA/protein and antibody supershifted complexes were observed as retarded bands in the gel.

Immunocytochemistry. MCF-7 cells were seeded in Lab-Tek Chamber Slides (Nalge Nunc International, Naperville, IL) at 100,000 cells/well in DME/F12 media supplemented with 5% FBS and after 14 h cells were transferred into serum free medium for 8-10 h. Cells were then transfected with iRNAs and after 36-44 h, the media chamber was detached and the remaining glass slides were washed in Dulbecco's PBS. After washing, the glass slides were fixed with cold (-20°C) methanol for 10 min, then slides were washed in 0.3% Tween/PBS for 5 min (2X) before blocking with 5% rabbit or goat serum in antibody dilution buffer (stock solution: 100 ml PBS-Tween, 1 g BSA, 45 ml glycerol, pH 8.0) for 1 h at 20°C. After removal of the blocking solution, rabbit Sp1-PEP2 or goat Lamin B1 polyclonal antibodies were added in antibody dilution buffer (1:200) and incubated for 12 h at 4°C. Slides were washed for 10 min with 0.3%Tween in 0.02 M PBS (3X) and incubated with FITC conjugated

anti-rabbit or anti-goat secondary antibodies (1:1000 dilution) for 2 h at 20°C. Slides were then washed for 10 min in 0.3%Tween/PBS (4X). Slides were mounted in ProLonged antifading medium (Molecular Probes, Inc., Eugene, OR) and cover slips were sealed using Nailslicks nail polish (Noxell Corp., Huntvalley, MD). Fluorescence imaging was performed using Carlzeiss Axiophoto 2 (Calzeiss. Inc., Thornwood, NY). Images were captured using Adobe Photoshop 5.5 using identical camera settings.

Chromatin Immunoprecipitation Assay (ChIP). Cells were transfected with iSp1 or iGL2 for 36 h, and then treated with Me_2SO. MCF-7 cells were then collected, suspended in 1X PBS with 1 mM PMSF, and formaldehyde was added to the medium to give a 1% solution which was incubated with shaking for 10 min at 20°C. Glycine was then added (0.125 M) and, after further incubation for 10 min, cells were collected by centrifugation and washed with PBS and 1 nM PMSF. Cells were then resuspended in swell buffer (85 mM potassium chloride, 0.5% NP-40, 1 nM PMSF, 5 μg/ml leupeptin and aprotinin at pH 8.0), homogenized, and nuclei were isolated by centrifugation at 1500 x *g* for 30 s. Nuclei were then resuspended in sonication buffer (1% SDS, 10 nM EDTA, 50 mM Tris at pH 8.1), and sonicated for 45-60 s to obtain chromatin with appropriate fragment lengths (500-1000 bp). The sonicated extract was then centrifuged at 15,000 x *g* for 10 min at 0°C, aliquoted and stored at -70°C until used. The crosslinked chromatin preparations were diluted in buffer (1% Triton X, 100 mM sodium chloride, 0.5% SDS, 5 mM EDTA and Tris at pH 8.1), and 20

μl of Ultralink protein A or G or A/G beads was added per 100 μl chromatin, and incubated for 4 h at 4°C. Beads were collected by centrifugation, and salmon sperm DNA, specific antibodies, and 20 μl Ultralink beads were added to the supernatant, and the mixture incubated for 6 h at 4°C. An aliquot was treated at 65°C to reverse the crosslinks, extracted with phenol:chloroform and DNA was precipitated with ethanol. This aliquot was used as an input control. Immunoprecipitated samples were then centrifuged; beads were resuspended in dialysis buffer, vortexed for 5 min at 20°C, and centrifuged at 15,000 x *g* for 10 s. Beads were then resuspended in immunoprecipitation buffer (11 mM Tris, 500 mM lithium chloride, 1% NP-40, 1% deoxycholic acid at pH 8,0) and vortexed for 5 min at 20°C. Procedures with the dialysis and immunoprecipitation buffers were repeated (3-4X), and beads were then resuspended in elution buffer (50 nM $NaHCO_3$, 1% SDS, 1.5 μg/ml sonicated salmon sperm DNA), vortexed, incubated at 65°C for 15 min, and supernatants were then isolated by centrifugation and incubated at 65°C for 6 h to reverse protein-DNA crosslinks. Wizard PCR kits (Promega) were used for additional DNA cleanup. A portion of the purified immunoprecipitated DNA and 0.2% of the input control were used for αdCTP^{32} incorporation PCR. One quarter of a microliter of α-dCTP^{32} (3000 Ci/mmol) was added to a 25 μl PCR reaction (3% Me_2SO), 1 M betaine, 1.5 mM magnesium chloride) and subjected to one cycle of 95°C x 5 min, 5 cycles of 95°C x 30 s, 60°C x 30 s, 5 cycles of 95°C x 30 s, 55°C x 30 s, 72°C x 30 s, and

5 cycles of 95°C x 30 s, 48°C x 30 s, 72°C x 30 s followed by one cycle at 72°C for 4 min. Reactions were loaded on a 10-15% non-denaturing acrylamide gel; the gel was then dried and exposed to a phosphor screen for 24 h. The primers used for PCR of the GC-rich region of $pSp1_3$ are indicated below.

pxp2 luc	Fw (6128)	5' – GTTTGTCCAAACTCATCAATG – 3'
	Rv (105)	5' – CTTTATGTTTTTGGCGTCTTC – 3'

RESULTS

iRNA for Sp1 (iSp1) specifically decreases nuclear Sp1 protein levels in ER-positive human breast cancer cells. Results of preliminary studies indicate that iSp1 and lamin (iLMN) were most effective at decreasing cellular protein levels by treating cells for 36-44 h with 0.75 μg of the duplex oligonucleotides. The results illustrated in Figures 20A and 20B show that transfection of iSp1 in MCF-7 cells significantly decreased Sp1 protein by approximately 60% in nuclear extracts, whereas immunoreactive Lamin B1 and ERα levels were unchanged. In contrast, transfection of iLMN decreased Lamin B1 but not Sp1 or ERα protein levels, thus demonstrating the specificity of the iRNAs. The results summarized in Figure 1C confirm that iSp1 (but not iLMN) also significantly decreased Sp1 protein in ERα-positive ZR-75 cells. The effects of iRNAs on nuclear protein levels were also investigated in gel mobility shift assays using extracts from MCF-7 or ZR-75 cells (Figs. 21A and 21B) and a consensus GC-rich oligonucleotide [^{32}P]Sp1 that binds Sp1 and other Sp1 family proteins.

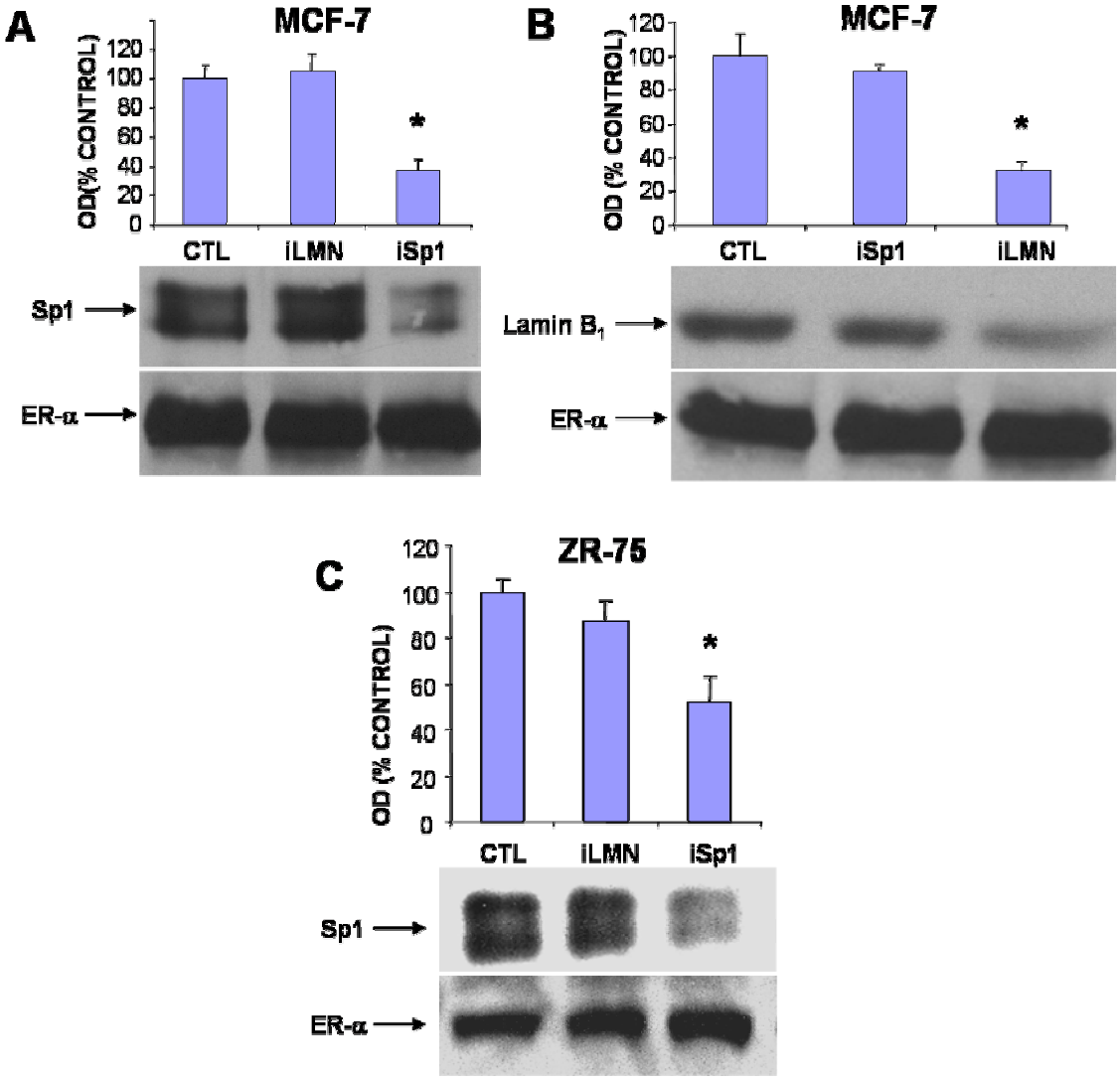

Fig. 20. **Interfering RNA for Sp1 (iSp1) decreases Sp1 protein in MCF-7 and ZR-75 cells.** [A] Effects on Sp1 protein in MCF-7 cells. Cells were transfected with iSp1 and iLMN and nuclear extracts were analyzed for Sp1 and ERα proteins by Western blot analysis as described in the Materials and Methods. Results are means ± SD for 3 replicate determinations for each treatment group, and a significant ($p < 0.05$) decrease in Sp1 protein levels was observed. [B] Effects on Lamin B1 in MCF-7 cells. Cells were treated as described in [A], and Lamin B1 and ERα proteins were detected by Western blot analysis. Treatment with iLMN significantly ($p < 0.05$) decreased Lamin B1 protein. [C] ZR-75 cells. Experiments were carried out as described in MCF-7 cells [A], and iSp1 significantly ($p < 0.05$) decreased Sp1 protein in ZR-75 cells.

Incubation of nuclear extracts from MCF-7 cells with [^{32}P]Sp1 gave a profile of retarded bands (lane 2) associated with Sp1- and Sp3-DNA complexes (571); the intensity of the former complex was decreased after incubation with unlabeled Sp1 oligonucleotide (lane 5) and supershifted with Sp1 antibodies (lane 6). In cells transfected with iSp1, there was a decrease in retarded band intensity (lane 4), whereas iLMN did not affect retarded band intensities. The results obtained for ZR-75 cells (Fig. 21B) were similar to those observed in MCF-7 cells and confirms the effectiveness and specificity of iSp1 for selectively decreasing Sp1 protein in breast cancer cells.

We have also used a chromatin immunoprecipitation assay to further investigate the *in situ* effects of iSp1 on Sp1-DNA interactions. MCF-7 cells were cotransfected with iSp1 or iGL2 and a construct containing three tandem GC-rich Sp1 binding sites ($pSp1_3$), and after 36-44 h, cells were treated with formaldehyde to crosslink DNA-bound proteins. After immunoprecipitation with Sp1 or Sp3 antibodies and removal of the crosslinks, PCR was used to identify the GC-rich region of $pSp1_3$ as part of the immunoprecipitable complexes. The results showed that iSp1 decreased interaction of Sp1 with the GC-rich promoter compared to that observed in cells transfected with iGL2, whereas the intensity of PCR products were similar for Sp3 immunoprecipitable complexes. Thus, results of Western blots, gel mobility shift and chromatin immunoprecipitation assays demonstrate a significant (40-60%) decrease in Sp1 protein in breast cancer cells transfected with iSp1.

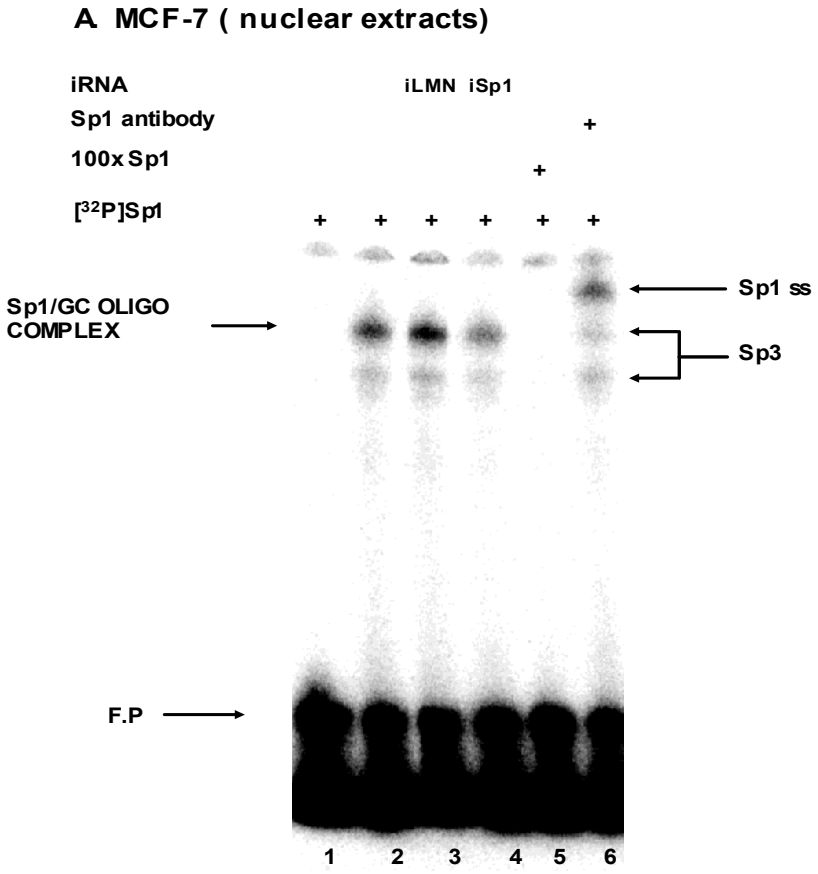

Fig. 21. **Binding of [^{32}P] Sp1 with nuclear extracts from breast cancer cells treated with iSp1 or iLMN.** MCF-7 [A] or ZR-75 [B] cells were treated with solvent, iSp1 or iLMN, and binding of nuclear extracts to [^{32}P] Sp1 was determined in gel mobility shift assays as described in the Materials and Methods. [C] ChIP assay. MCF-7 cells were transfected with $pSp1_3$ and iSp1 or iGL2, and analysis of Sp1 and Sp3 immunoprecipitable complexes associated with the transfected GC-rich construct were determined by ChIP/PCR as described in the Materials and Methods.

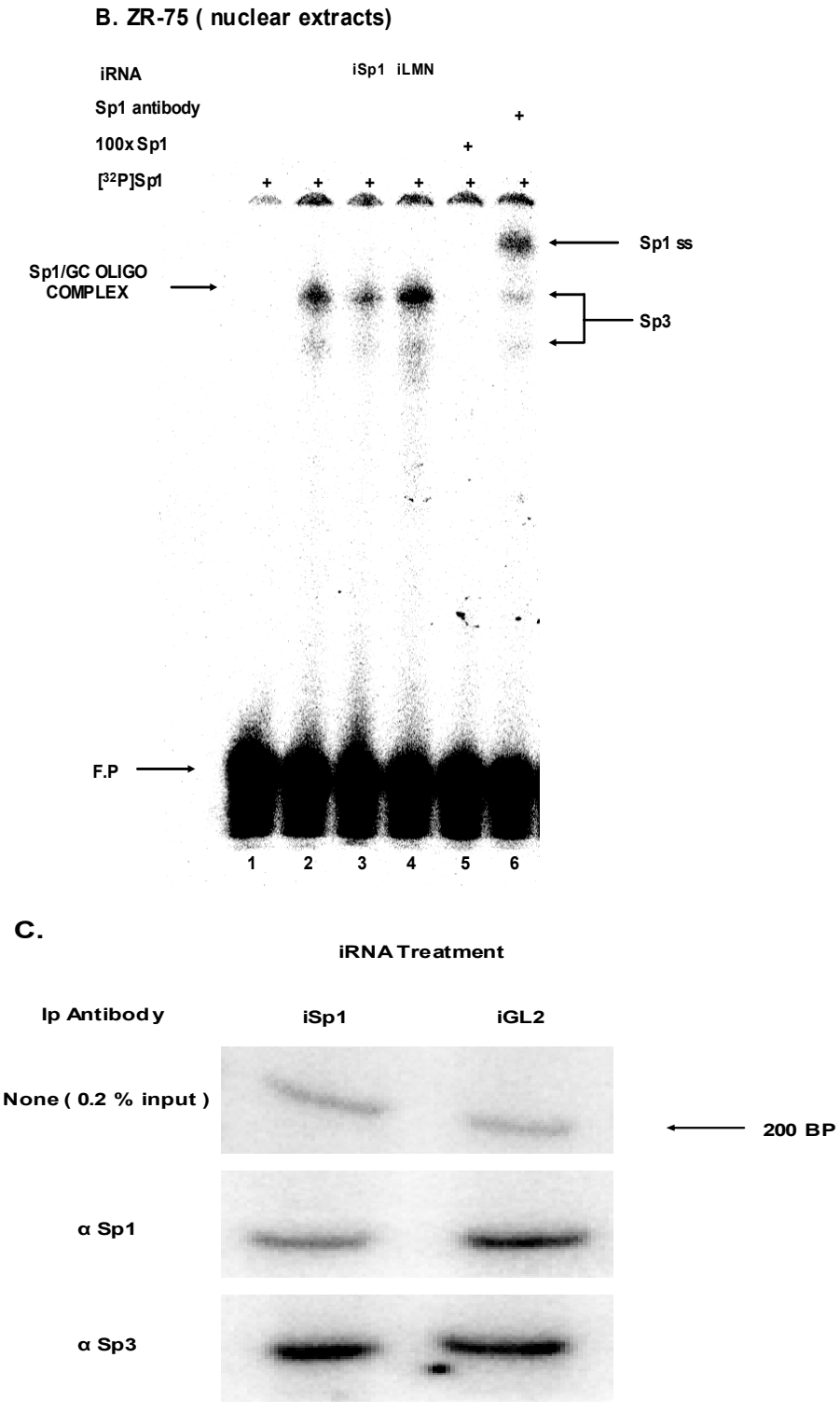

Fig. 21. Continued.

Sp1 protein expression, Sp1 and ERα/Sp1-mediated transactivation in MCF-7 cells transfected with iSp1. Transfection with lipofectamin results in >40-60% transfection efficiency in MCF-7 cells suggesting that iSp1 is highly effective in decreasing Sp1 expression in transfected cells. This was further investigated in MCF-7 cells by immunofluorescence analysis of Sp1 or lamin protein in MCF-7 transfected with iSp1 or iLMN (Fig. 22). Panels A and E are control panels where the primary antibody for lamin (A) or Sp1 (E) has been omitted. Panel C is a control for lamin (iLMN) showing immunofluorescence of Lamin B and phase contrast (panel B). In cells transfected with iLMN, most of the cells exhibited either significantly decreased Lamin B expression (transfected cells) or lamin expression was unchanged (non-transfected cells). Sp1 staining was observed in untreated MCF-7 cells (panel F) or in cells transfected with iLMN (panel G); however, in cells transfected with iSp1, there was a marked decrease of Sp1 staining in most cells, whereas the non-transfected cells were essentially unchanged. These data demonstrate that transfected iSp1 but not iLMN were highly effective in decreasing cellular expression of Sp1 and this accounts for the decreases in Sp1 protein in nuclear extracts (Figs. 20 and 21).

The results in Figure 23A summarize the effects of iLMN, iGL2 and iSp1 on luciferase activity in MCF-7 cells transfected with $pSp1_3$ and the iRNAs. iLMN did not significantly decrease activity, whereas iGL2 which is targeted to the luciferase mRNA and iSp1 both inhibited luciferase activity.

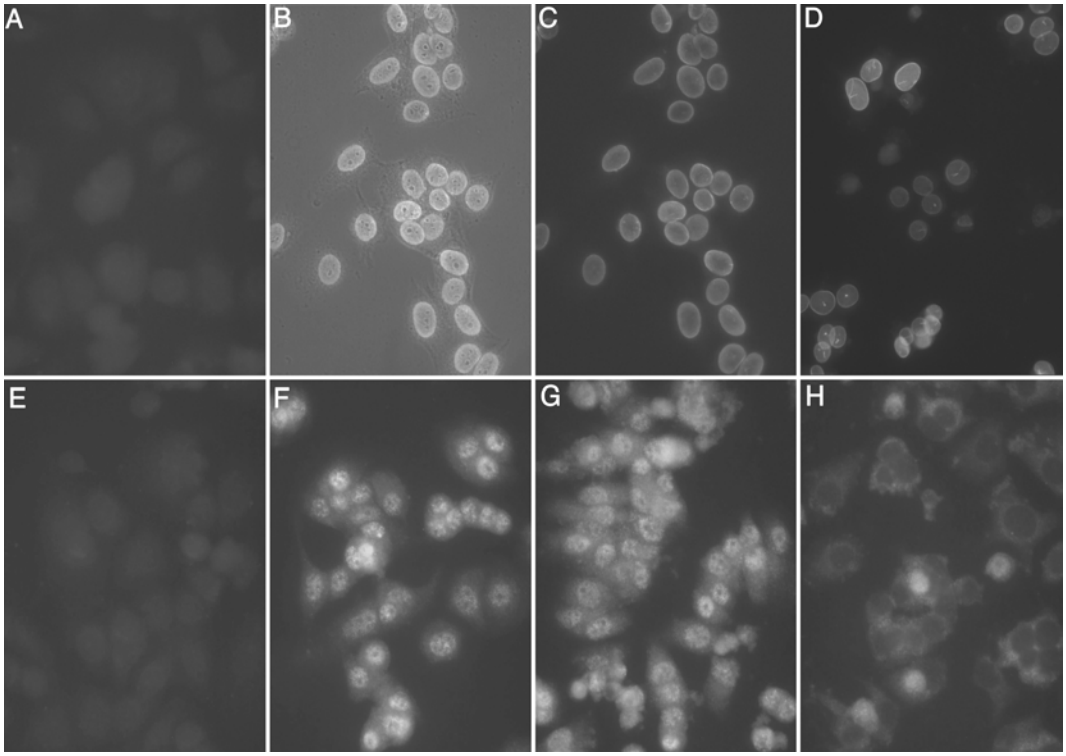

Fig. 22. **Immunofluorescence of Sp1 and Lamin B in MCF-7 cells transfected with iSp1 and iLMN.** MCF-7 cells were untreated (A, E), transfected with iSp1 (H), iLMN (D, G), and stained with Sp1 (F-H) or Lamin B (B-D) antibodies. Immunofluorescence was determined as described in the Materials and Methods.

In this study (Figs. 23A and 23B), there was a >60-77% decrease in basal activity in cells transfected with iSp1. Moreover, E2 induced luciferase activity in MCF-7 cells transfected with $pSp1_3$ as previously described (571), and in cells cotransfected with iSp1, there was a >80% decrease in hormone-induced transactivation. Thus, iSp1 inhibited both basal and E2-induced luciferase activity in MCF-7 cells transfected with $pSp1_3$. In contrast, hormone-induced transactivation in MCF-7 cells transfected with $pERE_3$ was not affected by cotransfection with iLMN or iSp1, whereas iGL2 decreased activity in cells treated with DMSO or E2 (Fig. 4C). Thus, iSp1 specifically blocks hormone-induced transactivation in cells transfected with $pSp1_3$ but not $pERE_3$.

iSp1 inhibits hormone-induced MCF-7 cell cycle progression.

Promoter regions in several genes associated with cell proliferation contain E2-responsive GC-rich motifs (340-343,383,513,514,567-571); however, the role of ERα/Sp1 in mediating cell growth can only be inferred from these studies. The role of Sp1 in hormone-induced cell cycle progression was further investigated to determine the effects of iSp1 and iGL2 (a control) on distribution of MCF-7 cells in G_0/G_1, G_2-M and S phases after treatment with solvent (Me_2SO) or 20 nM E2 for 18-20 h (Fig. 24). At this time point, iRNA for Sp1 increased the % of solvent-treated cells in G_0/G_1 from 75.3 to 78.3% and decreased the % in S phase (from 15.1 to 12.1).

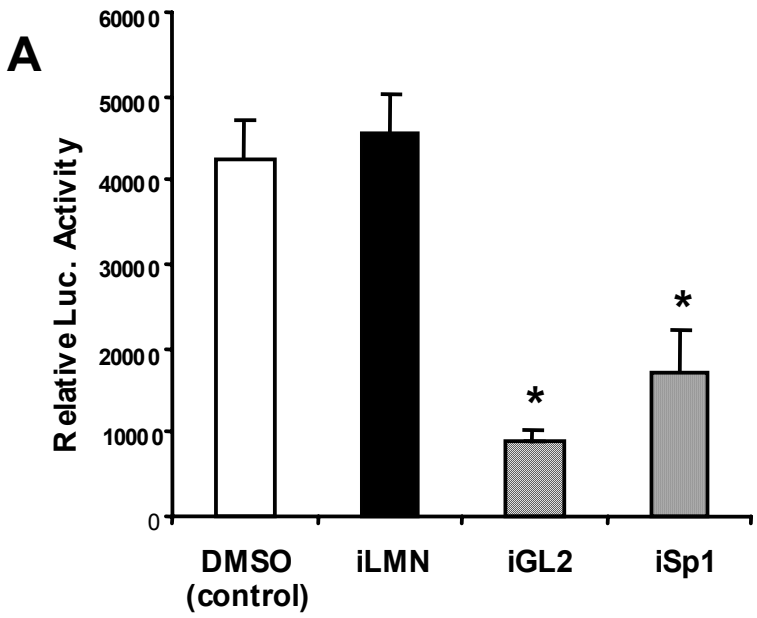

Fig. 23. **Effects of iLMN, iSp1 and iGL2 on luciferase activity in MCF-7 cells transfected with $pSp1_3$ and treated with Me_2SO or E2.** [A] Effects of inhibitor RNAs on basal activity. MCF-7 cells were transfected with $pSp1_3$ alone or in combination with iLMN, iGL2 or iSp1, and treated with Me_2SO. Luciferase activity was determined as described in the Materials and Methods. [B] iSp1-mediated inhibition of transactivation in cells transfected with $pSp1_3$. Cells were transfected with $pSp1_3$ and iSp1, treated with Me_2SO or 10 nM E2, and luciferase activity was determined as described in the Materials and Methods. [C] Effects of iSp1 on cells transfected with $pERE_3$. Cells were transfected with $pERE_3$ and iLMN, iGL2 or iSp1, treated with Me_2SO or 10 nM E2, and luciferase activity was determined as described in the Materials and Methods. Results summarized in [A], [B] and [C] are means $\pm$ SD for 3 replicate determinations for each treatment group and significant ($p < 0.05$) decreases in activity are indicated by an asterisk.

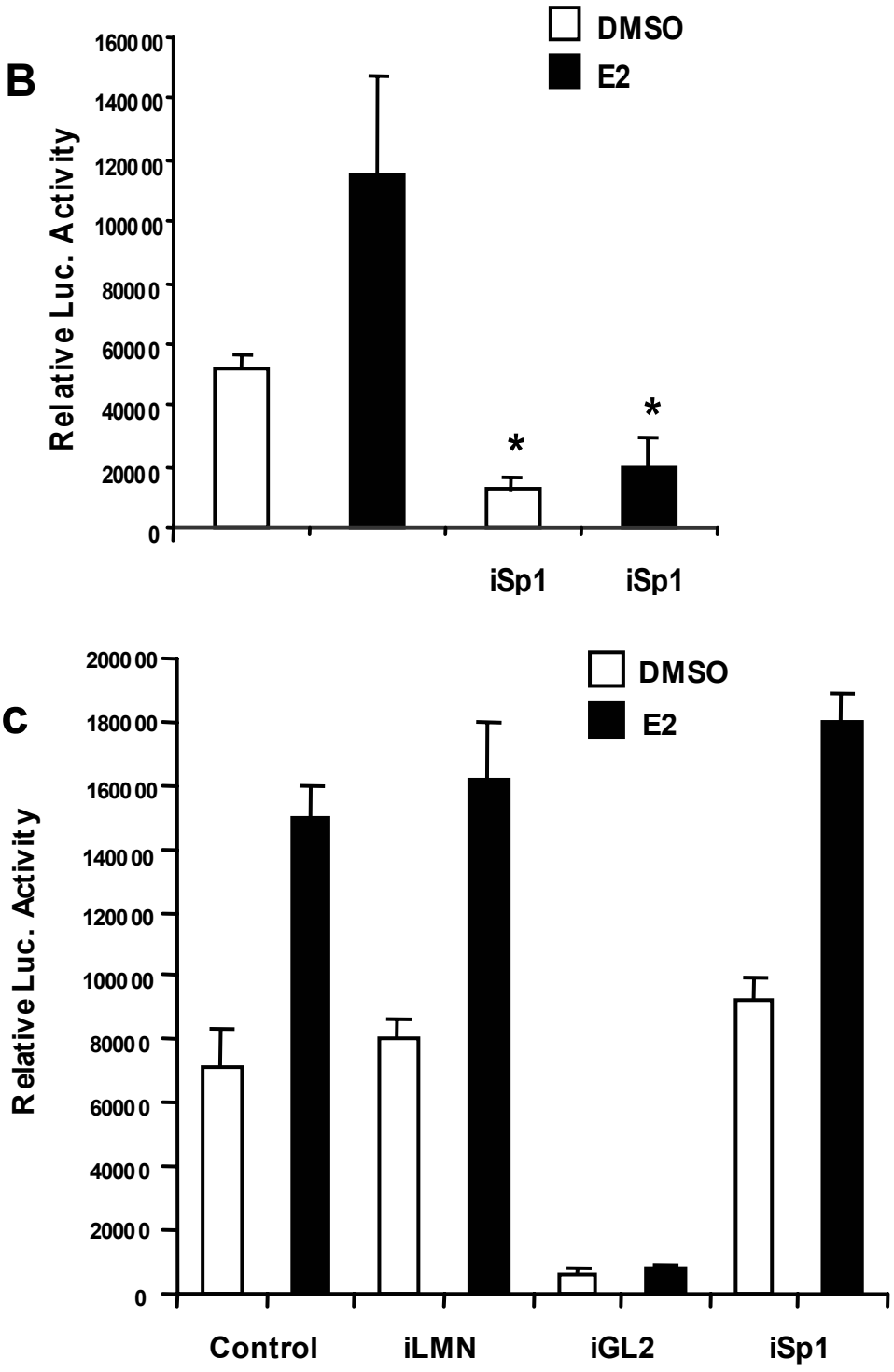

Fig. 23. Continued.

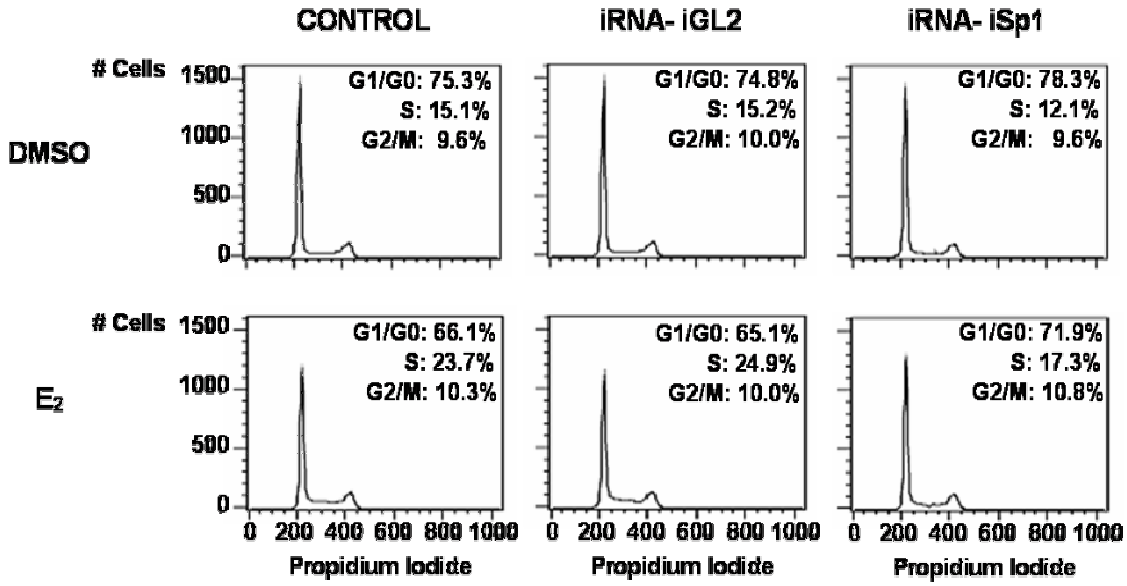

Fig. 24. **Effects of iSp1 on hormone-induced cell cycle progression in MCF-7 cells.** Serum-starved MCF-7 cells were treated with Me_2SO or E2 alone or cotransfected with iGL2 and iSp1, and the % distribution of cells in G_1/G_0, S and G_2/M were determined by FACS analysis as described in the Materials and Methods. Similar results were observed in a duplicate analysis.

In a parallel study in untreated cells at an earlier time point (8-10 h), a 5% decrease in cells in S phase and a similar increase in cells in G_0/G_1 was observed . More dramatic changes were observed for the effects of iSp1 on E2-induced proliferation of MCF-7 cells. For example, in cells treated with Me_2SO or 20 nM E2, the % of cells in G_0/G_1:S phase was 75.3:9.57% or 66.1:23.7%, respectively, showing a dramatic increase in $G_0/G_1 \rightarrow$ S progression after treatment with E2, and this has been observed in other studies (579,580). In contrast, the % of cells in G_0/G_1:S phase in cells treated with iSp1 was 71.9:17.3% indicating that hormone-induced cell cycle progression was markedly decreased by ablating cellular expression of Sp1 protein, whereas transfection of the control iGL2 did not affect cell cycle progression. These results demonstrate for the first time that Sp1 protein and ERα/Sp1-mediated transactivation are important for hormone-induced proliferation of MCF-7 cells.

DISCUSSION

The development of genetic technologies to regulate or delete expression of endogenous genes has been extensively used to probe the role of specific genes on biological function. For example, the generation of knock-out/knock-in mice and the overexpression of genes in transgenic animal models has provided unique insights on gene function in normal physiology and disease processes. RNA interference by double-stranded RNA involves the sequence-specific post-transcriptional silencing of genes which has been widely described and used in

plants and animals (46,575-577). It has recently been shown that small interfering RNA duplexes (21 to 25 nucleotides) targeted to specific genes can now be introduced into mammalian cells in culture to decrease RNA/protein expression (46,67,89,575-578). Elbashir and coworkers recently reported iRNA duplexes for endogenous and exogenous genes decreased their corresponding protein and/or protein-dependent activities in several mammalian cell lines including NIH 3T3, HeLa, COS-7 and 293 cells (67).

This study has used the iRNA approach for investigating the role of Sp1 protein in the growth and hormone-responsiveness of MCF-7 human breast cancer cells. Although Sp1 is important for basal transcription of genes involved in cell growth, expression of several cell cycle regulated genes such as dihydrofolate reductase, hypoxanthine/guanine phosphoribosyl transferase were unaffected in gd 8.5 day-old embryos (256). In contrast, transfection of GC-rich Sp1 oligonucleotide decoys into A549 human lung adenocarcinoma and U251 human glioblastoma cells blocked expression of several genes with GC-rich promoters and suppressed cell growth. This approach and others that target GC-rich sequences suggest that Sp1 protein may play an important role in cell growth (294,581); however, these techniques lack specificity since multiple Sp family proteins bind GC-rich motifs that may influence the function of other DNA bound transcription factors. Research in this laboratory has identified E2-responsive GC-rich motifs in promoters of several genes involved in cell proliferation, and these include cyclin D1, thymidylate synthase, c-fos, E2F1,

bcl2, and DNA polymerase α (340-343,383,513,514,567-571). Several approaches were previously used to demonstrate the role of ERα/Sp1 as a transcription factor complex, and this study was designed to further investigate this non-classical mechanism of estrogen action and its involvement in hormone-induced transactivation and cell proliferation. The results in Figures 20-22 clearly demonstrate that transfected iSp1 was highly effective for decreasing expression of Sp1 protein in nuclear extracts and not surprisingly, immunofluorescence studies indicate that Sp1 protein is barely detectable in transfected cells (Fig. 22). The high efficiency of iSp1 for ablating Sp1 protein in transfected cells was observed in MCF-7 cells cotransfected with iSp1 and $pSp1_3$, an E2-responsive GC-rich construct that serves as a surrogate for other GC-rich E2-responsive gene promoters (Fig. 23). In these transfection studies, iSp1 significantly decreased both basal and E2-induced luciferase activities confirming the role of ERα/Sp1 in ligand-activated transcription.

Treatment of growth-arrested MCF-7 cells with E2 results in cell cycle progression which is characterized by a decrease in cells in G_0/G_1 and an increase in cells in S phase (579,580) (Fig. 24). In untreated cells, iSp1 further increased the percentage of cells in G_0/G_1 (from 75.3 to 78.3%) and decreased the number of cell in S phase (from 15.1 to 12.1%). Since FACS analysis was carried out on the total cell population (transfected and non-transfected), the response of MCF-7 cells to transfected iSp1 demonstrates the important role of Sp1-regulated genes in basal growth of these cells. The effects of iSp1 were

more dramatic in reversing hormone-induced cell cycle progression and blocking a high proportion of these cells from progression to S phase. These data are consistent with results of previous studies showing that cyclin D1 and other genes important for cell proliferation are regulated by ERα/Sp1 (341-343,568,571). Future studies will use iRNAs to further investigate the role of Sp1, other Sp-like proteins and coregulatory factors on the growth of MCF-7 and other hormone-dependent cell lines and to identify key genes that are integral for these responses.

ARYL HYDROCARBON RECEPTOR GENE SILENCING WITH SMALL INHIBITORY RNA DIFFERENTIALLY MODULATES Ah-RESPONSIVENESS IN MCF-7 AND HepG2 CANCER CELLS *

The aryl hydrocarbon (AhR) is a ligand-activated nuclear transcription factor that is a member of the PAS and basic helix-loop-helix protein families (356,364,582). The transcriptionally active nuclear AhR complex is a heterodimer of the AhR and AhR nuclear translocator (ARNT) proteins which interact with genomic *cis*-acting dioxin responsive elements (DREs) in the CYP1A1 and other Ah-responsive genes (363,583,584). The ARNT protein which was initially identified as a partner for the AhR (585) is also called hypoxia-inducible factor 1β (HIF-1β) and many hypoxia-induced genes are regulated by the HIF1α:HIF1β complex interacting with hypoxia responsive elements (HREs) (586). The AhR was initially identified as the intracellular receptor for the environmental toxicant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) which binds with high affinity to the AhR (346). Interactions of TCDD and related halogenated aromatic compounds with the AhR mediate their

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diverse species-/strain-, tissue- and age-specific biochemical, toxic, carcinogenic and anticarcinogenic responses (347,377,378).

The physiological role of the AhR-ARNT heterodimer has been investigated in transgenic knockout mice which do not express the AhR protein (370-372). Not surprisingly, these knockout animals do not respond to the prototypical TCDD-induced biochemical (e.g. CYP1A1 induction) and toxic responses (587); however, the three strains of mice deficient in the AhR exhibit both common and different phenotypes. These mice typically have problems in liver development, poor fecundity, and weight loss suggesting that the AhR-ARNT complex regulate constitutive functions in the absence of exogenous ligand. These results are consistent with reports showing that AhR-ARNT alone may act as a transcription factor; however, this would not exclude a role for an unknown endogenous ligand (588-590).

The AhR also binds with moderate to low affinity to chemoprotective phytochemicals such as indole-3-carbinol, flavonoids and carotenoids which exhibit both AhR agonist and antagonist activities (591-594). Research in this laboratory has identified selective AhR modulators (SAhRMs) which exhibit minimal AhR-mediated toxicities but inhibit 17β-estradiol (E2)-induced gene expression and mammary tumor growth in rodent models (400,401,595,596). The molecular mechanisms of inhibitory AhR-estrogen receptor (ER) crosstalk may be complex and dependent on multiple factors including cell context. This study investigates AhR-ERα crosstalk and other AhR-mediated pathways using

small interfering RNA (siRNA) for the AhR which selectively degrades AhR mRNA and decreases AhR protein expression and function in breast cancer cells. Decreased expression of the AhR in MCF-7 breast cancer cells resulted in an increase in the percentage of cells in S phase and a decrease in G_0/G_1, suggesting that in the absence of exogenous ligand, the AhR suppresses growth of this cell line. In contrast, degradation of the AhR in HepG2 liver cancer cells decreases $G_0/G_1 \rightarrow$ S phase progression indicating a role for the AhR in enhancing growth of this cell line, and this is associated with decreased expression of cyclin D1, cyclin E, cdk2 and cdk4. We also observed that in MCF-7 cells transfected with siRNA for the AhR TCDD exhibits estrogenic activity and this complements results of a previous study in AhR-deficient MCF-7 cells (597). Thus, selective gene silencing of the AhR in breast and liver cancer cells illustrates the utility of this approach for investigating cellular mechanisms and function of the gene targeted for degradation.

MATERIALS AND METHODS

Cell lines, chemicals and biochemicals. MCF-7 and HepG2 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). DME/F12 with and without phenol red, 100X antibiotic/antimycotic solution, propidium idodide, and E2 were purchased from Sigma (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Intergen (Purchase, NY) [γ-^{32}P]ATP (300ci/mmol) was obtained from NEN Life Science Products (Boston, MA). T4-polynucleotide kinase was purchased from Roche Molecular Biochemicals

(Indianapolis, IN). Antibodies for proteins were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The $pDRE_3$ and $pERE_3$ construct contain three consensus DRE and ERE motifs, respectively; oligonucleotides containing these motifs were linked to the bacterial luciferase gene and cloned into *Bam*HI-*Hind*III cut XP-2 plasmid obtained from ATCC. Lysis buffer, luciferase reagent, and RNase were obtained from Promega Corp. (Madision, WI). siRNA duplexes were prepared by Dharmacon Research (Lafayette, CO) and targeted coding regions of the AhR (1416 to 1434), ARNT (445 to 463), lamin A/C (608 to 626), and GL2 (luciferase) (153 to 171). The siRNA duplexes used in this study are indicated below. Scrambled iRNA was derived from a message transcribed from the chloroplast genome of *Euglena gracilis* (Accession #70810, position 24750-24768).

GL2

5' – CGU ACG CGG AAU ACU UCG ATT
TT GCA UGC GCC UUA UGA AGC U – 5'

LMN

5' – CUG GAC UUC CAG AAG AAC ATT
TT GAC CUG AAG GUC UUC UUG U – 5'

Scramble

5' – GCG CGC UUU GUA GGA UUC GTT
TT CGC GCG AAA CAU CCU AAG C – 5'

AhR

5' – UAC UUC CAC CUC AGU UGG CTT
TT AUG AAG GUG GAG UCA ACC G – 5'

ARNT

5' – CCA UCU UAC GCA UGG CAG UTT
TT GGU AGA AUG CGU ACC GUC A – 5'

Transfection of MCF-7 and HepG2 cells and preparation of nuclear extracts. Cells were cultured in 6-well plates in 2 ml DME/F12 medium supplemented with 5% FBS. When cells were approximately 50-60% confluent, siRNA duplexes and/or reporter gene constructs were transfected using Oligofectamine Reagent (Invitrogen, Carlsbad, CA). Based on results of preliminary studies, 7 μl of 20 μM stock solution of siRNAs were transfected in each well to give a final concentration of 140 nM. Cells were harvested 48-56 h after transfection by manual scraping in 1X lysis buffer (Promega). Whole cell extracts were frozen in liquid nitrogen for 30 s, vortexed for 30 s, and centrifuged at 12,000 x *g* for 1 min to give lysates that were assayed for luciferase activity using luciferase assay reagent (Promega). β-Galactosidase activity was determined using Tropix Galacto – Light Plus assay system (Tropix, Bedford, MA) in a Lumicount Micro-well plate reader (Packard Instrument Co.). Nuclear extracts were prepared using 1X lysis buffer as mentioned before in the previous section. Aliquots of supernatant were stored at -80°C and used for gel shift assays.

Western immunoblot. Forty-eight h after transfection, cells were washed once with PBS and collected by scraping in 200 μl of lysis buffer [50 mM HEPES, 0.5 M sodium chloride, 1.5 mM magnesium chloride, 1 mM EGTA, 10% (v/v) glycerol, 1% Triton X-100, and 5 μl/ml Protease Inhibitor Cocktail (Sigma)]. The lysates were prepared as mention early in the previous section. Proteins

were detected by incubation with polyclonal primary antibodies Sp1 (PEP2), lamin A/C (N-18), AhR (N-19), ARNT1 (C-19), CYP1A1 (G-18), cyclin D1 (M-20), cyclin E (C-19), cdk2 (M-2), cdk4 (C-22), Rb (C-15) and p27 (C-19) followed by blotting with horseradish peroxidase-conjugated anti-rabbit (for Sp1, cyclin D1, cyclin E, cdk2, cdk4 and p27), anti-goat (for lamin A, CYP1A1, AhR and ARNT) or anti-mouse (for Rb) secondary antibody. Blots were then exposed to chemiluminescent substrate (NEN Life Science Products) and placed in Kodak X-Omat AR autoradiography film. Band intensities were determined by a scanning laser densitometer (Sharp Electronics Corp., Mahwah, NJ) using Zero-D Scanalytics software (Scanalytics Corp., Billerica, MA).

FACS analysis. Cells were transfected with siRNAs for AhR or scramble RNA and, after 36 h, cells were synchronized in serum-free media for 24 h, treated with Me_2SO or 20 nM E2, 20 nM TCDD or TCDD plus E2 for 18-20 h in serum-free medium. Cells were then trypsinized and approximately 2 x 10^6 cells were centrifuged, resuspended after removal of trypsin in 1 ml of staining solution containing 50 μg/ml propidium iodide, 4 mM sodium citrate, 30 units/ml RNase and 0.1% TX-100, pH 7.8). Cells were incubated at 37°C for 10 min, then prior to FACS analysis, sodium chloride was added to give final concentration of 0.15 M. Cells were analyzed as mentioned in the previous section and data analysis was performed in ModFit LT (Verity Software House, Topsham, ME) using PI-width versus PI-area to exclude cell aggregates. FlowJo (Treestar,Inc., Palo Alto, CA) was used to generate plots shown in the Figures.

Electrophoretic Mobility Shift Assay (EMSA). Consensus DRE oligonucleotide was synthesized and annealed, and 5 pmol aliquots were 5'-end-labeled using T4 Kinase and [γ-^{32}P] ATP (597). A 30 μl EMSA reaction mixture contained approximately 100 mM KCl, 3 μg of nuclear protein, 500 ng salmon sperm DNA (Invitrogen), with or without unlabeled competitor oligonucleotide, and 10 fmol radiolabeled probe. After incubation for 20 min on ice, antibodies against AhR protein were added and incubated another 20 min on ice. Protein/DNA complexes were resolved by 5% polyacrylamide gel electrophoresis in 1X TBE (0.09 M Tris–base, 0.09 M boric acid, 2 mM EDTA, pH 8.3) at 120 V at 4°C for 2-3 h. Specific DNA/protein and antibody supershifted complexes were observed as retarded bands in the gel.

EROD activity. EROD activity was determined as described (598). Trypsinized cells were seeded in 48-well plates and grown to 50% confluency. Thirty-six h after transfection with siRNAs, cells were treated with Me_2SO or 10 nM TCDD for 18-20 h. Cells were then washed with PBS; 200 μl of PBS was added to each well and cells were incubated at 37°C for 2 min. Ethoxyresorufin (1.25 μg) was added to each well, incubated for 10 min at 37°C, and the reaction was stopped by adding 100 μl fluorescamine. EROD activity and protein concentration were determined on a Cytofluor™ 2350 plate reader as described (598). Each treatment was carried out in triplicate, and results are presented as means $\pm$ SD.

Statistical analysis. Statistical significance was determined by analysis of variance and Scheffe's test, and the levels of probability are noted. The results are expressed as means ± S.D. for at least 3 separate (replicate) experiments for each treatment.

RESULTS

MCF-7 breast cancer cells are ERα-positive and express the AhR and ARNT proteins (599,600). Results summarized in Figure 25A demonstrate that AhR levels are decreased in MCF-7 cells transfected with siRNA for the AhR, whereas levels were unchanged in control cells and cells treated with siRNA for lamin A. Sp1 protein is used as a loading control for these experiments since it is highly expressed and unaffected by treatment with E2 or AhR agonists (600). This experiment has been replicated several times and AhR protein levels are typically decreased by 60-80% in whole cell extracts depending on the transfection efficiency in the individual experiments. Results in Figure 25B demonstrate the specificity of the iRNAs and show that treatment with siRNA for lamin A decreases lamin A protein levels by approximately 65%, whereas siRNA for the AhR did not affect lamin protein. Using a similar approach, we also show that siRNA for ARNT specifically decreases ARNT protein expression in MCF-7 cells, whereas siRNA for lamin A did not affect levels of ARNT protein (Fig. 25C). MCF-7 cells were treated with siRNA for lamin A, AhR and ARNT, and nuclear extracts were incubated with ^{32}P-labeled DRE and analyzed by gel

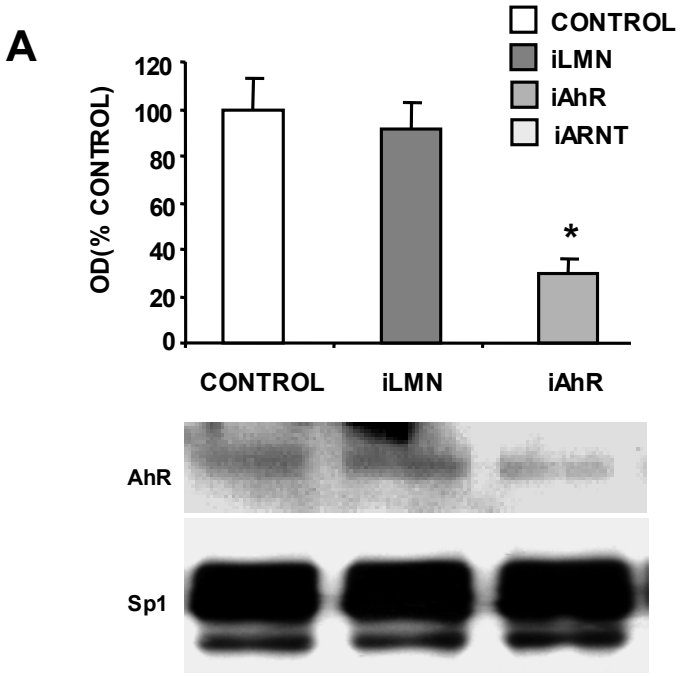


Fig. 25. **siRNAs for AhR (iAhR) and ARNT (iARNT) decrease their corresponding proteins in MCF-7 cells**. [A] Effects on AhR protein in MCF-7 cells. Cells were transfected with iLMN, iAhR and whole cell extracts were analyzed for AhR and Sp1 proteins by Western blot analysis as described in the Materials and Methods. Results are means ± SD for 3 replicate determinations for each treatment group, and a significant ($p < 0.05$) decrease in AhR protein levels was observed only in cells treated with iAhR. [B] Effects of siRNAs on lamin A in MCF-7 cells. Cells were treated as described in [A], and lamin A and Sp1 proteins were detected by Western blot analysis. Treatment with iLMN A significantly ($p < 0.05$) decreased lamin A protein. [C] Effects of siRNAs on ARNT protein. Experiments were carried out as described in [A], and iARNT significantly ($p < 0.05$) decreased ARNT protein in MCF-7 cells, whereas iLMN A did not affect ARNT protein levels. Sp1 protein serves as a loading and reference control protein that is not affected by the siRNAs used in this study.

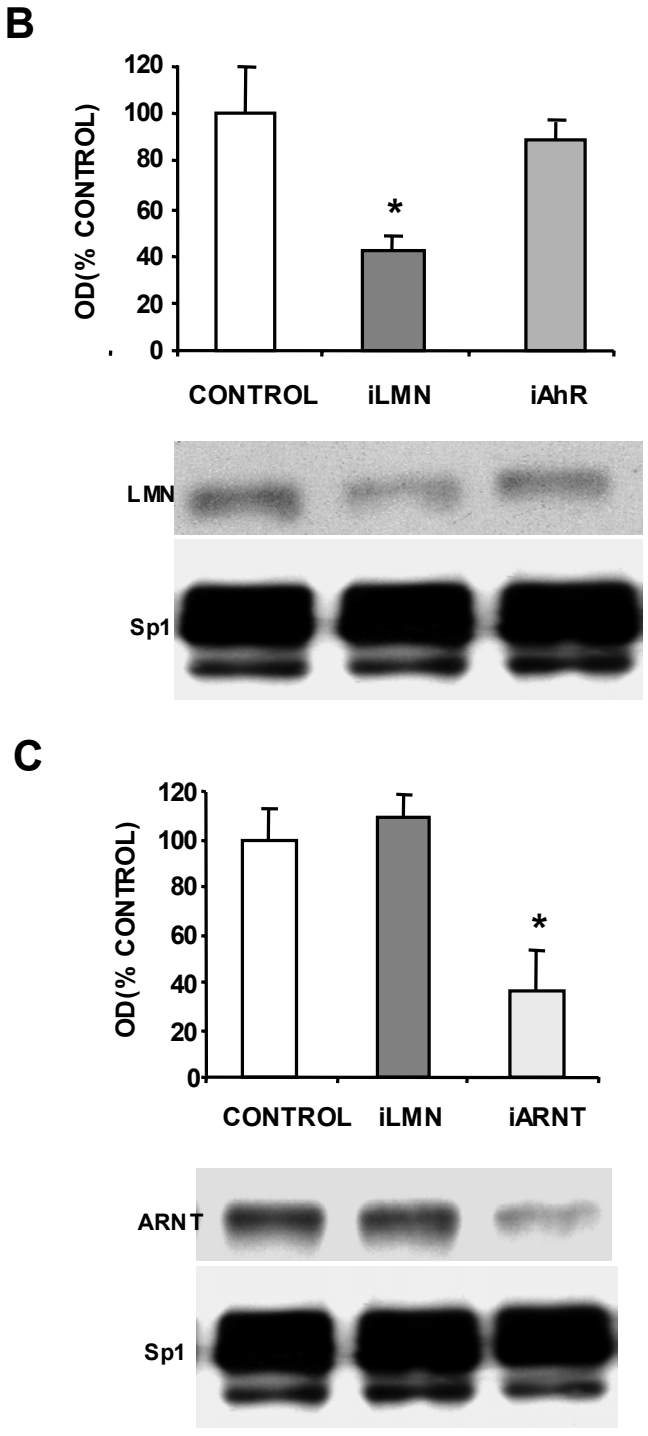


Fig. 25. Continued.

mobility shift assays (Fig. 26). In control cells and cells treated with siRNA for lamin A, a weak complex was observed after treatment with Me_2SO (lanes 2 and 4), and an intense retarded band was observed using nuclear extracts from cells treated with 10 nM TCDD (lanes 3, 5 and 9). In extracts from cells treated with siRNAs for AhR or ARNT, there was a marked decrease in retarded band intensities in extracts from Me_2SO- (lanes 6 and 10) and TCDD- (lanes 7 and 11) treated cells. The specifically-bound complex was decreased after incubation with unlabeled DRE (lane 8) and supershifted with AhR antibodies (lane 19). These results complement Western blot analyses of whole cell lysates (Figs. 25A – 25C) showing that siRNAs for AhR and ARNT decrease expression of their corresponding proteins in MCF-7 cells.

TCDD induces CYP1A1 mRNA and protein levels in multiple cells/tissues (363) including MCF-7 cells as indicated in Figure 27A. In cells treated with siRNA for lamin A, there was a slight decrease in CYP1A1 protein levels in the control and TCDD-induced response; however, after treatment with siRNA for AhR, CYP1A1 protein levels induced by TCDD were decreased by > 65%. In a separate experiment, the effects of siRNAs for lamin A and AhR on induction of CYP1A1-dependent EROD activity also showed that only siRNA for the AhR decreased induction of EROD activity by TCDD (Fig 27B). A complementary study used an Ah-responsive construct containing three tandem consensus DREs linked to a luciferase reporter gene.

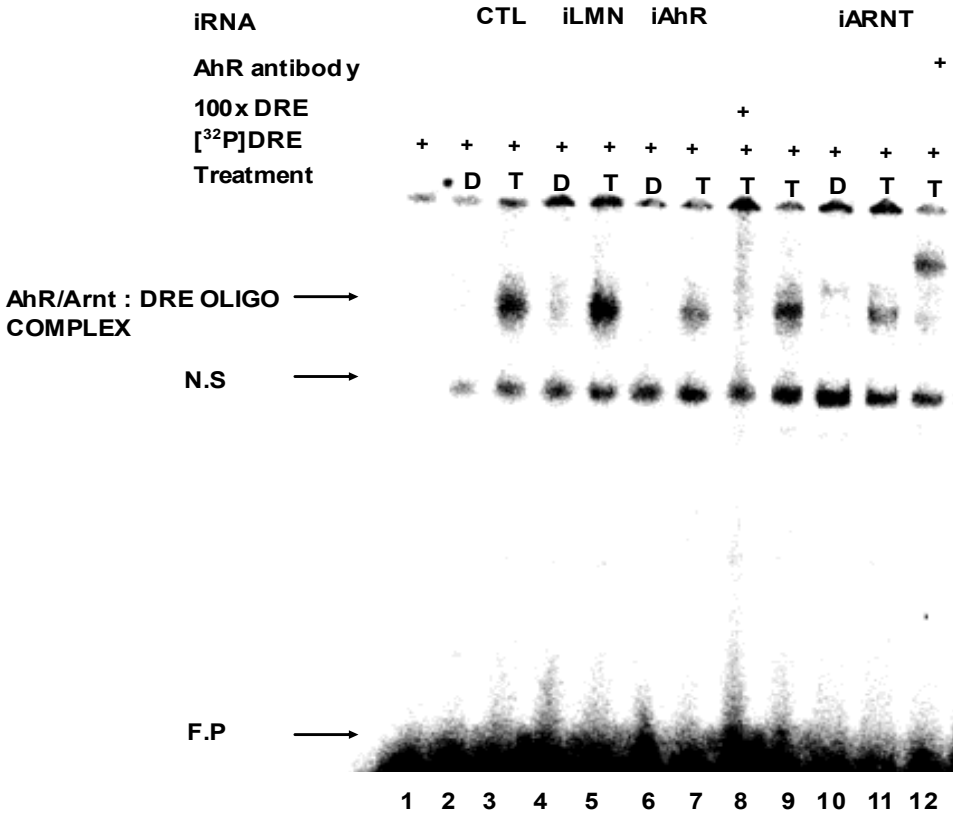


Fig. 26. **Binding of [^{32}P]DRE with nuclear extracts from breast cancer cells treated with iLMN, iAhR or iARNT.** MCF-7 cells were treated with Me_2SO (D) or TCDD (T) and transfected with C, iAhR, iARNT or iLMN, and binding of nuclear extracts to [^{32}P]DRE was determined in gel mobility shift assays as described in the Materials and Methods. Only iAhR or iARNT decreased intensity of the specifically bound AhR:ARNT-DRE complex (see arrow). Similar results were observed in duplicate experiments.

The results (Fig. 27C) showed that siRNAs for AhR and Arnt inhibited induction of luciferase activity by TCDD (compared to control cells), whereas siRNA for lamin A did not affect Ah-responsiveness. As a positive control, siRNA for GL2 (luciferase) inhibited luciferase activity in cells treated with solvent or TCDD.

Previous studies have demonstrated that TCDD and related AhR agonists inhibit expression of E2-induced genes and proliferation of ER-positive breast cancer cells (336,396,398,399,601,602). The results in Figure 28 summarize FACS analysis of the effects of Me_2SO (solvent control), E2, TCDD and their combination on MCF-7 cell cycle progression where interactions between the AhR- and ERα-mediated pathways are primarily directed at changes in the percentage of cells in G_0/G_1 and S phases. E2 induced a >11% increase in MCF-7 cells in S phase (compared to Me_2SO), whereas TCDD alone decreased the percentage of cells in S-phase and inhibited E2-induced $G_0/G_1 \rightarrow$ S phase progression. In solvent (Me_2SO)-treated MCF-7 cells transfected with siRNA for AhR, there was a >4.5% increase in cells in S phase compared to cells treated with Me_2SO alone (Fig. 27A). These results suggest that in the absence of exogenous ligand, the AhR inhibits $G_0/G_1 \rightarrow$ S phase progression of MCF-7 cells. E2 induced a >18% increase in cells in S phase in cells transfected with siRNA for the AhR and this was only decreased by 5% in cells cotreated with E2 plus TCDD.

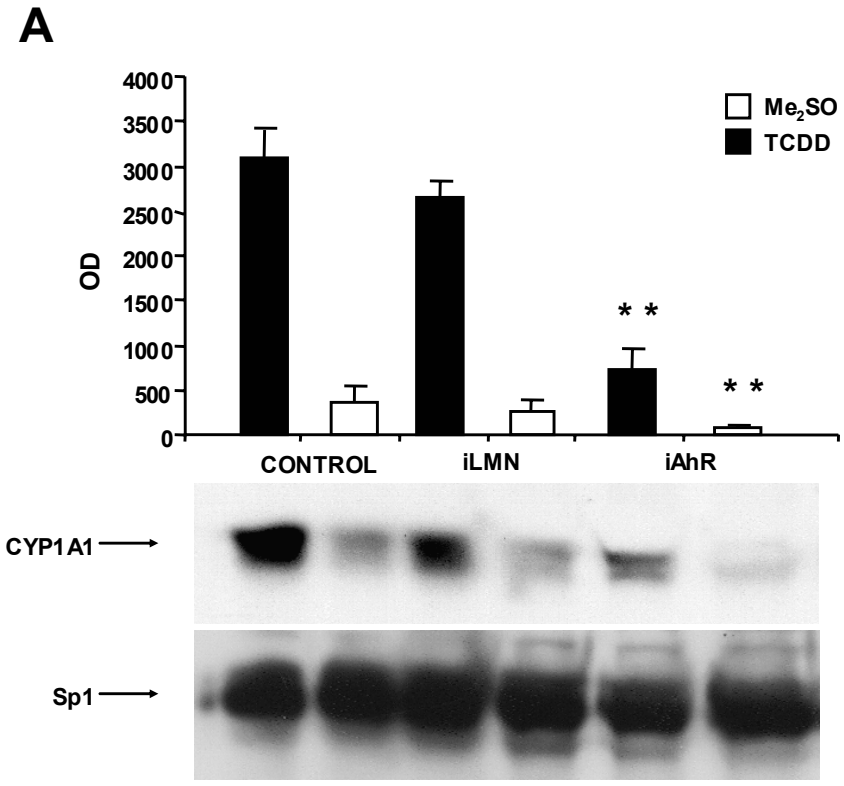
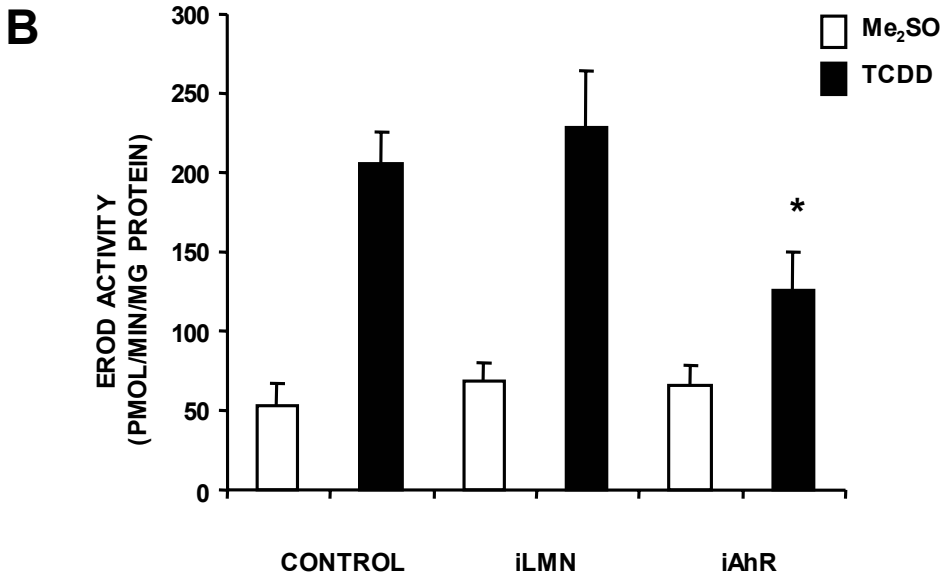


Fig. 27. **siRNA for the AhR (iAhR) inhibit TCDD-induced transactivation.** [A] CYP1A1 protein. Cells were transfected with iLMN or iAhR, treated with Me_2SO or 20 nM TCDD, and CYP1A1 protein was determined by Western blot analysis as described in the Materials and Methods. Significant ($p < 0.05$) decreases in activity compared to control cells are indicated with an asterisk. [B] EROD activity. The treatment groups were comparable to those outlined in Figure 3A, and EROD activity was determined as described in the Materials and Methods. Significant ($p < 0.05$) decreases in activity compared to control cells are indicated with an asterisk. [C] Luciferase activity. The treatment groups included those described in Figure 3A except that scrambled iRNA was used as control and iGL2 was also transfected. Cells were transfected with $pDRE_3$ and luciferase activity determined as described in the Materials and Methods. Significantly ($p < 0.05$) decreased activity compared to control cells is indicated with an asterisk. Results are exprssed as means $\pm$ SD for at least three replicate experiments for each treatment group.



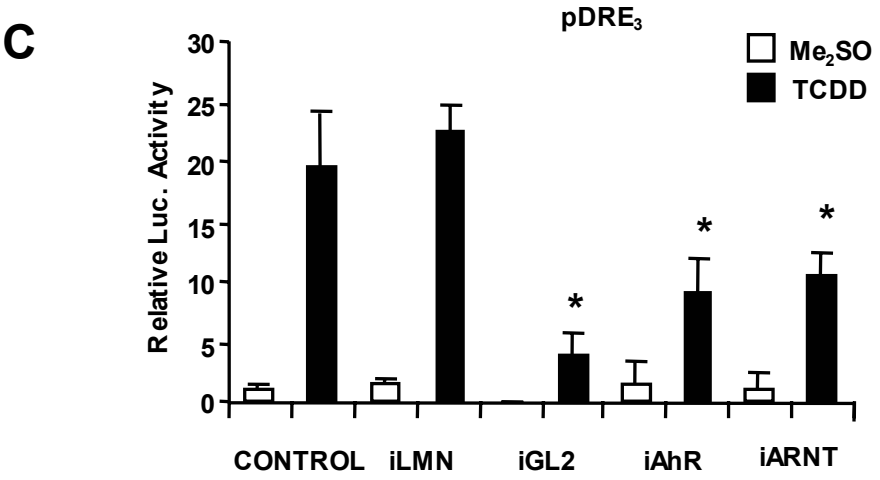


Fig. 27. Continued.

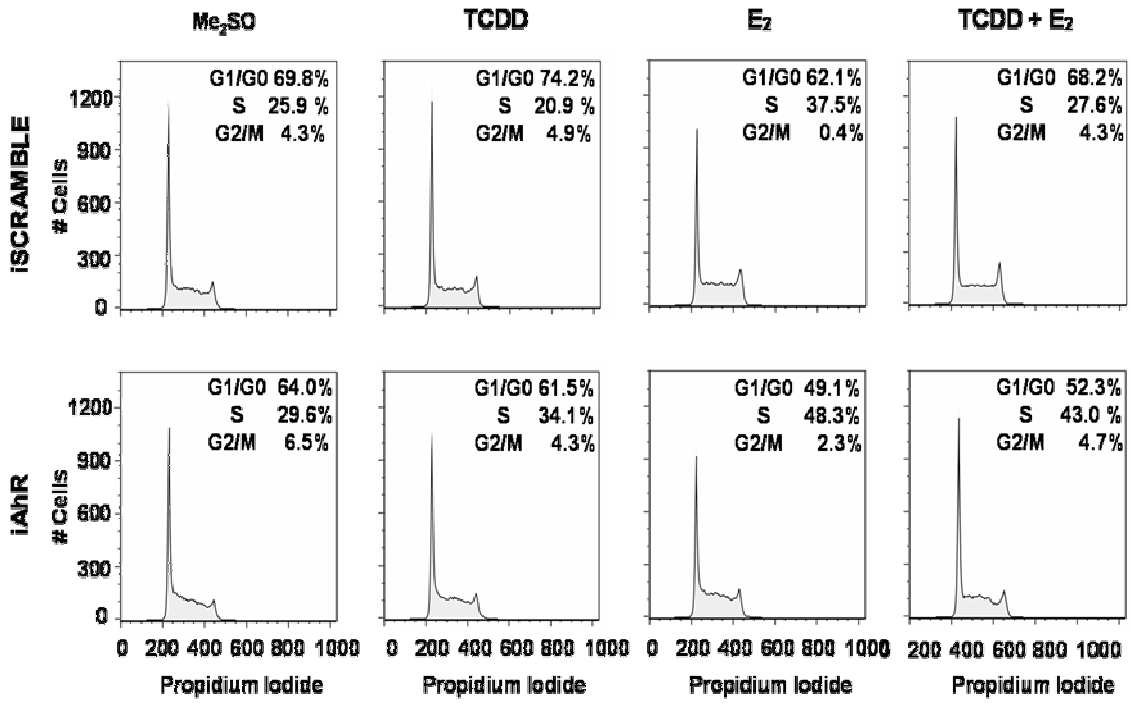


Fig. 28. **Effects of siRNA for the AhR on E2-, TCDD- and E2+TCDD-induced cell cycle progression in MCF-7 cells.** Serum-starved MCF-7 cells were treated with Me_2SO, 20 nM E2, 20 nM TCDD or TCDD+E2, transfected with scrambled RNA or iAhR, and the % distribution of cells in G_1/G_0, S and G_2/M were determined by FACS analysis as described in the Materials and Methods. Similar results were observed in a duplicate analysis.

These results confirm that the AhR is required for activation of growth inhibitory AhR-ERα crosstalk by TCDD (602). A comparison of the effects of TCDD alone in MCF-7 cells and in AhR-depleted cells treated with siRNA for the AhR indicates that TCDD induces AhR-independent $G_0/G_1 \rightarrow S$ phase progression and exhibits estrogen-like mitogenic activity. Therefore, the estrogenic activity of TCDD was further investigated in MCF-7 cells cotransfected with a construct containing three tandem EREs ($pERE_3$) and siRNAs for lamin A, luciferase and the AhR (Fig. 29). E2 induced luciferase activity in cells transfected with siRNA for lamin A or the AhR, and minimal activity was observed in cells transfected with siRNA for luciferase (iGL2). In wild-type Ah-responsive MCF-7 cells transfected with siRNA for lamin A, TCDD slightly decreased luciferase activity as previously observed using other E2-responsive constructs in MCF-7 cells (336,396,398,399). In contrast, TCDD significantly increased luciferase activity in cells cotransfected with $pERE_3$ and siRNA for the AhR, and this complemented the mitogenic activity of TCDD in these same AhR-deficient cells (Fig. 28). The estrogenic activity of TCDD (and E2) in AhR-deficient cells was inhibited after cotreatment with the antiestrogen ICI 182,780 (Fig. 29B); minimal interactions (TCDD plus ICI 182,780) were observed in cells transfected with siRNA for lamin A. Moore and coworkers (597) previously developed AhR-defective MCF-7 cells which express ARNT but low to non-detectable AhR protein.

Inhibitory AhR-ERα crosstalk was also not observed in this cell line, and treatment of these cells with TCDD caused an increase in cell growth and significantly induced reporter gene activity in cells transfected with an E2-responsive construct containing the ERE from the vitellogenin A2 gene promoter.

The growth inhibitory role of the endogenous AhR was in contrast to studies in AhR-deficient rodent liver cancer cells where AhR expression was associated with enhanced cell proliferation (588,590). The results in Figure 30A demonstrate that siRNAs for AhR and ARNT decrease expression of their respective proteins in Ah-responsive human HepG2 liver cancer cells, and induction of luciferase by TCDD in cells transfected with $pDRE_3$ was also decreased in cells cotransfected with the same siRNAs (Fig. 30B). siRNA for lamin A served as a control for these transfection studies and this oligonucleotide did not affect levels of AhR/ARNT protein or luciferase inducibility by TCDD. Similar results were observed using scrambled siRNA. FACS analysis of HepG2 cells transfected with scrambled siRNA or siRNA for the AhR (Fig. 30C) indicated that in AhR-deficient HepG2 cells, there was an 8% decrease in cells in S phase and a comparable increase in G_0/G_1. These results suggest that in HepG2 cells, the endogenous AhR enhances cell cycle progression as previously reported in rodent cancer cell lines (588,590).

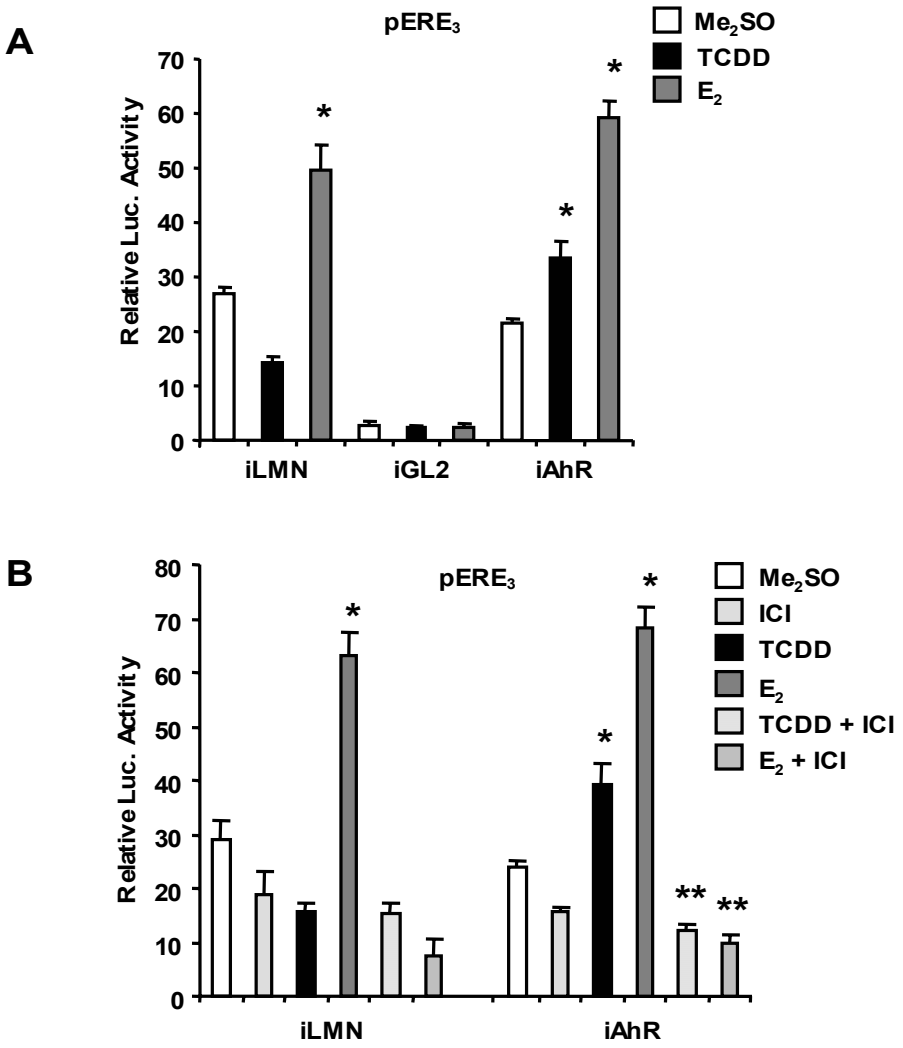


Fig. 29. **Effects of iLMN, iAhR and iGL2 on luciferase activity in MCF-7 cells transfected with pERE$_3$ and treated with Me$_2$SO, 30 nM E2, 30 nM TCDD, 1 μM ICI 182,780, or their combination.** [A] Effects of iAhR on induced luciferase activity. MCF-7 cells were cotransfected with pERE$_3$ along with iLMN, iGL2 or iAhR, and treated with Me$_2$SO, TCDD or E2. Luciferase activity was determined as described in the Materials and Methods and significant ($p < 0.05$) induction is indicated with an asterisk. [B] Antiestrogen inhibition of TCDD- and E2-induced transactivation. Cells were transfected with pERE$_3$ and iLMN or iAhR, treated with Me$_2$SO, E2, TCDD, ICI 182,780 or combinations, and luciferase activity was determined as described in the Materials and Methods. Significant ($p < 0.05$) induction is indicated with an asterisk and significant inhibition by ICI 182,780 is also indicated (**). Results summarized in [A] and [B] are means ± SD for 3 replicate determinations for each treatment group.

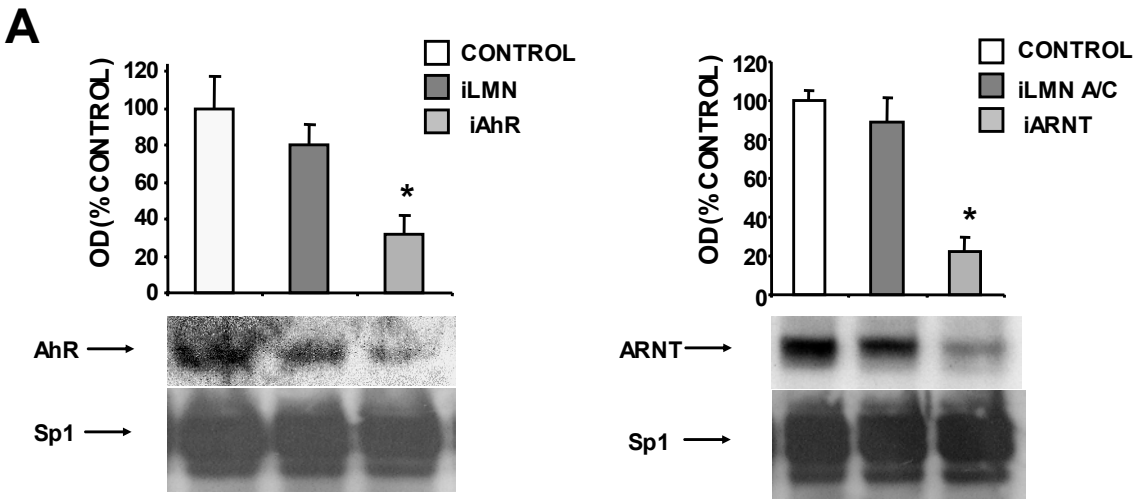
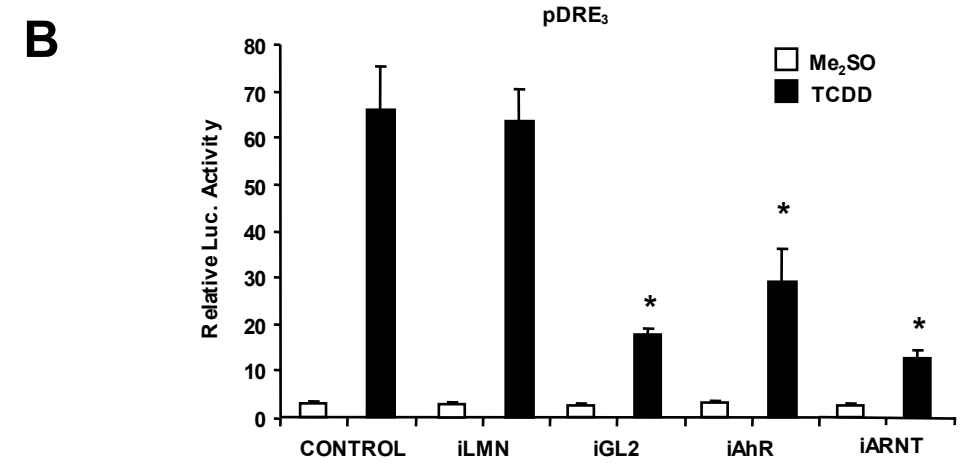


Fig. 30. **siRNAs for the AhR or Arnt decrease protein expression, TCDD-induced transactivation, and affect cell cycle progression in human HepG2 cells.** [A] Decreased protein expression. HepG2 cells were transfected with siRNA for AhR (iAhR), lamin A (iLMN), or ARNT (iARNT) and AhR, ARNT or Sp1 proteins were determined in the various treatment groups by Western blot analysis. [B] Decreased Ah-responsiveness. Cells were cotransfected with $pDRE_3$ and scrambled RNA (control), iLMN, iGL2, iAhR, or iARNT, treated with Me_2SO or 20 nM TCDD, and luciferase activity determined as described in the Materials and Methods. Results summarized in [A] and [B] are means $\pm$ SD for three replicate detemrinations for each treatment group, and significant ($p < 0.05$) decreases in activity are indicated by an asterisk. [C] Effects of siRNA for the AhR on cell cycle progression of HepG2 cells. HepG2 cells were transfected with scrambled RNA or iAhR, and the % distribution of cells in G_0/G_1, S and G_2/M were determined by FACS analysis as described in the Materials and Methods. Similar results were observed in a duplicate analysis.



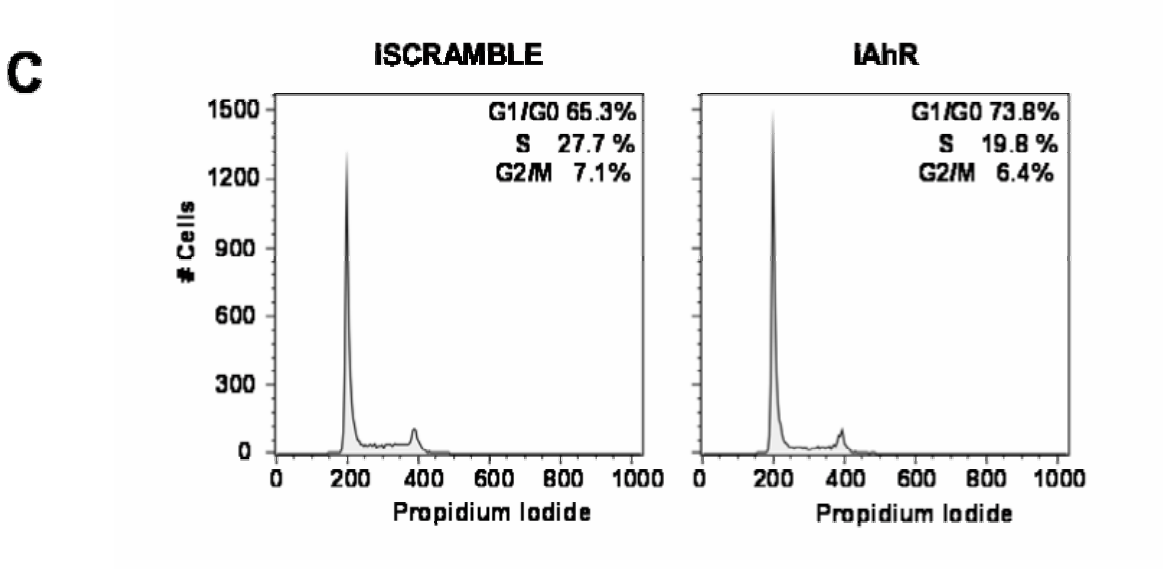


Fig. 30. Continued.

In contrast, in breast cancer cells (Fig. 28), the AhR is growth inhibitory and this demonstrates the importance of cell context on the role for the endogenous AhR in Ah-responsive breast and liver cancer cell lines.

Since decreased AhR expression in MCF-7 and HepG2 cells affected $G_1 \rightarrow S$ phase progression in both cell lines, we further investigated modulation of several key cell cycle regulatory proteins that are important in this phase of the cell cycle. The results in Figure 31 show that in HepG2 cells transfected with siRNA for the AhR, there were significant decreases in cyclin D1, cyclin E, cdk2 and cdk4 protein expression, whereas no significant changes in Rb or p27 proteins were observed. Immunoblot analysis showed low to non-detectable levels of p21 protein in HepG2 (and MCF-7) cells. Thus, decreased proliferation of HepG2 cells transfected with siRNA AhR is consistent with decreased expression of several proteins required for $G_1 \rightarrow S$ phase progression. In contrast, expression of these same proteins was unchanged in MCF-7 cells transfected with siRNA for the AhR. This suggests that other genes/proteins associated with increased proliferation of AhR-deficient MCF-7 cells must be affected, and these are currently being investigated.

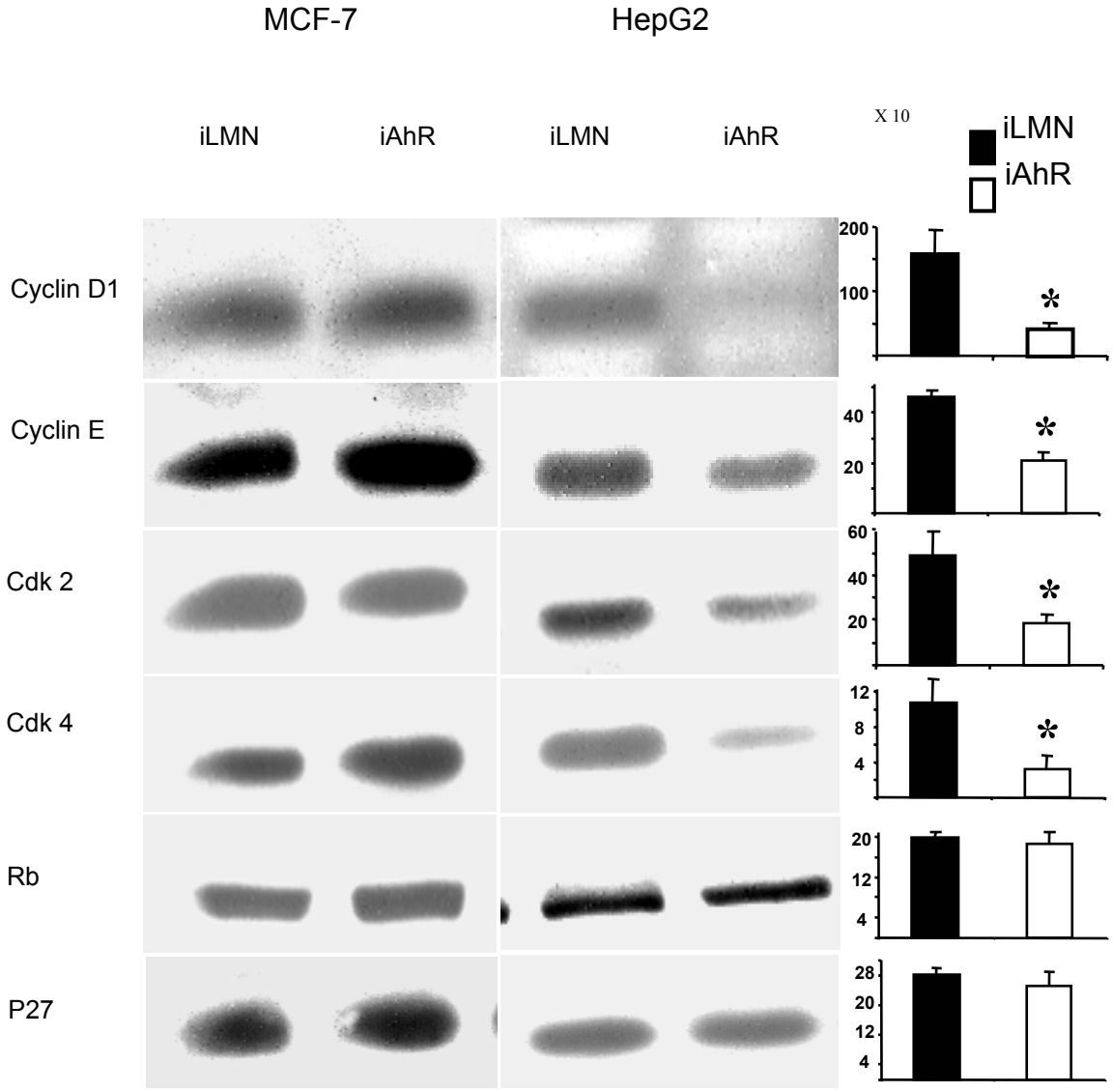


Fig. 31. **Effects of AhR gene silencing on cell cycle enzymes in MCF-7 and HepG2 cells**. MCF-7 or HepG2 cells were transfected with siRNA for lamin A (iLMN) or AhR (iAhR) as described for the FACS analysis experiments (Fig. 4 and Fig. 6C), and levels of cyclin D1, cyclin E, cdk2, cdk4, Rb and p27 proteins were determined in whole cell lysates by Western blot analysis as described in the Materials and Methods. Determinations were carried out in triplicate and, in HepG2 cells, relative protein levels in AhR-depleted cells compared to cells treated with iLMN are presented as means ± SD. Significant ($p < 0.05$) decreases in protein levels are indicated by an asterisk. Minimal to non-detectable p21 protein was detected in all groups (data not shown).

DISCUSSION

The development of transgenic animal models in which specific gene(s) have been ablated, overexpressed or conditionally expressed has provided unique insights into their physiological significance and roles in various diseases including cancer. Analogous approaches have been used for studies in mammalian cells using transiently or stably-transfected expression plasmids for specific genes or their antisense/dominant negative counterparts. RNA interference associated with double-stranded RNA which is rapidly processed into siRNAs has been identified in many eukaryotes (575,576,578). Recent studies have demonstrated that siRNA oligonucleotides can be successfully used for gene silencing in mammalian cells (67,89,92,96,603-605). Initial applications of this technique by Elbashir and coworkers (67) in HeLa, NIH3T3, COS-7 and 293 cells and subsequent studies in several different mammalian cell lines have demonstrated that gene silencing can target multiple genes and this approach has numerous applications (604). For example, research in this laboratory (603) has shown that siRNA for Sp1 decreases Sp1 protein expression in MCF-7 cells and also blocks E2-dependent transactivation of a GC-rich construct through interactions of ERα/Sp1. Moreover, silencing of Sp1 inhibited hormone-induced cell cycle progression of MCF-7 cells showing that ERα/Sp1-mediated genes play an important role in the growth of breast cancer cells.

In this study, we have successfully used siRNA for AhR to decrease AhR protein expression in MCF-7 cells, and siRNAs for lamin A and ARNT also silence their corresponding genes resulting in 60-80% decreased expression of their corresponding proteins (Figs. 25 and 26). AhR-mediated induction of CYP1A1 has been extensively investigated as a model for understanding the molecular mechanisms of AhR action (363), and the results in Figure 27 demonstrate siRNA for the AhR blocks induction of CYP1A1 protein, EROD activity and DRE-dependent reporter gene activity and siRNA for ARNT gave similar results in some of the assays. These data confirm the role of AhR:ARNT in mediating the induction of CYP1A1 and also illustrate that the siRNA approach can be used for targeting the AhR and ARNT.

Several studies report that TCDD inhibits E2-induced gene/reporter gene activity in breast cancer cells and inhibitory AhR-ERα crosstalk is also observed for cell proliferation and cell cycle progression (336,396-399,601,602). Treatment of MCF-7 cells with E2 significantly enhances G_0/G_1. Treatment of MCF-7 cells with E2 significantly enhances $G_0/G_1 \rightarrow$ S phase progression of ER-positive breast cancer cells, and this response is inhibited after cotreatment with TCDD (602). The results summarized in Figure 28 also show that E2 and E2 + TCDD primarily act on the $G_0/G_1 \rightarrow$ S phase of the cell cycle and that TCDD alone is growth inhibitory as previously reported (602). Not unexpectedly, in cells transfected with siRNA for the AhR, the inhibitory effects of TCDD on E2-induced $G_0/G_1 \rightarrow$ S phase progression were dramatically decreased as

demonstrated by FACS analysis of the whole cells (transfected plus untransfected). Moreover, in cells transfected with siRNA for the AhR and treated with Me_2SO (solvent), there was an increase in the percentage of cells in S phase and this was also observed in all the treatment groups in the AhR-depleted cells. These results show that in the absence of TCDD, the AhR is growth inhibitory in MCF-7 cells and this constitutes a function for the endogenous AhR in this cell line. Expression of several proteins required for $G_1 \rightarrow$ S phase progression were investigated in AhR-deficient MCF-7 cells (Fig. 31); significant changes in levels of cyclin D1, cyclin E, cdk2, cdk4, Rb or p27 (or p21) proteins were not observed. Currently, we are using microarrays to identify specific AhR-regulated genes that play a role in inhibiting breast cancer cell growth.

Phenotypic changes observed in AhR knockout mice suggest that the AhR complex exhibits exogenous ligand-independent activity as a transcription factor, and this is supported by other reports including studies in cell lines with defective or mutated AhR expression (588,590). Ma and Whitlock (588) showed that AhR-defected mouse Hepa 1 cells exhibited a different morphology, longer doubling times and a higher % of cells in G_0/G_1 compared to wild-type (AhR-positive) cells. Similar results were reported for AhR-defective rat hepatoma 5L cells which also exhibit an increased percentage of cells in G_0/G_1 compared to wild-type Ah-responsive cells (590). The cell context-dependent differences in endogenous AhR function in human breast cancer (growth inhibitory) versus

rodent liver cancer (growth promoting) cells was confirmed in this study using a human hepatoma cell line (HepG2) where siRNA for the decreased AhR protein expression, increased the % cells in G_0/G_1 and decreased cells in S phase (Fig. 30). The ligand-independent effects of the AhR in HepG2 cells was further investigated by determining expression of several proteins required for $G_1 \rightarrow S$ phase progression in HepG2 cells transfected with siRNA for the AhR (Fig. 31). In AhR-depleted HepG2 cells, there was significantly decreased expression of cyclin D1, cyclin E, cdk2 and cdk4, and this was consistent with the higher percentage of HepG2 cells in G_0/G_1 compared to cells expressing the AhR. These results suggest that these four genes/proteins may be regulated by the endogenous AhR in liver cancer cells, and the molecular mechanisms of ligand-independent AhR gene regulation are currently being investigated.

FACS analysis of AhR-deficient MCF-7 cells shows that treatment with TCDD resulted in a 14% decrease in cells in G_0/G_1 and a nearly comparable increase of cells in S phase. These data suggest that in AhR-deficient cells, TCDD exhibits mitogenic activity and, like E2, induces $G_0/G_1 \rightarrow S$ phase progression. The estrogen-like activity of TCDD was surprising; however, previous studies in AhR-deficient benzo[a]pyrene-resistant MCF-7 cells also showed that TCDD increased cell proliferation and reporter gene activity in cells transfected with an E2-responsive construct containing a cathepsin D gene promoter insert (597). The ER agonist activity of TCDD was confirmed in MCF-7 cells transfected with $pERE_3$ and siRNA for lamin (control) or AhR. In AhR-

deficient cells, both TCDD and E2 induced luciferase activity and these responses were inhibited by the antiestrogen ICI 182,780. Thus, TCDD activates both the AhR and ER in breast cancer cells; however, because of the high affinity of TCDD for the AhR, the ER agonist response is only observed in AhR-deficient cells. A previous report showed that indolo[3,2-b]carbazole, an acid catalyzed condensation product of the phytochemical indole-3-carbinol, was also an AhR and ER agonist in MCF-7 cells (606). Unlike TCDD, indolo[3,2-b]carbazole activated both pathways in Ah-responsive MCF-7 cells, and this may be due to the lower affinity of this compound for the AhR (591).

In summary, results of this study demonstrate that ligand-independent actions of the AhR on cell proliferation are dependent on cell context and both growth inhibitory (breast) and growth promoting (liver) functions can be observed in cancer cell lines. The results also demonstrate that TCDD exhibits estrogenic activity in AhR-deficient MCF-7 cells and it is possible that activation of ER signaling by TCDD may be the predominant response in cells with high ER/AhR protein ratios. Ongoing studies are focused on developing cancer cell lines that stably express specific siRNAs that can be used for investigating the function of other transcription factors.

Sp PROTEIN-DEPENDENT REGULATION OF VASCULAR ENDOTHELIAL GROETH FACTOR AND PROLIFERATION OF PANCREATIC CANCER CELLS

Pancreatic ductal adenocarcinoma (PDAC) is a major cause of cancer-related deaths in developed countries and it is estimated that in 2003, more than 30,000 new cases will be diagnosed in the United States (121).PDAC is a highly aggressive disease that invariably evades early diagnosis (415,417,607,608). The mean survival time for patients with metastatic disease is only 3 – 6 months, and the 1-year survival time for all pancreatic cancers cases is approximately 20-30% (121). Several factors are associated with increased risk for pancreatic cancer and these include chronic pancreatitis, prior gastric surgery, smoking, diabetes, exposure to certain classes of organic solvents, and radiation (412,418-420,426-429,609-616).

Heritable germline mutations in several genes are also associated with increased risks for pancreatic cancer (433,607,608,612,617,618). For example, Peutz-Jeghers, hereditary pancreatitis, familial atypical multiple melanoma (FAMM), familial breast cancer 2, and hereditary nonpolyposis colorectal cancer syndromes, which are linked to specific heritable gene mutations, markedly increase the risk for pancreatic cancer. Moreover, familial pancreatic cancer syndrome where there is at least one pair of first degree relatives also increases the risk for this disease. However, the gene(s) involved have not been

identified. In addition to heritable mutations, several acquired gene mutations have been identified in sporadic pancreatic tumors and typically these mutations lead to dysregulated growth and deficiencies in DNA repair (619-624). For example, the K-*ras* oncogene is primarily mutated in codon 12 in >90% of pancreatic tumors and the mutation results in a constitutively active form of *ras* which can lead to increased cell proliferation. Mutations in the cyclin-dependent kinase inhibitor *p16*, the tumor suppressor gene *p53*, and *SMAD4*, a downstream target of transforming growth factor β (TGFβ) also exhibit high mutation frequencies in pancreatic tumors (433,617).

Specificity protein 1 (Sp1) is expressed in pancreatic tumors and in pancreatic cells in culture, and there is evidence suggesting that Sp1 plays an important role in regulation of vascular endothelial growth factor (*VEGF*) expression in Panc-1 and other pancreatic cancer cells (297). Sp family proteins play a complex role in regulation of cancer cell growth and expression of genes required not only for growth but also apoptosis and angiogenesis (552). In this study, we used RNA interference to investigate the role of Sp proteins in *VEGF* expression and cell cycle progression. Our results show for the first time that Sp4 is expressed in pancreatic cancer cells and along with Sp1 and Sp3 plays an important role in regulating expression of VEGF. In contrast, Sp3 but not Sp1 or Sp4 was identified as a key regulator of $G_0/G_1 \rightarrow S$ phase progression and retinoblastoma (Rb) protein phosphorylation in Panc-1 cells, and this was linked to Sp3-dependent suppression of the cyclin-dependent kinase inhibitor *p27*.

MATERIALS AND METHODS

Cell lines, chemicals, biochemicals, constructs and oligonucleotides. Panc-1, HepG2, 22RV1, MCF7 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). DME/F12 with and without phenol red, 100× antibiotic/antimycotic solution, and propidium iodide were purchased from Sigma. Fetal bovine serum was purchased from Intergen (Purchase, NY). [γ-^{32}P]ATP (300Ci/mmol) was obtained from Perkin Elmer Life Sciences. Poly (dI-dC) and T4 polynucleotide kinase were purchased from Roche Molecular Biochemicals (Indianapolis, IN). Antibodies for Sp1, Sp3, Sp4, Rb, p27, cyclin D1, and cyclin E proteins were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Lysis buffer, luciferase reagent, and RNase were obtained from Promega Corp. (Madison, WI). Consensus GC-rich oligonucleotides and VEGF promoter constructs have previously been described (345,515). The consensus GT-box probe used in electrophoretic mobility shift assays (EMSA) was 5'-TCG AGA GGT GGG TGG AGT TTC GCG -3'. p27 ^{Kip1} promoter luciferase constructs p27 PF (-3568/-12), p27 No. 2 (-549/-12), and p27 Sac II (-311/-12) were kindly provided by Dr. Toshiyuki Sakai (Kyoto Prefectural University of Medicine, Japan). siRNA duplexes were prepared by Dharmacon Research (Lafayette, CO) and targeted coding regions of the Sp1 (1811-1833), Sp3 (1681-1701), Sp4 (1181-1201), lamin A/C (608 - 626), and luciferase (GL2) (153 - 171). Previous studies in this laboratory have reported

oligonucleotide sequences for Sp1, GL2 and lamin A/C siRNA (344,512,603) and the iRNA duplex for Sp3, Sp4 is given below.

Sp3 5' – GCGGCAGGUGGAGCCUUCACUTT
TTCGCCGUCCACCUCGGAAGUGA – 5'

Sp4 5' – GCAGUGACACAUUAGUGAGCTT
TTCGUCACUGUGUAAUCACUCG – 5'

Transfection of Panc- 1 cells and preparation of nuclear extracts. Cells were cultured in 6-well plates in 2 ml of DME/F12 medium supplemented with 5% fetal bovine serum. After 16-20 h when cells were 50-60% confluent, iRNA duplexes and/or reporter gene constructs were transfected using Oligofectamine Reagent (Invitrogen, Carlsbad, CA). The effects of iSp1, iSp3, and iSp4 on transactivation was investigated in Panc-1 cells cotransfected with (500 ng) different VEGF and p27 ^{Kip} constructs. Briefly, iRNA duplex was transfected in each well to give a final concentration of 50 nM. Cells were harvested 48-56 h and luciferase activity of lysates (relative to β-galactosidase activity) was determined (344,515,603). For EMSA assay, nuclear extracts were isolated as previously described (344,345,515,603). Aliquots were stored at -80 °C and used for gel shift assays.

Western immunoblot. Cells were washed once with PBS and collected by scraping in 200 µl of lysis buffer [50 mM HEPES, 0.5 M sodium chloride, 1.5 mM magnesium chloride, 1 mM EGTA, 10% (v/v) glycerol, 1% Triton X-100, and 5 µl/ml of Protease Inhibitor Cocktail (Sigma)]. Brain tissue was obtained from

B6C3F1C mice, washed with cold PBS and homogenized in 1x lysis buffer (Promega). The lysates from cells and brain tissues were incubated on ice for 1 h with intermittent vortexing followed by centrifugation at 40,000 *g* for 10 min at 4 °C. Equal amounts of protein from each treatment group were diluted with loading buffer, boiled, and loaded onto 10 and 12.5% SDS-polyacrylamide gel. Samples were electrophoresed and proteins were detected by incubation with polyclonal primary antibodies Sp1 (PEP2), Sp3 (D-20), Sp4 (V-20), lamin A/C (N-18), cyclin D1 (M-20), cyclin E (C-19), Rb (C-15), and p27 (C-19) followed by blotting with appropriate horseradish peroxidase-conjugated secondary antibody as previously described (344,515,603). After autoradiography, band intensities were determined by a scanning laser densitometer (Sharp Electronics Corporation, Mahwah, NJ) using Zero-D Scanalytics software (Scanalytics Corporation, Billerica, MA).

FACS analysis. Cells were transfected with iRNAs for Sp1, Sp3, Sp4 or GL2 and, after 48-56 h, cells were then trypsinized and ~2 × 10^6 cells were centrifuged and resuspended after removal of trypsin in 1 ml of staining solution containing 50 μg/ml propidium iodide, 4 mM sodium citrate, 30 units/ml RNase, and 0.1% Triton X-100, pH 7.8. Cells were incubated at 37 °C for 10 min, and then prior to FACS analysis, sodium chloride was added to give a final concentration of 0.15 M. Cells were analyzed as mention early in the previous section.

Electrophoretic Mobility Shift Assay (EMSA). Consensus Sp1 and GT-box oligonucleotides were synthesized and annealed, and 5-pmol aliquots were 5'-end-labeled using T4 kinase and [γ-^{32}P] ATP. A 30-µl EMSA reaction mixture contained ~100 mM KCl, 3 µg of crude nuclear protein, 1 µg poly (dI-dC), with or without unlabeled competitor oligonucleotide, and 10 fmol of radiolabeled probe. After incubation for 20 min on ice, antibodies against Sp1, Sp3 and/or Sp4 proteins were added and incubated another 20 min on ice. Protein-DNA complexes were resolved by 5% polyacrylamide gel electrophoresis as previously described (344,515,603). Specific DNA-protein and antibody-supershifted complexes were observed as retarded bands in the gel.

Immunocytochemistry. HepG2 and Panc-1 cells were seeded in Lab-Tek Chamber slides (Nalge Nunc International, Naperville, IL) at 100,000 cells/well in DME/F12 medium supplemented with 5% fetal bovine serum. Cells were then transfected with iRNAs, and after 48 h the media chamber was detached and the remaining glass slides were washed in Dulbecco's PBS. The immunostanning for Sp4 was determined essentially as previously described for Sp1 (603) and fluorescence imaging was performed using Carlzeiss Axiophoto 2 (Calzeiss, Inc., Thornwood, NY) and Adobe Photoshop 5.5 was used to capture the images.

Statistical analysis. Statistical significance was determined by analysis of variance and Scheffe's test, and the levels of probability are noted. The

results are expressed as means ± S.D. for at least three separate (replicate) experiments for each treatment.

RESULTS

Regulation of VEGF expression by Sp1 and Sp3. The contributions of Sp1 and Sp3 proteins on regulation of *VEGF* expression were investigated in Panc-1 pancreatic cancer cells that express high levels of VEGF (297). RNA interference was used to decrease expression of Sp1 or Sp3 in Panc-1 cells transfected with small inhibitory RNAs for lamin A/C (iLMN) (non-specific), Sp1 (iSp1) or Sp3 (iSp3) (344,515,603). The results show that transfected iSp1 oligonucleotide decreases (>50%) Sp1 protein in whole cell lysates and similar results were obtained using iSp3 (Fig. 32A). Both inhibitory RNAs were highly specific and did not affect other Sp proteins as previously reported (344,515,603). Gel mobility shift assays also confirmed that iSp1 and iSp3 decreased retarded bands associated with both proteins (Fig. 32B). The role of Sp1 and Sp3 in regulation of VEGF was investigated using a series of constructs containing different inserts from the *VEGF* gene promoter. The results (Fig. 32C and 1D) show that in Panc-1 cells transfected with pVEGF1, pVEGF5, pVEGF6 or pVEGF8 and cotransfected with iSp1 or iSp3, there was a decrease in luciferase activity that was dependent on the promoter insert. Significant inhibition of activity by iSp1 and iSp3 was observed for the 4 constructs; however, iSp1 was more effective using pVEGF1 which contains the -2018 to

+54 *VEGF* promoter insert. iSp3 was a more effective inhibitor using pVEGF5, pVEGF6 and pVEGF8 which contain -133 to +54, -67 to +54 and -66 to -47 *VEGF* promoter inserts, respectively. These data suggest a differential interaction of Sp1 and Sp3 with the *VEGF* promoter with preferential binding of Sp3 to the proximal GC-rich sites.

Sp4-dependent regulation of VEGF. Gel mobility shift assays (Fig. 32B) show that although Sp1 and Sp3 are bound to the consensus GC-rich oligonucleotide, supershift experiments with Sp1 and Sp3 antibodies show that some residual complex remains. Sp4 protein also binds GC/GT-rich oligonucleotides and is primarily expressed in the developing brain in the mouse with lower but detectable levels in many other tissues (273,297). Results in Figure 33A demonstrate that immunoreactive Sp4 protein can be detected in Panc-1 cells as well as brain tissue, MCF-7 breast, and 22Rv1 prostate human cancer cells. In contrast, Sp4 was not detected in the human HepG cancer cell line. Sp4 expression clearly activates pVEGF1 and pVEGF2 (Fig. 33B), and we have also observed activation of these VEGF constructs by Sp1 and Sp3 as previously reported (297).

Expression of Sp4 in Panc-1 cells was further investigated in gel mobility shift assays using nuclear extracts from Panc-1 cells and a ^{32}P-labeled GT-rich oligonucleotide (Fig. 33C, lanes 1 and 2, labeled oligonucleotide alone). The retarded band complex (lane 2) gave supershifted bands after coincubation with Sp1, Sp3 and Sp4 antibodies (lanes 3 - 5, respectively).

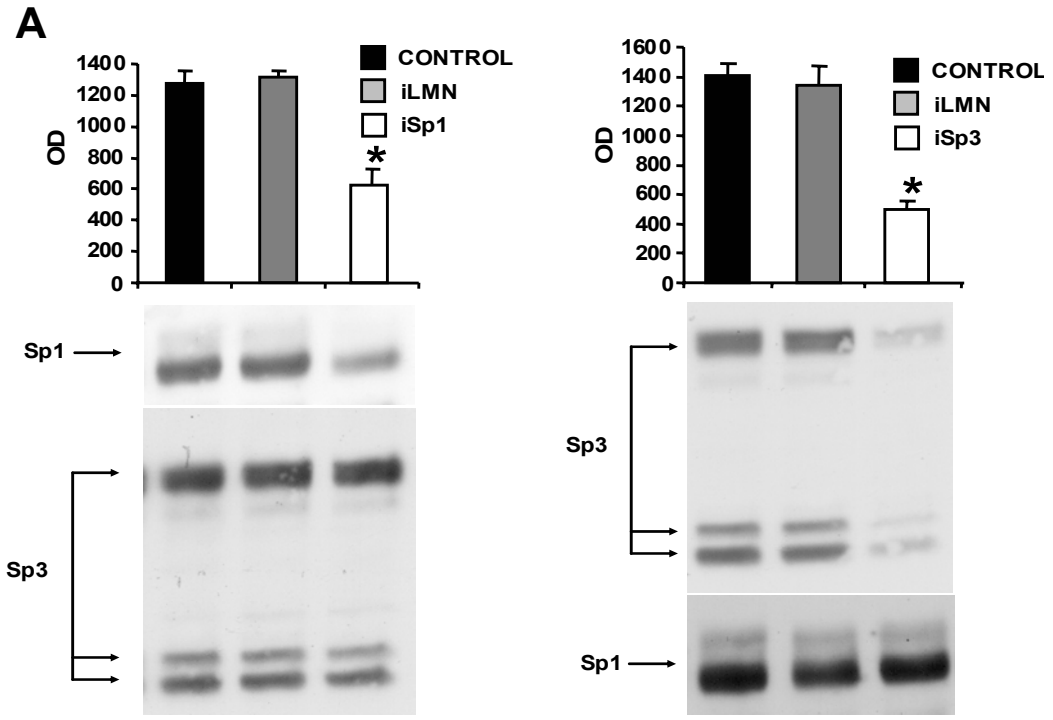


Fig. 32. **Activation of VEGF by Sp1 and Sp3 in Panc-1 cells.** [A] siRNAs for Sp1 and Sp3 downregulate their corresponding proteins in Panc-1 cells. Results are expressed as means ± SE for three separate experiments, and iSp1 and iSp3 significantly ($P < 0.05$, *) downregulated their corresponding proteins. [B] EMSA analysis of nuclear extracts from Panc-1 cells transfected with iLMN, iSp1 or iSp3. Specifically-bound complexes and supershifted complexes are indicated (arrows). [C and D] Transfection with iSp1, iSp3 or iLMN and various VEGF promoter constructs. Results are expressed as means ± SD for three separate experiments for each treatment group, and significant ($P < 0.05$, *) decreases in activity (compared to control) are indicated.

B

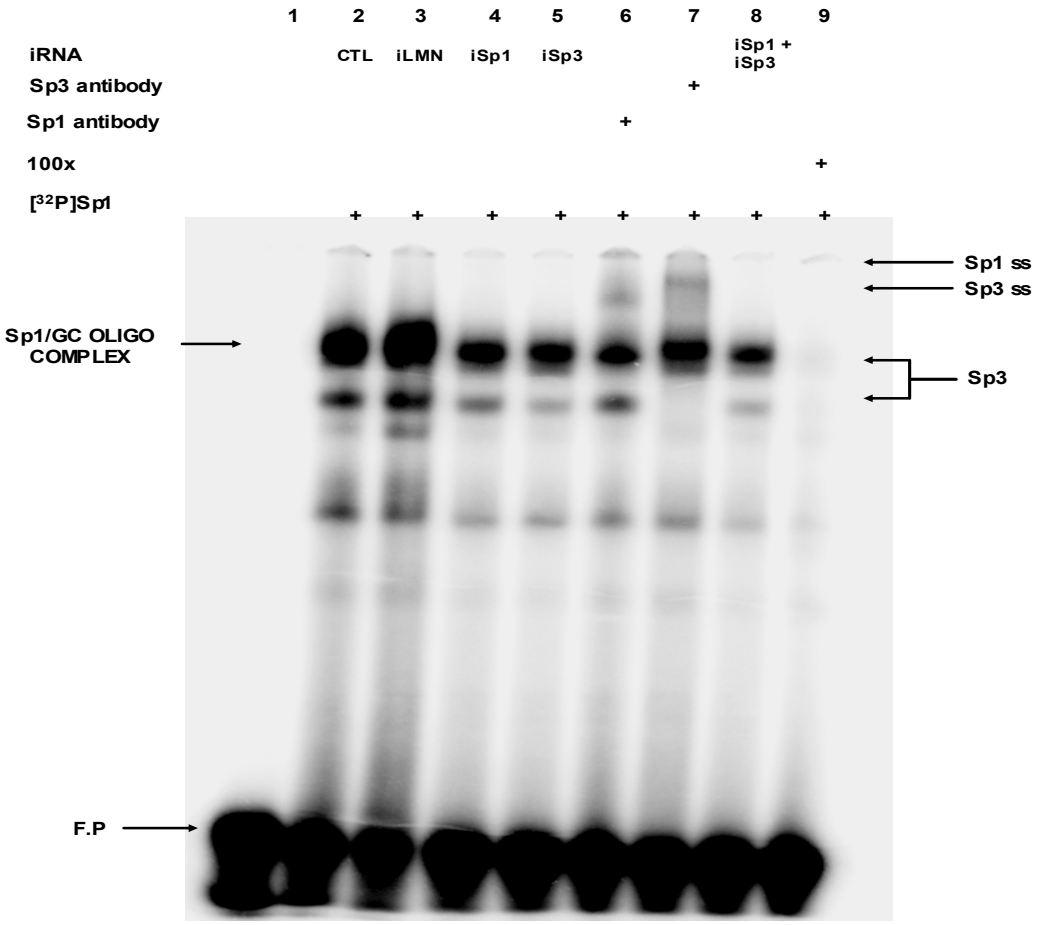


Fig. 32. Continued.

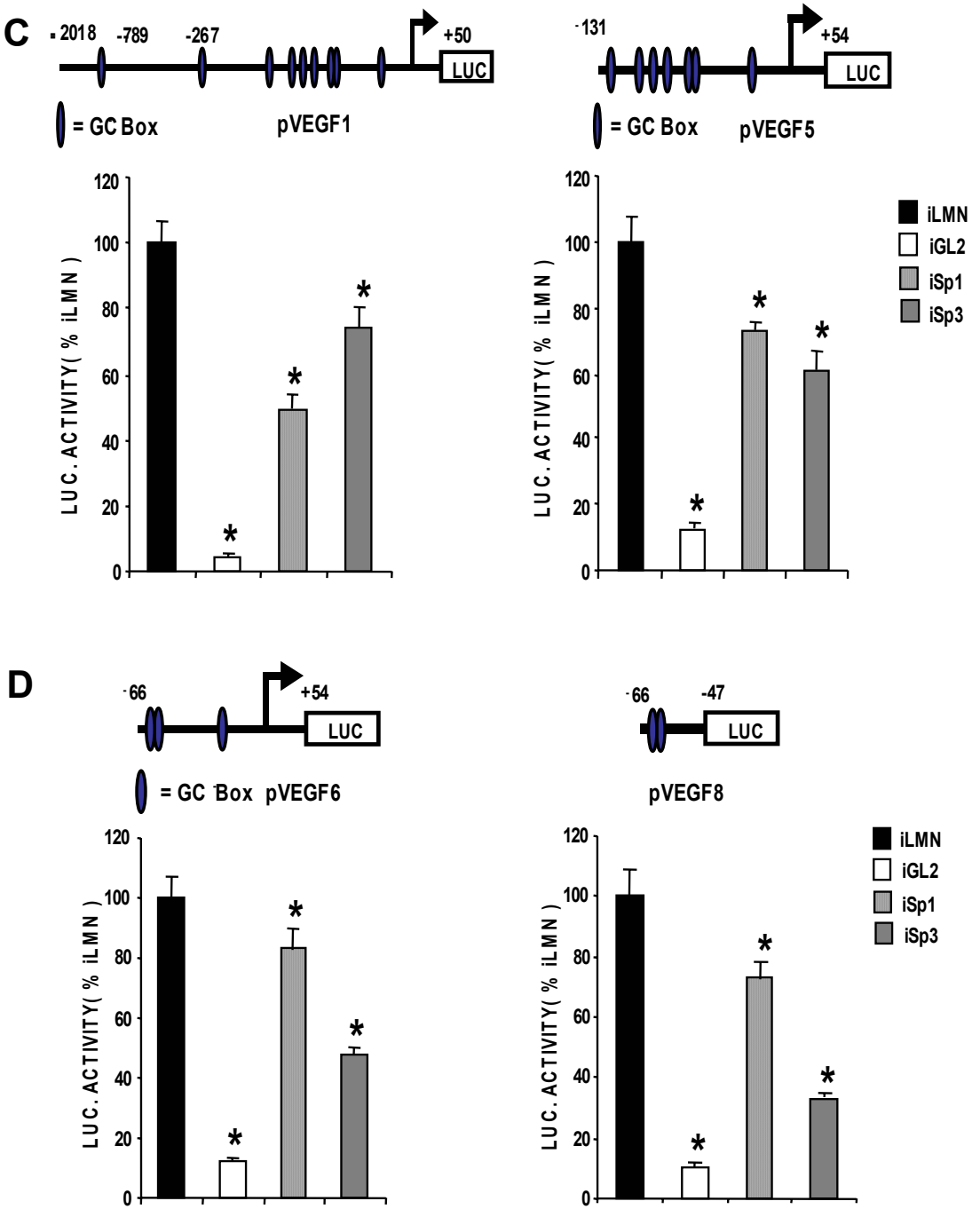


Fig. 32. Continued.

Incubation with Sp1 plus Sp3 antibodies did not completely supershift the major retarded band complex (lane 6); however, coincubation with Sp1, Sp3 plus Sp4 antibodies immunodepleted/supershifted the specifically bound bands (lane 7). Expression of Sp4 protein was also confirmed by immunostaining with Sp4 antibodies (Fig. 33D). Sp4 staining was observed in Panc-1 but not HepG2 cells. These results demonstrate that Panc-1 cells express Sp4 protein and the antibody supershift experiment (Fig. 33C) suggests that Sp1, Sp3 and Sp4 constitute the major Sp family proteins expressed in this cell line. Transfection of small inhibitory RNA for Sp4 (iSp4) specifically decreased immunoreactive Sp4 (but not Sp1) protein in Panc-1 cells. The effects of iSp4 on VEGF promoter constructs are summarized in Figure 2E. The results show that iSp4 inhibited (>50%) transactivation in Panc-1 cells transfected with pVEGF1, pVEGF5 and pVEGF6 suggesting that Sp4 also plays a major role in regulation of VEGF in this cell line.

Sp3 as a key regulator of cell cycle progression. Sp1 and other Sp proteins also regulate expression of multiple genes associated with cancer cell proliferation (552) and the effects of iSp1, iSp3 and iSp4 on growth of Panc-1 cells was determined by measuring retinoblastoma protein (Rb) phosphorylation as a downstream marker of cell growth (Fig. 34A). In cells transfected with iSp1 or iSp4, there were minimal changes in Rb phosphorylation compared to results in control cells (untreated) or cells transfected with iLMN.

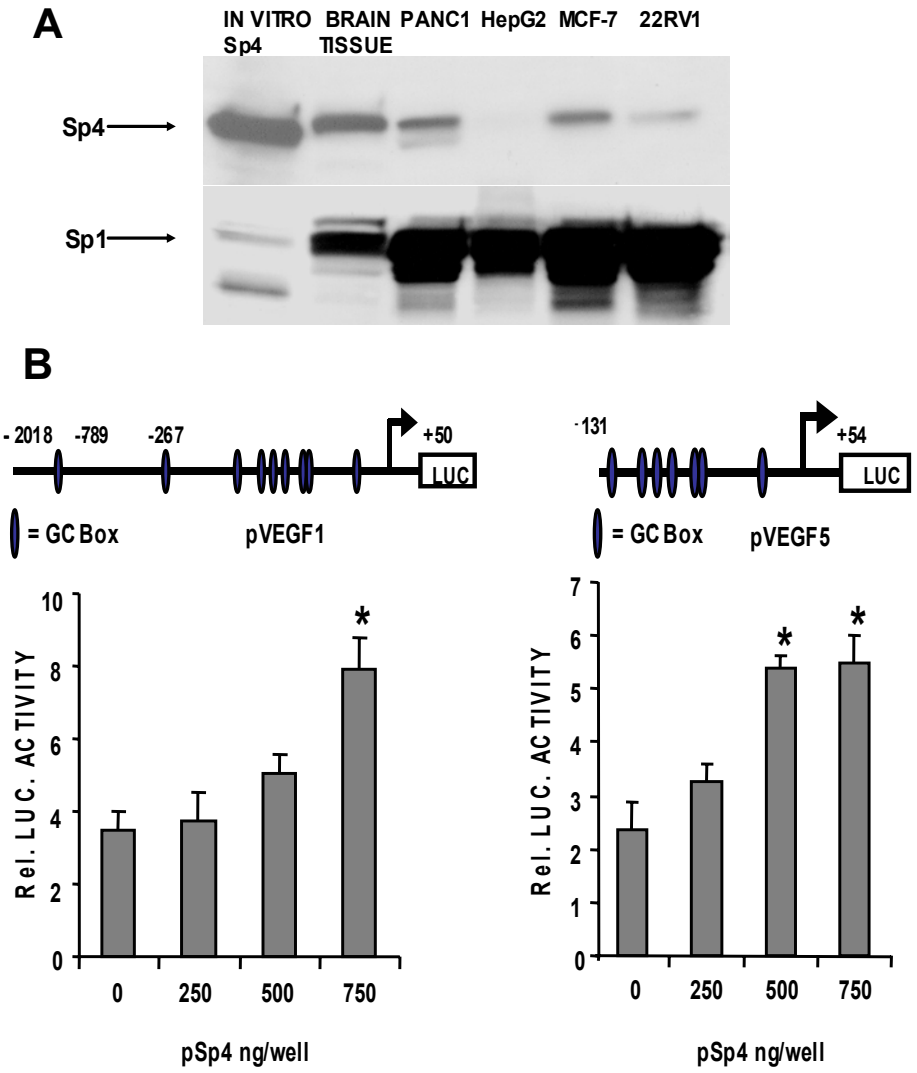


Fig. 33. **Sp4 expression in Panc-1 cells and regulation of VEGF.** [A] Western blots. Whole cell and mouse brain lysates were analyzed for Sp4 and Sp1 by Western immunoblot analysis. [B] Induction of VEGF promoter constructs by Sp4. Empty vector was used to ensure the same amount of DNA was transfected. Results are expressed as means ± SD for three replicate determinations for each treatment group, and significant ($P < 0.05$, *) induction is indicated. [C] EMSA assay. Gel mobility shifts were determined using ^{32}P-labeled GT-rich oligonucleotide, nuclear extracts from Panc-1 cells, and antibodies for Sp1, Sp3 and Sp4. [D] Immunostaining. Immunofluorescence was determined in HepG2 (a, b) and Panc-1 (c, d) cells stained with Sp4 primary antibody (b, d) or only with the secondary antibody (a, c). [E] Effect of iSp4 on VEGF expression. iSp4 significantly ($P < 0.05$, *) decreased Sp4 protein in whole cell lysates from Panc-1 cells. Results of transfection and Western blots are expressed as means ± SD for three replicate determinations for each treatment group, and significantly ($P < 0.05$, *) decreased reporter gene activity after cotransfection with iSp4 is indicated.

C

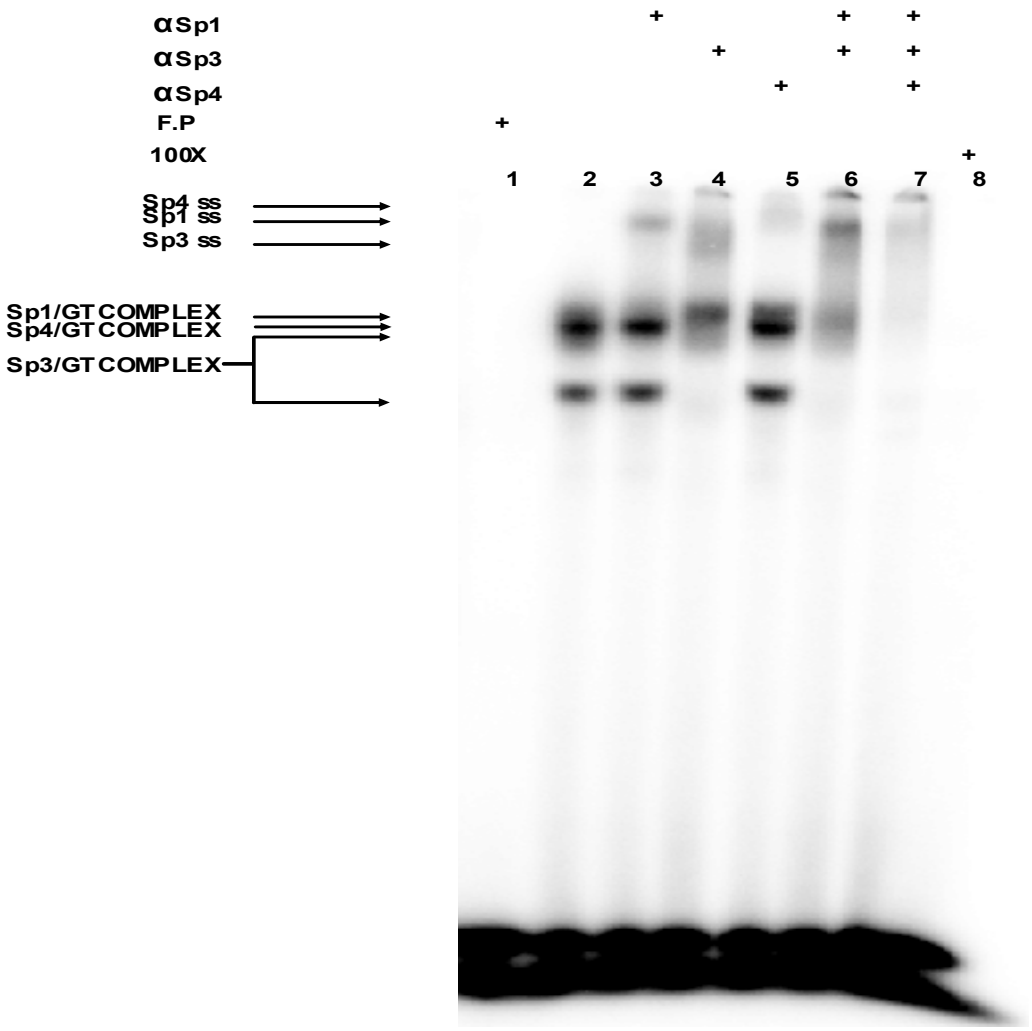


Fig. 33. Continued.

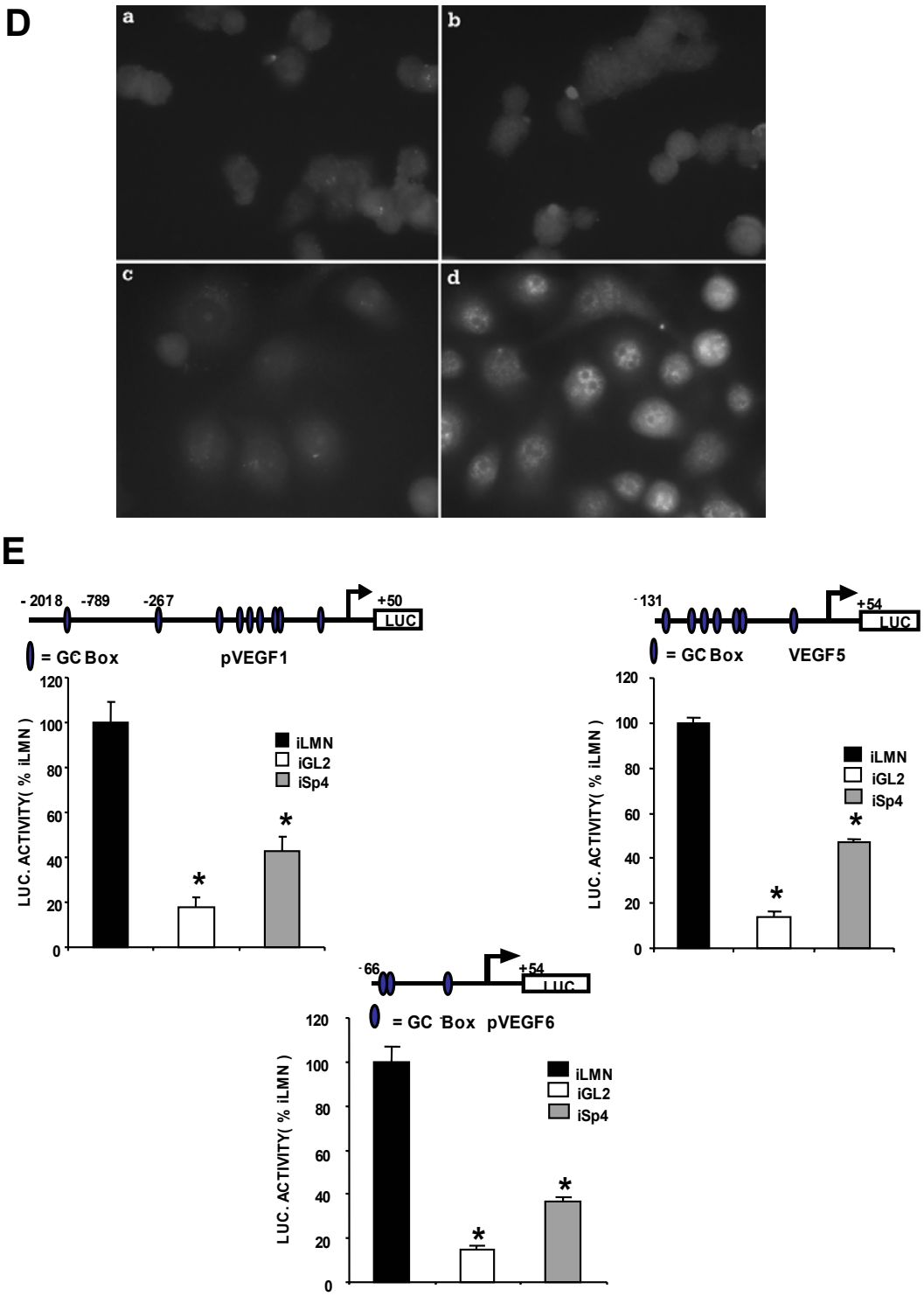


Fig. 33. Continued.

FACS analysis of cells transfected with iSp1 or iSp4 also showed that the % distribution of Panc-1 cells in G_0/G_1, S or G2/M phases of the cell cycle were comparable to those observed in control cells or cells transfected with nonspecific small inhibitory RNAs (iGL2). The effects observed for iSp3 clearly demonstrate that this specific Sp family protein plays an important role in Panc-1 cell growth. Transfection with iSp3 in Panc-1 cells markedly decreased Rb phosphorylation (Fig. 34B). Moreover, FACS analysis after transfection with iSp3 increased the percentage of cells in G_0/G_1 (62.96%) compared to control cells (42.86%) and decreased the percentage of cells in S-phase (15.58%) and G_2/M (21.45%) compared to control cells (32.97% and 24.18%, respectively). These data clearly show that iSp3 primarily inhibits $G_0/G_1 \rightarrow$ S phase progression and this is consistent with decreased Rb phosphorylation in Panc-1 cells transfected with iSp3 (Fig. 34A).

These results suggest that Sp3 plays a critical role in regulation of genes required for $G_0/G_1 \rightarrow$ S phase progression, and the identities of the target gene products were investigated by Western blot analysis of whole cell lysates from Panc-1 cells transfected with iSp3. The results demonstrate that loss of Sp3 protein did not affect levels of cyclin D1 or cyclin E protein; however, there was a dramatic increase of *p27* expression (Fig. 35A). Only minimal effects on p21 and other proteins involved in $G_0/G_1 \rightarrow$ S phase of the cell cycle were observed. The role of Sp3 on *p27* expression was further investigated using constructs containing *p27* promoter inserts linked to a luciferase reporter gene.

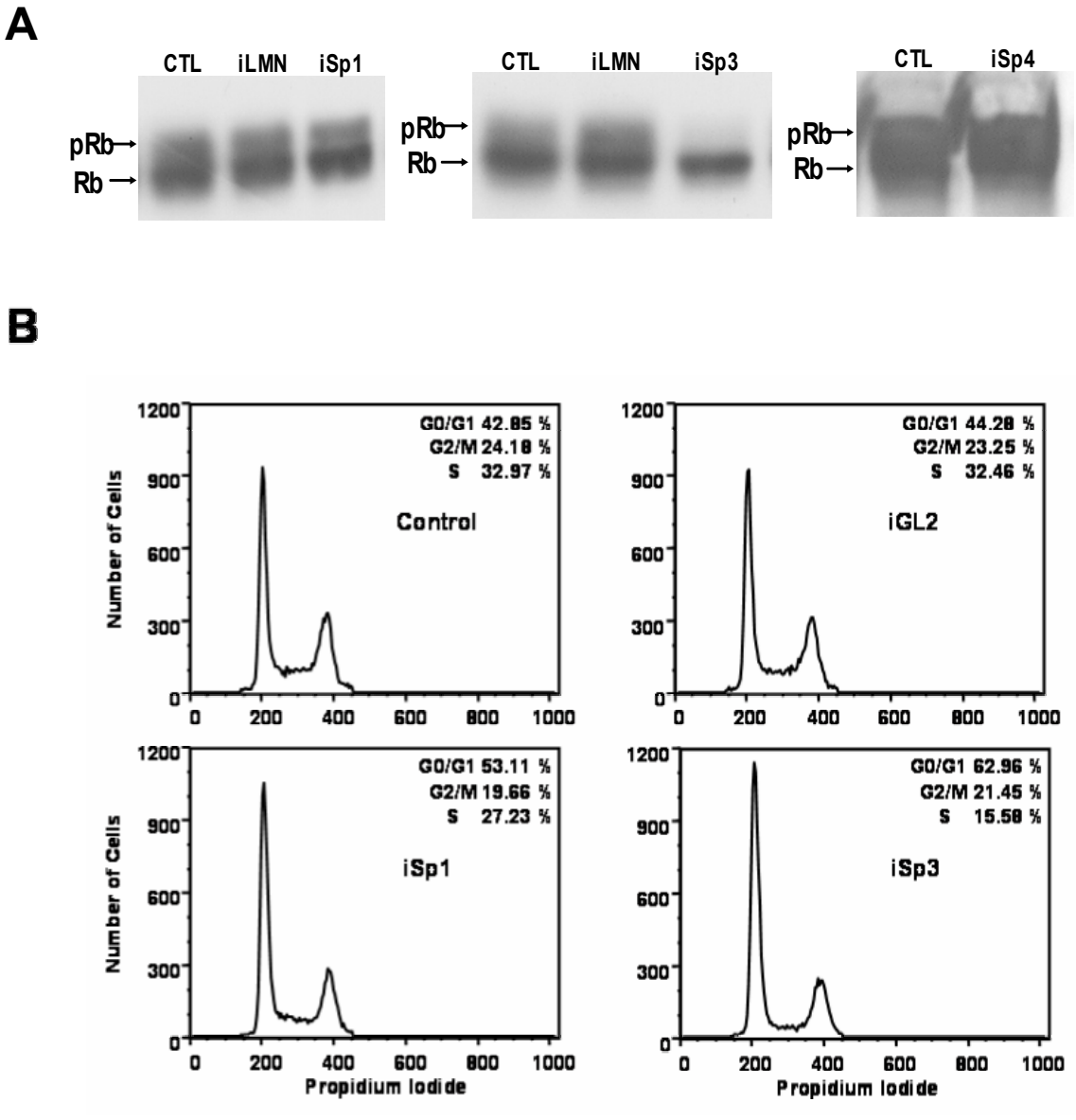


Fig. 34. **Role of Sp proteins in Panc-1 cell proliferation.** [A] Rb phosphorylation. Immunoblot analysis of Rb and phosphor-Rb (pRb) was determined in whole cell lysates of Panc-1 cells transfected with iSp1, iSp3 or iSp4. [B] FACS analysis. Cells were transfected with iSp1, iSp3 or iSp4, and their subsequent distribution in different phases of the cell cycle was determined by FACS analysis. Results obtained for Sp1 and Sp4 (data not shown) were comparable, and the effects of iSp1, iSp3 or iSp4 gave similar results in duplicate analyses.

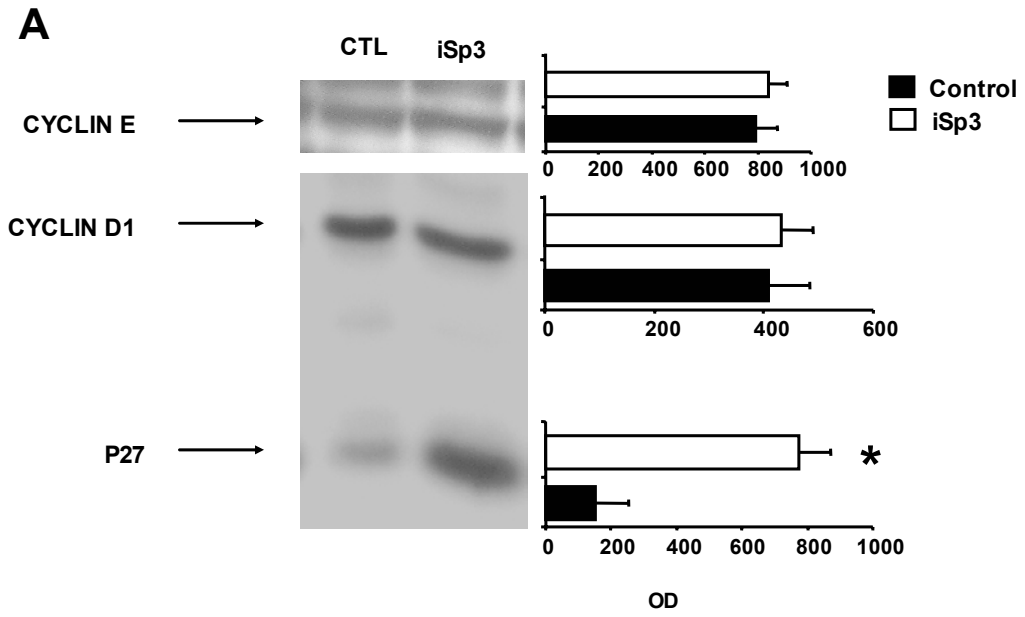


Fig. 35. **Sp3-dependent regulation of *p27* in Panc-1 cells.** [A] Western blot analysis. Cells were transfected with iSp3 or control (no oligonucleotide), and whole cell lysates were determined by Western blot analysis. Levels of other proteins associated with $G_0/G_1 \rightarrow S$ phase progression were unchanged (data not shown). Results are expressed as means $\pm$ SD for three replicate determinations for each treatment group, and significantly ($P < 0.05$, *) increased levels are indicated. [B] p27 promoter activity. Panc-1 cells were transfected with p27 promoter constructs and iLMN, iSp1 or iSp3. Results are expressed as means $\pm$ SD for three replicate determinations for each treatment group, and significantly ($P < 0.05$, *) increased activity is indicated. [C] Immunostaining of p27 and phospho-Rb. Panc-1 cells were transfected with iGL2 (panels c and d) or iSp3 (panels e and f) and immunostained with phospho-Rb (panels c and e) or p27 (panels d and f) antibodies. Panels a and b represent cells stained only with the secondary antibody.

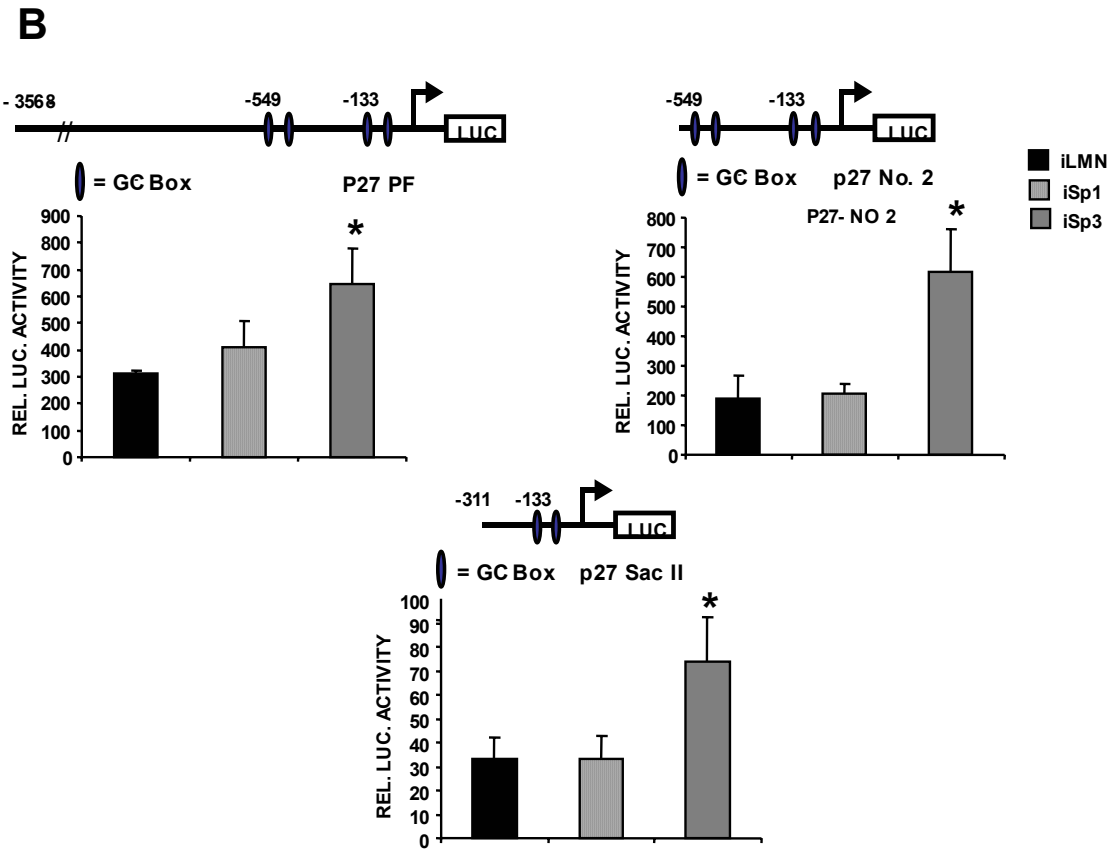


Fig. 35. Continued.

C

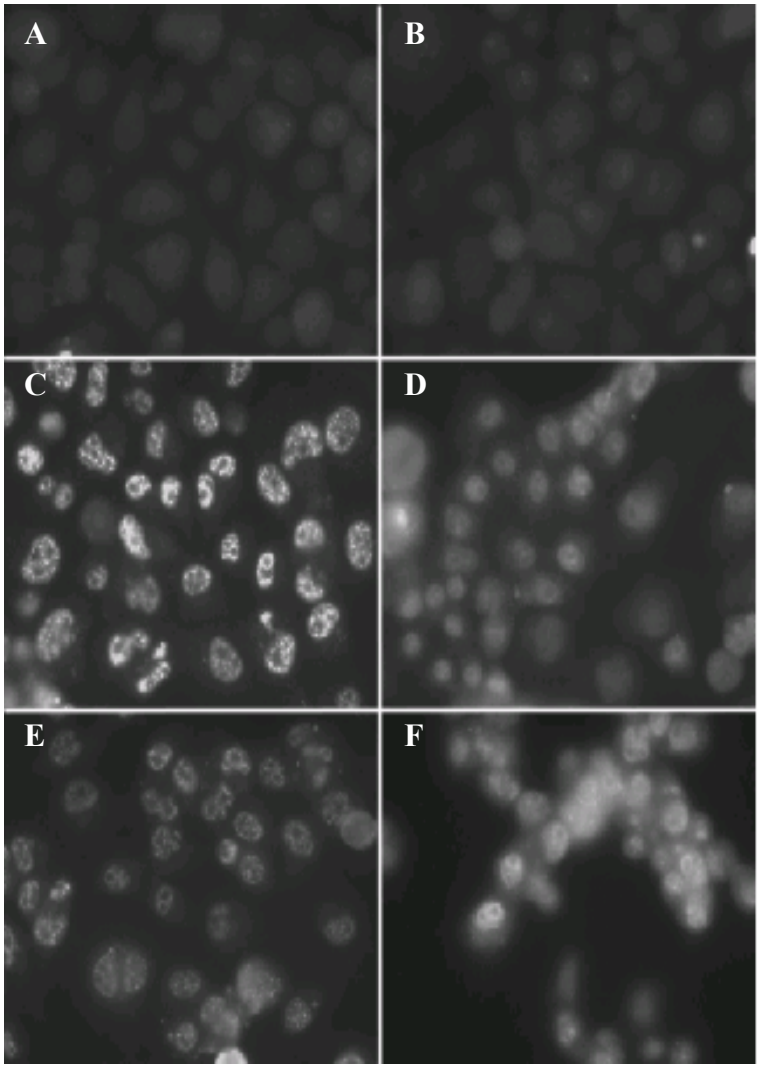


Fig. 35. Continued.

The results (Fig. 35B) clearly show that iSp3 but not iSp1 increases transactivation in Panc-1 cells transfected with the p27 constructs which contain distal and proximal GC-rich sites and confirms the critical role of Sp3 in regulating growth of Panc-1 cells through inhibition of *p27* expression. Confirmation that iSp3 results in upregulation of p27 is given in Figure 35C where knockdown of Sp3 results in upregulation of p27 protein (panel F) and downregulation of pRb (panel E) compared to cells transfected with the non-specific control siRNA for luciferase (iGL2) (panels C and D).

DISCUSSION

Pancreatic cancer is a complex and devastating disease which is usually detected in advanced stages or after metastases. Not surprisingly, current chemotherapies for this disease have limited efficacy and the 1 and 5 year survival times are approximately 21 and 5%, respectively (121,415). Development of new strategies for detection and treatment of this disease will depend on several factors which include a more comprehensive understanding of critical genes and pathways that control pancreatic tumor growth. PDAC cell lines have been developed for *in vitro* studies, and Panc-1 cells were derived from a primary tumor (625) and exhibit K-*ras*, *p53* and *p16* mutations which are typically observed in PDAC (433,618-624).

Previous studies reported that Panc-1 and other pancreatic cancer cell lines expressed high levels of Sp1 and Sp3 proteins, and Sp1 protein levels correlated with VEGF protein and VEGF promoter activity (297). The results are consistent with other studies showing that basal expression of *VEGF* is due, in part, to interactions of Sp1 with proximal GC-rich sites in the *VEGF* gene promoter (345,483,515). Studies in this laboratory have shown that the -131 to -47 region of the promoter is important for basal and estrogen-inducible expression of VEGF/VEGF promoter constructs through estrogen receptor/Sp protein interactions (345,515). Moreover, in ZR-75 breast cancer cells, hormone-induced transactivation of VEGF was due to both ERα/Sp1 and ERα/Sp3 as determined in RNA interference assays (515). As previously reported (297), Panc-1 cells expression both Sp1 and Sp3 (Fig. 32A and B), and transfection of iSp1 and iSp3 specifically decreases expression of both proteins (Fig. 32C). Based on results of gel mobility shift assays (Fig. 32B), there was evidence for expression of at least one additional protein that bound the GC-rich oligonucleotide (Fig. 32B). Subsequent Western blot, gel mobility shift and immunostaining assays showed that Sp4 protein is also expressed in Panc-1 cells (Fig. 33) and ongoing studies indicate that Sp4 is widely expressed in pancreatic cancer cell lines. Since all three Sp family proteins can potentially regulate *VEGF* expression in Panc-1 cells, we further investigated activation of *VEGF* promoter constructs in cells transfected with iSp1, iSp3 or iSp4. The results show that all three proteins cooperatively activate *VEGF* (Figs. 32C and

D and 33E) in Panc-1 cells and thereby expands the role of Sp family proteins in regulation of VEGF.

Sp proteins also regulate genes required for cancer cell proliferation (297), and we used RNA interference assays with iSp1, iSp3 and iSp4 to investigate their role in Panc-1 cell growth. Transfection with iSp1 or iSp4 followed by FACS analysis indicated that distribution of the cells in G_0/G_1, G_2/M or S phase and Rb phosphorylation was not significantly affected by either protein. In contrast, after transfection with iSp3, there was a decrease in Rb phosphorylation (Figs. 34A and 35C), a significant increase in cells in G_0/G_1, and a decrease in S-phase, suggesting that progression of Panc-1 cells through $G_0/G_1 \rightarrow S$ is Sp3-dependent (Fig. 34B). Subsequent analysis of cell cycle proteins in Panc-1 cells transfected with iSp3 showed that knockdown of Sp3 protein resulted in increased p27 protein and *p27* reporter gene expression (Fig. 35A - C). These data suggest a novel mechanism for Panc-1 cell growth which is determined, in part, by Sp3-dependent suppression of the cyclin-dependent kinase inhibitor *p27*. The strong inhibitory effects of Sp3 on the *p27* promoter contrasts to Sp3-dependent transactivation of VEGF (Fig. 32C and D) and illustrates the promoter-dependent inhibitory and activating responses linked to this transcription factor in Panc-1 cells. Currently, we are further investigating the relative expression and functions of Sp family proteins in other pancreatic cancer cells and tumors to gain further insights on tumor growth and angiogenesis and on identifying specific cellular targets for chemotherapy.

SUMMARY

The development of genetic technologies to regulate or delete expression of endogenous genes has been extensively used to probe the role of specific genes in biological and pathological pathways. RNA interference associated with dsRNA that is rapidly processed into siRNA has been identified in many eukaryotes and siRNA oligonucleotides can be successfully used for gene silencing in mammalian cells. We have focused on applications of siRNA techniques to investigate the role of several genes in pathways associated with growth and progression of breast, liver, and pancreatic cancer cells.

Two estrogen receptor (ER) positive breast cancer cell lines have been used to investigate the molecular mechanisms of the ligand-dependent activation of ERα/Sp1. 17β-estradiol (E2)-dependent transactivation of a GC-rich construct through interactions with ERα/Sp1 was blocked by siRNA for Sp1. In addition, silencing of Sp1 inhibits hormone-induced cell-cycle progression of MCF-7 cells, showing that genes activated by ERα/Sp1 play an important role in the growth of breast cancer cells.

siRNA for the aryl hydrocarbon receptor (AhR) and estrogen receptor (ER) were used to investigate inhibitory AhR-ERα crosstalk in breast cancer cells. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) inhibits E2-induced gene/reporter gene activity in breast cancer cells and this inhibitory crosstalk is observed for cell proliferation and cell cycle progression. siRNA for the AhR not

only inhibited TCDD-induced CYP1A1 gene expression, but also abrogated AhR-ER crosstalk in breast cancer cells. Moreover it was also shown that the endogenous AhR functions as an inhibitorof cell growth in human breast cancer cells whereas the AhR enhances liver cancer cell growth demonstrating important cancer cell context-dependent effects of the AhR. Interestingly, in the absence of the AhR, TCDD was estrogenic suggesting that cellular ER/AhR ratios may dictate receptor specific activation by this compound.

Using pancreatic cancer cells, we have investigated the role of several Sp family proteins in regulating angiogenesis and growth of pancreatic cancer cells. This study has shown for the first time that Sp4 is expressed in different pancreatic cancer cell lines and Sp4, Sp1 and Sp3 coordinately activate vascular endothelial growth factor (VEGF) in pancreatic cancer cells. The specificity of RNAi was critical for delineating the individual and cooperative roles of these Sp proteins in regulating VEGF expression. In contrast, Sp3 is the key regulator of the cell cycle progression through a novel pathway that involves suppression of cyclin-dependent kinase inhibitor p27 expression. These results suggest that Sp3 and other Sp family proteins regulate angiogenesis and proliferation of pancreatic cancer cells and current studies are investigating these pathways in other pancreatic cancer cell lines.

Finally, along with the rapidly growing literature on using siRNA as a functional genomic tool, there is emerging evidence that siRNAs may represent novel therapeutic agents for cancer treatment when optimized local and

systemic delivery systems are available. Our laboratory and others are developing strategies for delivery of siRNAs to specific target tissue and this will facilitate the *in vivo* applications and the therapeutic benefits of this technology.

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