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RNA Interference and cyclooxygenase-2 (COX-2) regulation in colon cancer cells

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Summary

Despite new methods and combined strategies, conventional cancer chemotherapy still lacks specificity and induces drug resistance. Gene therapy can offer the potential to obtain the success in the clinical treatment of cancer and this can be achieved by replacing mutated tumour suppressor genes, inhibiting gene transcription, introducing new genes encoding for therapeutic products, or specifically silencing any given target gene. Concerning gene silencing, attention has recently shifted onto the RNA interference (RNAi) phenomenon. Gene silencing mediated by RNAi machinery is based on short RNA molecules, *small interfering RNAs* (siRNAs) and *microRNAs* (miRNAs), that are fully o partially homologous to the mRNA of the genes being silenced, respectively. On one hand, synthetic siRNAs appear as an important research tool to understand the function of a gene and the prospect of using siRNAs as potent and specific inhibitors of any target gene provides a new therapeutical approach for many untreatable diseases, particularly cancer. On the other hand, the discovery of the gene regulatory pathways mediated by miRNAs, offered to the research community new important perspectives for the comprehension of the physiological and, above all, the pathological mechanisms underlying the gene regulation. Indeed, changes in miRNAs expression have been identified in several types of neoplasia and it has also been proposed that the overexpression of genes in cancer cells may be due to the disruption of a control network in which relevant miRNAs are implicated. For these reasons, I focused my research on a possible link between RNAi and the enzyme cyclooxygenase-2 (COX-2) in the field of colorectal cancer (CRC), since it has been established that the transition adenoma-adenocarcinoma and the progression of CRC depend on aberrant constitutive expression of COX-2 gene. In fact, overexpressed COX-2 is involved in the block of apoptosis, the stimulation of tumor-angiogenesis and promotes cell invasion, tumour growth and metastatization.

On the basis of data reported in the literature, the first aim of my research was to develop an innovative and effective tool, based on the RNAi mechanism, able to silence strongly and specifically COX-2 expression in human colorectal cancer cell lines. In this study, I firstly show that an siRNA sequence directed against COX-2 mRNA (*siCOX-2*), potently downregulated COX-2 gene expression in human umbilical vein endothelial cells (HUVEC) and inhibited PMA-induced angiogenesis *in vitro* in a specific, non-toxic manner. Moreover, I found that the insertion of a specific cassette carrying anti-COX-2 shRNA sequence (*shCOX-2*, the precursor of siCOX-2

previously tested) into a viral vector (pSUPER.retro) greatly increased silencing potency in a colon cancer cell line (HT-29) without activating any interferon response. Phenotypically, COX-2 deficient HT-29 cells showed a significant impairment of their *in vitro* malignant behaviour. Thus, results reported here indicate an easy-to-use, powerful and high selective virus-based method to knockdown COX-2 gene in a stable and long-lasting manner, in colon cancer cells. Furthermore, they open up the possibility of an *in vivo* application of this anti-COX-2 retroviral vector, as therapeutic agent for human cancers overexpressing COX-2.

In order to improve the tumor selectivity, pSUPER.retro vector was modified for the shCOX-2 expression cassette. The aim was to obtain a strong, specific transcription of shCOX-2 followed by COX-2 silencing mediated by siCOX-2 only in cancer cells. For this reason, H1 promoter in basic pSUPER.retro vector [pS(H1)] was substituted with the human *Cox-2* promoter [pS(COX2)] and with a promoter containing repeated copies of the TCF binding element (TBE) [pS(TBE)]. These promoters were chosen because they are particularly activated in colon cancer cells. COX-2 was effectively silenced in HT-29 and HCA-7 colon cancer cells by using enhanced pS(COX2) and pS(TBE) vectors. In particular, an higher siCOX-2 production followed by a stronger inhibition of *Cox-2* gene were achieved by using pS(TBE) vector, that represents not only the most effective, but also the most specific system to downregulate COX-2 in colon cancer cells.

Because of the many limits that a retroviral therapy could have in a possible *in vivo* treatment of CRC, the next goal was to render the enhanced RNAi-mediate COX-2 silencing more suitable for this kind of application. Xiang and et al. (2006) demonstrated that it is possible to induce RNAi in mammalian cells after infection with engineered *E. Coli* strains expressing *Inv* and *HlyA* genes, which encode for two bacterial factors needed for successful transfer of shRNA in mammalian cells. This system, called "*trans-kingdom*" RNAi (tkRNAi) could represent an optimal approach for the treatment of colorectal cancer, since *E. Coli* in normally resident in human intestinal flora and could easily vehicled to the tumor tissue. For this reason, I tested the improved COX-2 silencing mediated by pS(COX2) and pS(TBE) vectors in the tkRNAi system. Results obtained in HT-29 and HCA-7 cell lines were in high agreement with data previously collected after the transfection of pS(COX2) and pS(TBE) vectors in the same cell lines. These findings suggest that tkRNAi system for COX-2 silencing, in particular mediated by pS(TBE) vector, could represent a promising tool for the treatment of colorectal cancer.

Flanking the studies addressed to the setting-up of a RNAi-mediated therapeutical strategy, I proposed to get ahead with the comprehension of new molecular basis of human colorectal cancer. In particular, it is known that components of the miRNA/RNAi pathway may be altered during the progressive development of colorectal cancer (CRC), and it has been already demonstrated that some miRNAs work as tumor suppressors or oncomiRs in colon cancer. Thus, my hypothesis was that overexpressed COX-2 protein in colon cancer could be the result of decreased levels of one or more tumor suppressor miRNAs.

In this thesis, I clearly show an inverse correlation between COX-2 expression and the human *miR-101(1)* levels in colon cancer cell lines, tissues and metastases. I also demonstrate that the *in vitro* modulating of *miR-101(1)* expression in colon cancer cell lines leads to significant variations in COX-2 expression, and this phenomenon is based on a direct interaction between *miR-101(1)* and COX-2 mRNA. Moreover, I started to investigate *miR-101(1)* regulation in the hypoxic environment since adaptation to hypoxia is critical for tumor cell growth and survival and it is known that COX-2 can be induced directly by hypoxia-inducible factor 1 (HIF-1). Surprisingly, I observed that COX-2 overexpression induced by hypoxia is always coupled to a significant decrease of *miR-101(1)* levels in colon cancer cell lines, suggesting that *miR-101(1)* regulation could be involved in the adaption of cancer cells to the hypoxic environment that strongly characterize CRC tissues.

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Chapter I

Cyclooxygenases (Prostaglandin Endoperoxide H Synthases)

1. Cyclooxygenase Isozymes

Prostaglandin endoperoxide H synthases (PGHSs) catalyze the conversion of arachidonic acid and O_2 to PGH_2, the committed step in prostanoid biosynthesis. Before 1991, only one PGHS had been described, the isozyme now called PGHS-1, COX-1 (for cyclooxygenase-1) or the *constitutive* enzyme. At that time Simmons and Herschman and their colleagues discovered mRNAs whose expression was induced in chicken and mouse fibroblasts in response to *src* and tumor-promoting phorbol esters, respectively, and which encoded proteins having 60% amino acid sequence identity with PGHS-1. Subsequent work has shown that the new protein, called PGHS-2, COX-2 or the *inducible* isoform, is very similar to PGHS-1 in structure but differs substantially from PGHS-1 with respect to its pattern of expression and its biology. The reason for the existence of the two PGHS isozymes is unknown. However, PGHS-1 and -2 are often coexpressed in the same cell and may act as parts of separate prostanoid biosynthetic systems that function somewhat independently to channel prostanoids to the extracellular milieu and the nucleus, respectively. Nonsteroidal anti-inflammatory drugs (NSAIDs) represent one of the most highly utilized classes of pharmaceutical agents in medicine. All NSAIDs act through inhibiting prostaglandin synthesis, a catalytic activity possessed by two distinct COX isozymes encoded by separate genes. The discovery of COX-2 launched a new era in NSAID pharmacology, resulting in the synthesis, marketing, and widespread use of COX-2 selective drugs. These pharmaceutical agents have quickly become established as important therapeutic medications with potentially fewer side effects than traditional NSAIDs. Additionally, characterization of the two COX isozymes is allowing the discrimination of the roles each play in physiological processes such as homeostatic maintenance of the gastrointestinal tract, renal function, blood clotting, embryonic implantation, parturition, pain, and fever. Of particular importance has been the investigation of COX-1 and -2 isozymic functions in cancer, dysregulation of inflammation, and Alzheimer's disease. More recently, additional heterogeneity in COX-related proteins has been described, with the finding of variants of COX-1 and COX-2 enzymes. These variants may function in tissue-specific physiological and pathophysiological processes and may represent important new targets for drug therapy.

1.1 Prostaglandins and Cyclooxygenase

Prostaglandins, potent bioactive lipid messengers derived from arachidonic acid (AA), were first extracted from semen, prostate, and seminal vesicles by Goldblatt and von Euler in the 1930s and shown to lower blood pressure and cause smooth muscle contraction. Bergström and colleagues purified the first prostaglandin isomers during the 1950s and 60s, and in 1964, van Dorp et al. and Bergstrom et al. independently identified AA, a 20-carbon tetraenoic fatty acid (C20:4Ω6) as the precursor to prostaglandins. The cyclooxygenase reaction through which AA is enzymatically cyclized and is oxygenated to yield endoperoxide-containing prostaglandin G_2 (PGG_2) was later identified by Samuelsson and colleagues (Hamberg and Samuelsson, 1973; Hamberg et al., 1974) (Fig.1). The enzyme, cyclooxygenase (COX) that catalyzes this cyclooxygenation reaction also reduces a hydroperoxyl in PGG_2 to a hydroxyl to form PGH_2 via a separate peroxidase active site on the enzyme. Isomerases and oxidoreductases produce various bioactive prostaglandin isomers using PGH_2 as substrate as shown in **Fig.1**.

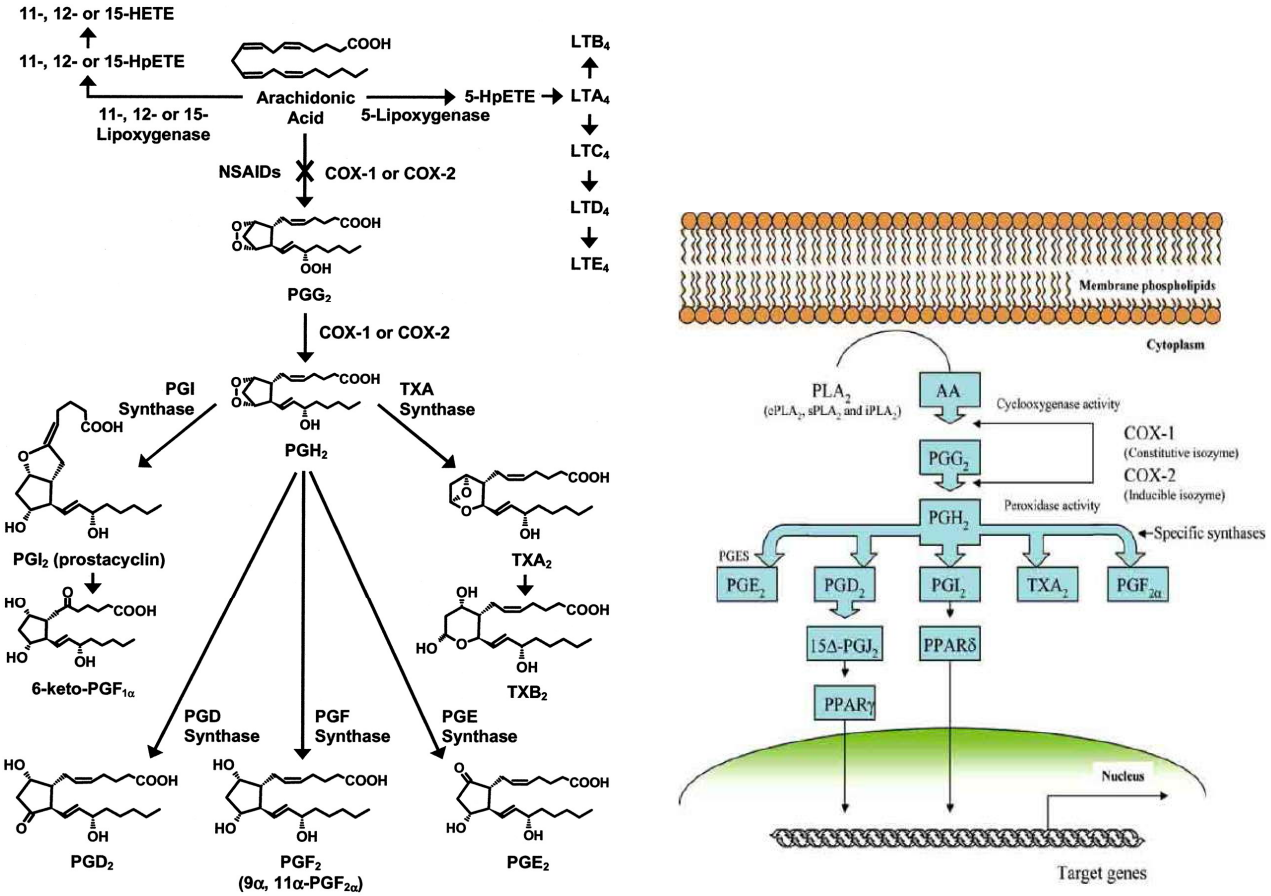


Figure 1. The arachidonic acid cascade.

Until 1976 (Hemler and Lands, 1976; Miyamoto et al., 1976), when purified COX preparations were first described, tissue homogenates were used as a source of COX enzyme activity, which was frequently referred to at that time as a prostaglandin synthetase. Because the COX enzyme reaction does not require ATP, the nomenclature was later changed to synthase. The COX enzyme, also known as prostaglandin H synthase (PGHS) or prostaglandin endoperoxide synthase (E.C. 1.14.99.1), was identified as the major enzyme in the oxidative conversion of AA to PGG_2 and PGH_2 (Smith and Lands, 1972; Hamberg et al., 1974), with seminal vesicles of sheep, bovines being a rich enzyme source (Smith and Lands, 1972). Thus, purification of PGHS enzyme to homogeneity was first achieved from the sheep (Hemler and Lands, 1976; van der Ouderaa et al., 1977) and bovine (Miyamoto et al., 1976; Ogino et al., 1978) seminal vesicles. This purified enzyme migrated in the region of approximately 67 kDa in SDS-polyacrylamide gel electrophoresis and contained cyclooxygenase and peroxidase activities, which were later found to be at separate sites (Marshall and Kulmacz, 1988). Since detergents such as Tween 20 were needed to solubilize the enzyme, it was classified as an integral microsomal membrane protein. In 1971, John Vane used a cell-free homogenate of guinea pig lung to demonstrate that aspirin, indomethacin, and salicylate, popular nonsteroidal anti-inflammatory drugs (NSAIDs), were inhibitors of this enzyme—thus defining the mechanism of action of this important class of drugs.

1.2 Early Evidence for Multiple Cyclooxygenases

Researchers, beginning in the early 1970s, speculated on whether there was more than one COX enzyme. Flower and Vane (1972) postulated the existence of an acetaminophen-inhibitable COX activity that was in dog brain but not in rabbit spleen. The same year, two catalytically distinct prostaglandin synthase activities were reported to be present in acetone powder extracts of sheep vesicular glands (Smith and Lands, 1972). Studies of autoinactivation rates of COX, inhibition by NSAIDs, and time course profiles of PGE_2 and PGF_2 synthesis led Lysz and colleagues (1982, 1988) to propose that rabbit and mouse, but not rat brain, contained two forms of COX. It was, however, through the study of prostaglandin induction by mitogens and proinflammatory agents, as well as prostaglandin down-regulation by glucocorticoids, that the most provocative data regarding the potential of more than one COX were obtained. The phenomenon that was observed by many laboratories was that prostaglandin synthesis and release in some situations, such as in activated platelets, occurs within a few minutes after stimulation. In other cases, such as in mitogen-stimulated fibroblasts, prostaglandin synthesis may take hours to occur. In 1985, Habenicht and colleagues (1985) reported that platelet-derived growth factor treatment of Swiss 3T3 cells resulted in an early (10 min) and a late (2–4 h) peak in induction in prostaglandin synthesis. Only the late

peak was blocked by cycloheximide, leading to the conclusion that platelet-derived growth factor-stimulated PG synthesis occurred by "direct effects on the PG-synthesizing enzyme system, one involving a protein synthesis-independent mechanism and another that requires rapid translation of cyclooxygenase". The activities revealed in Habenicht's early study were indicative of an endogenous COX enzyme (COX-1) and an inducible enzyme (COX-2). Many other laboratories at this time did similar studies on induction of prostaglandin synthesis, but with only nucleic acid and antibody probes to the seminal vesicle form of COX to work with, many investigators observed the paradoxical phenomenon that, in many instances, prostaglandin induction occurred without an increase in the seminal vesicle COX—an enzyme which was found to be present in most cells and tissues investigated (DeWitt and Smith, 1988). Frequently only marginal increase in seminal vesicle COX was observed despite robust increase in PG synthesis. Similar anomalies in which PG synthesis and seminal vesicle COX did not coincide were observed with regard to the action of glucocorticoids, which strongly decreased PG synthesis but typically had little to no effect on seminal vesicle COX levels. Various postulates were proposed that were consistent with these observed phenomena, the most common of which was that changes in substrate delivery were responsible for these fluctuations in PG synthesis. In 1989, Rosen et al. used low-stringency Northern blot hybridization with an ovine seminal vesicle COX cDNA as probe to detect a 4.0-kb RNA, in addition to the known 2.8-kb mRNA encoding seminal vesicle COX. This 4.0-kb mRNA was inducible and paralleled induction of enzyme activity. These investigators concluded that "the larger mRNA may encode for a cyclooxygenase" encoded by a distinct gene. In 1990, Needleman and colleagues (Fu et al., 1990) studying lipopolysaccharide (LPS) stimulation of monocytes concluded that these "cells may contain two pools of COX, each with a differential sensitivity to LPS or DEX (dexamethasone)." During this time, Young, Macara, and colleagues (Han et al., 1990) identified, by using giant two-dimensional protein gel electrophoresis, proteins immunoreactive with COX-1 antibodies that were induced in v-*src*-transformed cells. The evidence in these and other early studies was consistent with distinct inducible and constitutive COX isozymes encoded by separate genes but was also compatible with other explanations.

1.3 Studies of Cell Division and the Discovery of Cyclooxygenase-2

The answer to the mechanism of how COX enzyme activity rapidly increases PG induction in inflammation and in other physiological contexts came from studies of cell division. In the late 1980s, Simmons et al. (1989) and Herschman (Varnum et al., 1989) and colleagues independently identified immediate-early genes in fibroblast-like cells activated by mitogens. Genes found by Simmons in chicken (Simmons et al., 1989; Xie et al., 1991) and mouse (Simmons et al., 1991)

were activated by the v-*src* oncogene, phorbol esters and serum. Herschman used Swiss 3T3 cells to identify tetradecanoyl-13-phorbol acetate-inducible sequences (or TIS genes), which were also induced by other mitogens (Varnum et al., 1989; Kujubu et al., 1991). In 1991, each laboratory independently reported that one of their sequences encoded a new inducible COX enzyme. Also contributing to the identification of COX-2 in 1991, Young and colleagues (O'Banion et al., 1991) reported a partial predicted sequence of COX-2 from a murine cDNA. The inducible enzyme cloned in these studies is now most frequently referred to as COX-2 and the seminal vesicle form of the enzyme as COX-1. Herschman and colleagues expressed the mouse TIS10 cDNA in heterologous cells and showed that increased prostaglandin E_2 synthesis was induced by this cDNA (Fletcher et al., 1992). Ectopic overexpression was also done by Young's laboratory (O'Banion et al., 1992) and Meade et al. (1993), who also demonstrated the importance of the 3'-untranslated region of the COX-2 mRNA in governing overexpression of the enzyme. Using mouse COX-2 sequences as probe, Hla and Neilson (1992) identified and published the sequence of the human homolog of TIS10/CEF147, which they named COX-2. Overexpression of human COX-2 cDNA in Cos cells also induced COX enzymatic activity, similar to that of TIS10, and this activity was inhibited by nonsteroidal anti-inflammatory drugs (Hla and Neilson, 1992). The human COX-2 cDNA was widely expressed as an inducible gene in nonimmortalized vascular and inflammatory cells.

1.4 Structure of Cyclooxygenase-1 and Cyclooxygenase-2

Pure preparations of the COX-1 enzyme obtained from seminal vesicles were instrumental in the elucidation of the primary structure of this enzyme by molecular cloning. Both the N terminus and the internal sequences following limited trypsin digestion of the sheep seminal enzyme were reported (Chen et al., 1987). Roth and colleagues (1975), using sheep and bovine seminal vesicle enzyme preparations, showed that aspirin acetylated the COX enzyme. The region of the active site residues and the determination of the serine acetylated by aspirin were elucidated by sequencing ^{3}H-aspirin-labeled peptides of the sheep seminal vesicle enzyme (Roth et al., 1980; DeWitt et al., 1990); however, molecular cloning by three different laboratories ultimately elucidated the complete primary structure of the COX-1 enzyme (DeWitt and Smith, 1988; Merlie et al., 1988; Yokoyama et al., 1988). Sequence analysis of COX-1 cDNAs indicated that they contained an open reading frame of ~1.8 kb, which contained all the polypeptide sequences from protein microsequencing efforts (Roth et al., 1980, 1983; DeWitt and Smith, 1988), including the aspirin acetylation site (DeWitt et al., 1990). These data strongly suggested that the isolated cDNA clone encoded the sheep seminal vesicle COX enzyme. In addition to ovine COX-1, the gene for the human homolog of this enzyme was also cloned (Yokoyama and Tanabe, 1989). These cloning

efforts were followed by the demonstration that the cDNA for sheep seminal vesicle COX exhibited both cyclooxygenase and peroxidase activities upon ectopic overexpression in mammalian and insect cells (DeWitt et al., 1990; Funk et al., 1991; Meade et al., 1993). Mutagenesis experiments were conducted to identify critical residues for catalysis, such as the heme coordination sites, aspirin acetylation sites, etc. (Shimokawa et al., 1990; Shimokawa and Smith, 1992). Northern blot analysis with cDNA probes of COX-1 identified a major mRNA species of 2.8 kb and a minor species of 5.2 kb in human endothelial cells (Hla, 1996). Further sequence analysis of a human endothelial cell-derived cDNA, which encoded the 3'-end of the 5.2-kb mRNA, indicated that the 5.2-kb cDNA and the 2.8-kb cDNAs represent alternatively polyadenylated mRNA species with differing lengths of the 3'-UTR (Hla, 1996). These alternative polyadenylation states were also confirmed in cDNAs encoding the 3' of the 5.2-kb mRNA from a human megakaryoblastic cell line (Plant and Laneuville, 1999). The predicted amino acid sequences of COX-2 cloned in chicken and mammals showed it to possess approximately 60% amino acid identity with COX-1 (Simmons et al., 1991). COX-1 and COX-2 were found to be approximately 600 amino acids in size in all species. The COX-1 and/or -2 cDNA sequences from many organisms, including bony and cartilaginous fish, birds, and mammals have been characterized. Furthermore COX genes appear to be expressed in invertebrates, such as coral and sea squirts, where two COXs have been identified in two different species of each phylum (Valmsen et al., 2001, Jarving et al., 2004). These data suggest that the cyclooxygenase pathway was present in early invertebrate speciation in the animal kingdom. From an evolutionary standpoint COX-1 and COX-2 appear to have resulted from a gene duplication event that occurred early in or before vertebrate speciation. Cyclooxygenases in unicellular organisms, insects, or the plant kingdom have not been identified; however, COX enzymes have recently been determined to be members of a larger fatty acid oxygenase family that includes pathogen-inducible oxygenases (PIOXs). These latter enzymes have been identified in monocotyledon and dicotyledon plants, *Caenorhabditis elegans*, and bacteria (*Pseudomonas*). Like COXs, PIOXs oxygenate polyunsaturated fatty acids using molecular oxygen (Sanz et al., 1998; Hamberg et al., 1999; Koeduka et al., 2000). They also introduce a hydroperoxyl moiety into the fatty acid, which is introduced at the α carbon by PIOXs to form 2*R*-hydroperoxy fatty acids. Generation of α-peroxyl-fatty acids by PIOXs has been proposed to be a signaling response in these organisms to activate genes needed for antipathogen defense (Sanz et al., 1998). COXs and PIOXs share approximately 30% sequence identity, and PIOXs contain conserved critical residues needed for fatty acid oxygenation seen in COX. Sequence similarity to COXs in the region of the Tyr385 has also been found in the enzyme linoleate diol synthase (LDS) from fungus (Oliw et al., 1998; Su et al., 1998; Hörnsten et al., 1999). This enzyme is a homotetrameric ferric hemeprotein that

catalyzes the dioxygenation of linoleic acid to (8*R*)-hydroperoxylinoleate and isomerization of this latter compound to (7*S*,8*S*)-dihydroxylinoleate. Like COXs, the enzyme is known to form ferryl intermediates and a tyrosyl radical. PIOXs and LDSs are clearly related to peroxidases; however, there is no evidence that PIOXs or LDS possess peroxidase activity. These findings lead to the conclusion that PIOXs, LDS, and COXs each represent distinct subfamilies of fatty acid oxygenases that are descended from ancient peroxidases. If they descend from a common peroxidase progenitor, LDS, PIOXs and COXs have additionally gained hydrophobic pockets for binding and oxygenation of fatty acid substrates; however, the PIOX and LDS branches of this fatty acid oxygenase family have a degenerate peroxidase active site, that likely functions solely to activate the enzyme. PIOXs and LDSs, therefore, are predicted to perform primarily the dioxygenation reaction typical of the COX active site. Since PIOXs and LDS are found in plants, bacteria, fungus, and lower animals, the fatty acid oxygenase progenitor of COXs and PIOXs is predicted to exist very early in evolution, underscoring the concept that generation of oxygenated fatty acids by these enzymes represents an evolutionarily ancient mechanism of cell signaling. Recently a cyclooxygenase enzyme from the protozoan *Entamoeba histolytica* has been identified that lacks structural similarity with other COXs, PIOXs, or LDS enzymes, but makes PGE_2 from arachidonic acid (Dey et al., 2003). Therefore, prostaglandin-synthesizing enzymes distinct from the COX lineage characterized in vertebrates, coral, and sea squirts appear to have arisen during speciation of some organisms. Landmark studies by Garavito and colleagues (Picot et al., 1994) elucidated the tertiary and quaternary structure of COX-1. Early studies in the 1970s showed that COX-1 was likely a dimer and was tightly bound to microsomal membranes; however, the topology of the enzyme in microsomal membranes was unknown. At crystallographic resolution, Garavito's studies described COX-1's distinct domains for dimerization, membrane binding, and catalysis. A fourth domain, the N-terminal signal peptide, which is clearly evident in the primary structure of COX-1, was not observed because this sequence is cotranslationally cleaved from the nascent polypeptide by microsomal signal peptidase. Crystallographic structures of COX-2 have been obtained by Luong et al. (1996), Bayly et al. (1999), and Kurumbail et al. (1996) and show striking similarity with COX-1. In fact, all known COX enzymes share the same functional domains. Outstanding reviews of COX structure and enzyme kinetics have recently been written (Garavito and DeWitt, 1999; Marnett, 2000; Smith et al., 2000) and thus only the essential aspects of these topics needed to understand the pharmacology of NSAIDs are discussed here. The structures of COX-1 and COX-2 predict that both enzymes are located in the lumen of the nuclear envelope and endoplasmic reticulum. Structural aspects of each of the four domains (**Figs. 2** and **3**) of COX-1 and COX-2 lead to this conclusion.

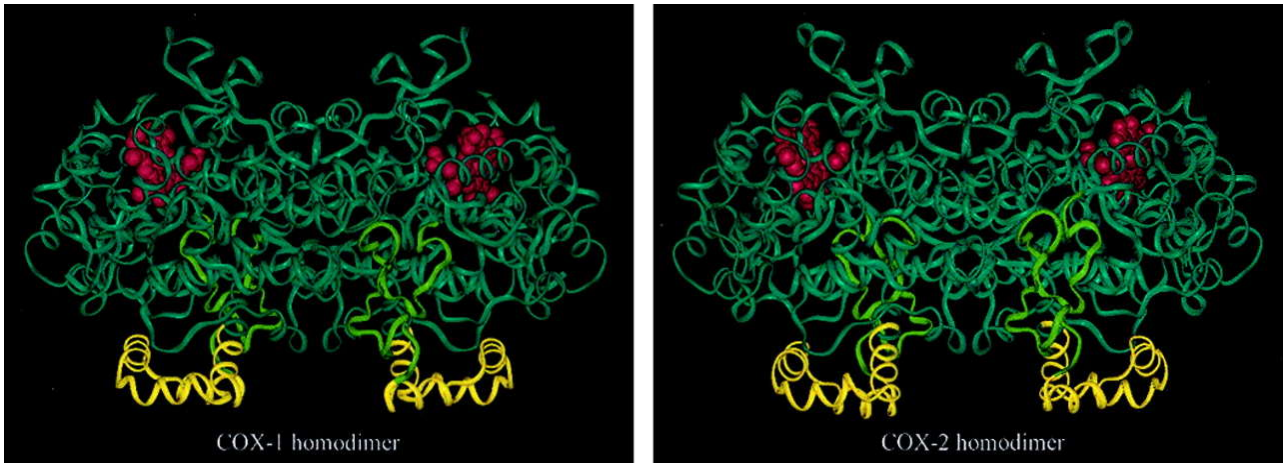


Figure 2. Crystallographic structures of ovine COX-1 (left) and murine COX-2 (right) homodimers.

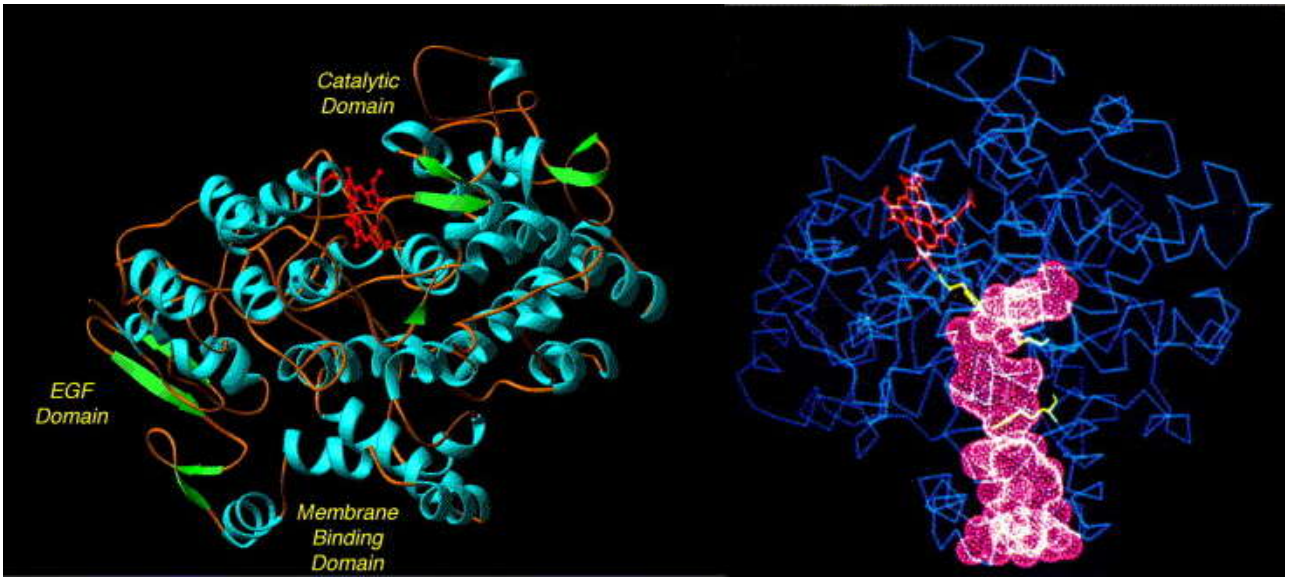


Figure 3. Structure of a single subunit of COX.

1. Amino-Terminal Signal Peptide. Nascent COX-1 and COX-2 polypeptides are directed into the lumen of the endoplasmic reticulum by amino-terminal signal peptides. Although cleaved from the nascent polypeptide, these hydrophobic peptides show a size difference between COX-1 and COX-2 that, until recently, has been of unknown biological significance. The signal peptide for COX-1 is always 22 to 26 amino acids in length with a large hydrophobic core comprised of four or more leucines or isoleucines. COX-2's signal peptide is 17 amino acids long in all species and appears to be less hydrophobic. *In vitro* translation experiments demonstrate that COX-1 is rapidly translocated into the lumen of canine pancreatic microsomes, whereas COX-2 is inefficiently translocated (Xie et al., 1991). Immediately following the signal peptide in COX-1 are eight amino acids that are not found in COX-2. The function of this sequence is unknown. Recently, as described below, variants

of COX-1 have been identified in which retention of all or part of intron-1 results in a retained signal peptide in COX-1, altering the biological properties of the enzyme (Chandrasekharan et al., 2002). Also, one coral isozyme has seven amino acids inserted in the same location; however, in this case, the insertion appears to change the location of the cleavage site in COS-7 cells rather than to affect retention of the signal peptide (Jarving et al., 2004).

2. Dimerization Domain. COX-1 and COX-2 dimers are held together via hydrophobic interactions, hydrogen bonding, and salt bridges between the dimerization domains of each monomer. Heterodimerization of COX-1 and COX-2 subunits does not occur. The dimerization domain is encoded by approximately 50 amino acids near the amino terminus of the proteolytically processed protein. Three disulfide bonds hold this domain together in a structure reminiscent of epidermal growth factor. A fourth disulfide bond links the dimerization domain with the globular catalytic domain. The presence of disulfide bonds, which require an oxidizing environment, is consistent with the location of COXs inside the lumen of the nuclear envelope, ER, or Golgi, which have redox states that are significantly more oxidized than cytosol.

3. Membrane Binding Domain. COX isozymes associate with the intraluminal surface of microsomal membranes in an unusual fashion. Rather than employing transmembrane spanning sequences or covalently bound lipids for attachment, COX isozymes contain a tandem series of four amphipathic helices, which creates a hydrophobic surface that penetrates into the upper portion of the luminal side of the hydrophobic core of the lipid bilayer. These helices are encoded by approximately 50 amino acids found immediately carboxy-terminal to the dimerization domain. The helices allow COX dimers to float on the surface of the lumen of the ER/nuclear envelope, with the majority of the protein protruding into the luminal space of these compartments. The membrane binding domain also forms the mouth of a narrow hydrophobic channel that is the cyclooxygenase active site.

4. Catalytic Domain. Carboxy-terminal to the membrane binding domain in COX primary structures is the catalytic domain, which comprises 80% (approximately 480 amino acids) of the protein and contains two distinct enzymatic active sites.

a. Peroxidase Active Site. The catalytic domain is globular with two distinct intertwining lobes. The interface of these lobes creates a shallow cleft on the upper surface of the enzyme (i.e., the surface furthest from the membrane) where the peroxidase active site is located and where heme is bound. Coordination of the heme is via an iron-histidine bond involving His388 in sheep COX-1. (All numbering hereafter uses sheep COX-1 as reference.). Other important interactions

between the protoporphyrin also occur, and specific amino acids that may function in coordinating PGG_2 have been identified (Malkowski et al., 2000; Thuresson et al., 2001). The geometry of heme binding leaves a large portion of one side of the heme exposed in the open cleft of the peroxidase active site for interaction with PGG_2 and other lipid peroxides.

b. Cyclooxygenase Active Site. The cyclooxgyenase active site is a long, narrow, dead-end channel of largely hydrophobic character whose entrance is framed by the four amphipathic helices of the membrane binding domain. The channel extends approximately 25 Å into the globular catalytic domain and is on average about 8 Å wide (Picot et al., 1994). However, significant narrowing of the channel is observed where arginine 120, one of only two ionic residues found in the COX active site, protrudes into the channel and forms a hydrogen bonded network with glutamate 524 (the other ionic residue in the channel) and tyrosine 355. Arginine 120 is essential for binding substrates and carboxylate-containing NSAIDs in COX-1. In contrast, this residue is unessential in binding substrate in COX-2 (Rieke et al., 1999), where it also appears to be nonessential in coordinating carboxylate-containing NSAIDs (Greig et al., 1997).

The upper portion of the channel, or catalytic pocket, contains tyrosine 385 that forms a tyrosyl radical, abstracts hydrogen from the pro-S side of carbon 13 of AA, and creates an activated arachidonyl radical that undergoes the cyclization/oxygenation reaction shown in **Fig. 4**.

a **b**

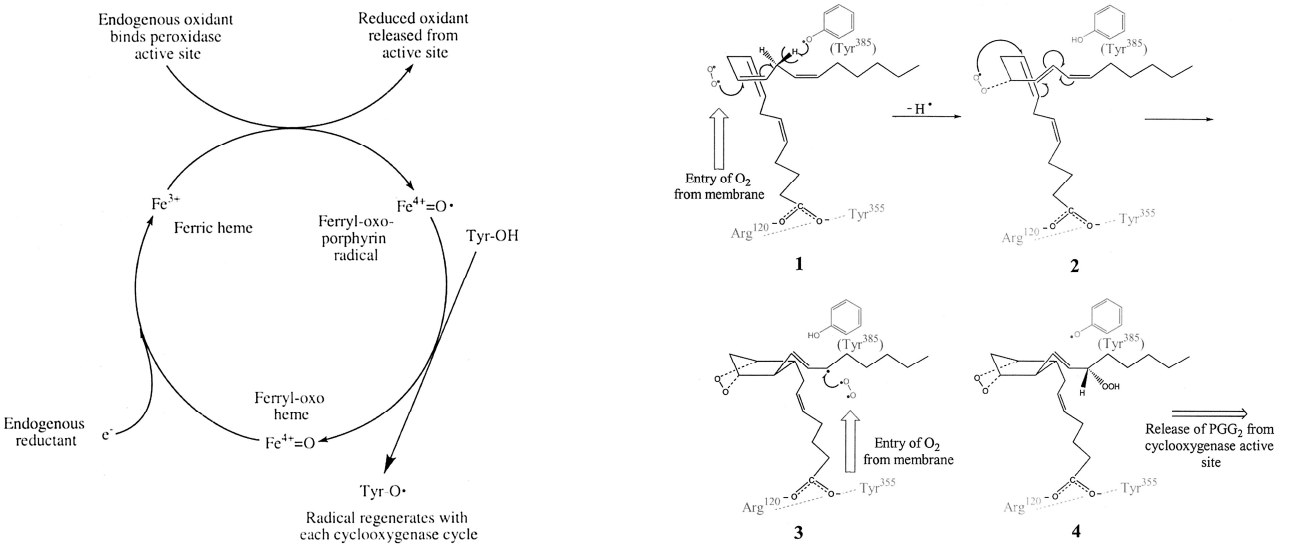


Figure 4. Priming of COX enzymes by formation of a tyrosyl radical (**a**). Catalytic steps in cyclooxygenation of arachidonic acid (**b**).

Also in the hydrophobic pocket is Ser530, which is transacetylated by aspirin. The hydroxyl of serine 530, together with valine 349, appears to be essential in governing the stereochemistry of oxygen attack at carbon 15 in the production of PGG_2 (Schneider et al., 2002); however, its acetylation prevents abstraction of hydrogen from AA in COX-1 by sterically preventing AA from binding productively in the active site (Rowlinson et al., 2000). In contrast, abstraction of hydrogen does occur in acetylated COX-2, but cyclization of the arachidonyl radical and formation of the endoperoxide does not occur, yielding 15-*R*-hydroxyeicosotetraenoic acid (15*R*-HETE) rather than PGH_2 (Holtzman et al., 1992). A crucial structural difference between the active sites of COX-1 and COX-2 is a substitution of isoleucine 523 in COX-1 for a valine in COX-2. This single difference opens a hydrophobic outpocketing in COX-2 that can be accessed by some COX-2-selective drugs (Kurumbail et al., 1996). There are other changes in residues that are near but do not line the COX active site, so-called second shell residues, that result in subtle changes and a slightly enlarged COX-2 active site relative to COX-1 (Kurumbail et al., 1996; Luong et al., 1996). The evolutionary conservation of an enlarged cyclooxygenase active site in COX-2 relative to COX-1 may be essential to the recognition of bulkier substrates by COX-2. Anandamide (arachidonylethanolamide) and 2-arachidonylglycerol are endocannabinoids that are efficiently oxidized by COX-2 to endocannabinoid-derived prostanoids (Kozak et al., 2002). COX-2 utilizes these bulkier substrates as efficiently as arachidonic acid, and the resulting endoperoxide can be utilized by downstream isomerases (Kozak et al., 2002). The function of these prostanoid-like oxidized endocannabinoids is unknown but may represent new biological roles of COX-2. The endocannabinoid analog methandamide upregulates COX-2 expression, further linking this enzyme with metabolism of endocannabinoids (Gardner et al., 2003; Ramer et al., 2003). Elegant studies done collaboratively by the laboratories of Garavito and Smith (Malkowski et al., 2000; Thuresson et al., 2001) have succeeded in defining the productive structure of COX-1 with its substrate AA as well as with eicosapentaenoic and linoleic acids. COX-1 binds AA in an extended L shape, its carboxylate forming both a salt bridge with the guanidinium group of arginine 120 and also a hydrogen bond with tyrosine 355. The remainder of the fatty acid makes more than 50 mostly hydrophobic interactions with 19 amino acid residues, which position substrate for hydrogen abstraction and facilitate conversion to PGG_2 rather than to HETEs (Thuresson et al., 2001). Two molecules of oxygen for the bisoxygenation reaction and hydroperoxidation reaction that yield the endoperoxide and hydroperoxide moieties, respectively, have been postulated to diffuse into the COX active site from the direction of the membrane, thus resulting in the observed fact that attack of oxygen at carbon 11, to eventually result in the PGG_2 endoperoxide, occurs from the opposite or anterofacial orientation from that of hydrogen abstraction at carbon 13. At the carboxy terminus of the catalytic

domain of COX-1 and COX-2 are modified versions of the KDEL sequence that act as a signal for retention of proteins in the endoplasmic reticulum (Song and Smith, 1996). Additionally, COX-2 has an 18-amino acid sequence located next to this retention signal. This structure, which is not found in COX-1, is not fixed in crystallographic studies, and its function is unknown. The above structural features are consistent with localization of COX isozymes inside the lumen of the ER, a fact that is further supported by numerous studies using fluorescence and immuno-electron microscopy (Song and Smith, 1996; Liou et al., 2000); however COX-1 has been found by Weller and colleagues to be localized to lipid bodies in leukocytes and other cells (Bozza et al., 1996). Lipid bodies in these cells are rapidly formed following treatment with platelet-activating factor (PAF), nonesterified fatty arachidonate, or other fatty acids (Bozza and Weller, 2001) and are induced in endothelial cells by hypoxia (Scarfo et al., 2001). Unlike the ER, which contains a lumen, the structure of lipid bodies is less defined and may contain a central core of neutral lipids surrounded by a monolayer of phospholipid, which is thought to be derived from the cytosolic side of the ER bilayer (Murphy and Vance, 1999). In addition to containing COX-1, lipid bodies have also been shown to be rich in other lipid-metabolizing enzymes (Bandeira-Melo et al., 2001). In addition to lipid bodies, COX-1 has been localized to unusual filamentous structures in endothelial ECV304 cells (Liou et al., 2000), and COX-2 was localized to caveolin-1-containing vesicles in bovine arterial endothelial cells treated with phorbol ester (Liou et al., 2000) or human fibroblasts treated with either phorbol ester or IL-1. COX-1 and COX-2 have been identified by a number of laboratories to traffic within the nucleus following a variety of stimuli (Coffey et al., 1997; Neeraja et al., 2003). How extraluminal COX isozymes might structurally and enzymatically differ from their intraluminal counterparts or result in differential targeting of prostaglandins (e.g., to the nucleus) is currently unknown.

1.5 Variants of Cyclooxygenase Isoenzymes

Recently it has become clear that the transcriptome and proteome is significantly larger than the genome. Much of the discrepancy is due to alternative splicing. The first COX-1 splice variant was identified by Diaz in 1992 from a cDNA clone that contained the complete coding region for human lung COX-1; however, the cDNA contained an in-frame removal, due to alternative splicing, of the last 111 base pairs encoded by exon 9. This deletion eliminated the *N*-glycosylation site at residue 409, which had previously been shown by others to be essential for proper folding of the enzyme and for enzyme activity. Differential expression of this variant relative to COX-1 was observed following treatment of human lung fibroblasts with transforming growth factor-β, IL-1β, TNFβ, serum, and phorbol esters. Human myometrium was found to express this transcript at low levels

that do not change during parturition (Moore et al., 1999). A second COX-1 variant, which lacks exon 1 and instead contains part of intron 2, was identified in a rat tracheal cell line (EGV-6). This transcript was expressed at low levels; however, more than 90% of the COX-1 transcripts in this cell line are in this variant form. Primary rat tracheal epithelial cells and fibroblasts were also found to contain the variant transcript, but at only 1% of the level of COX-1 mRNA. Because this transcript lacks exon 1, which contains the initiating codon for translation, it has been considered to encode a nonsense COX protein (Kitzler et al., 1995). Interestingly, however, studies of the rat gastrointestinal tract show differential expression of this variant relative to COX-1 in aging stomach (Vogiagis et al., 2000). Moreover, expression of this variant was elevated in colorectal tumors, and its expression was reduced following treatment with NSAIDs. One of these, termed by the authors COX-3, consists of the COX-1 mRNA that retains intron-1. Intron-1 is small in all mammalian COX-1 genes thus far characterized. In dogs, it is 90 nucleotides in length and represents an in-frame insertion into the portion of the COX-1 open reading frame encoding the N-terminal hydrophobic signal peptide. The COX-3 variant produces protein containing the encoded intron-1 sequence when expressed in insect cells. The protein possesses reduced prostaglandin synthesis activity relative to COX-1, but analgesic/antipyretic drugs such as acetaminophen and dipyrone preferentially inhibit this activity. Evolutionary comparisons show that intron-1 is of similar size in all species but is not always in frame as in canines. For example, it is out of frame in humans and rodents and would require additional mechanisms such as the use of alternative splice sites, ribosomal frameshifting, or RNA editing to make a functional protein (Chandrasekharan et al.,). Other COX-1 splice variants recently identified encode PCOX-1 (partial COX-1) proteins (Chandrasekharan et al., 2002). PCOX-1 variants exhibit in-frame deletion of exons 5 through 8. This deletion results in the removal of 219 amino acids from the catalytic domain corresponding to amino acids 119–337 in COX-1. Two forms of PCOX-1 are known, PCOX-1a and PCOX-1b. PCOX-1a contains intron-1 whereas this sequence is removed by splicing in PCOX-1b. The deleted portion of PCOX-1 proteins contains structural helices HE, H1, H2, H3, H5, and part of H6, which constitute part of the cyclooxygenase and peroxidase catalytic sites. Consequently, PCOX-1 proteins do not make prostaglandins (Chandrasekharan et al., 2002); however, the critical proximal ligand to heme is not deleted and, therefore, PCOX-1, like their distant relatives PIOX and linoleate diol synthase, may be fatty acid oxidases or isomerases. It is important to note that the intron/exon placements in mammalian COX-1 and COX-2 genes are strictly conserved except for intron-1 in COX-1. COX-2 genes lack this intron. Therefore, it is possible that a PCOX-2 protein exists that would be analogous to PCOX-1b; however, a PCOX-2a could not exist because COX-2 lacks the equivalent of intron-1 in COX-1. Exons 2 through 5 and 7 in COX-1 and exons 2 through 4 and 6 in

COX-2 genes all have the potential of producing in-frame deletions if excised during pre-mRNA splicing. Simultaneously skipping exons 6 and 8 in COX-1 or exons 5 and 7 in COX-2 transcripts also produces in-frame deletions. Thus many different splice variants of COX-1 and COX-2 can be generated by exon skipping that produce proteins that potentially contain a heme binding site. In addition to the above splice variants that affect the coding region of COX-1, a number of alternatively polyadenylated transcripts are known. COX-1 in some human cells and tissues (e.g., endothelial cells) is expressed as three transcripts of 2.8, 4.5, and 5.2 kb (Hla, 1996). The 2.8-kb transcript encodes COX-1 and is the most abundant of these mRNAs. The 4.5-kb transcript has been poorly characterized. The 5.2-kb transcript arises by read-through of the consensus polyadenylation site and termination at another consensus termination site that is approximately 2.7 kb downstream (Plant and Laneuville, 1999). The 5.2-kb transcript was expressed at highest levels in human bladder and colon where its level exceeded that of the 2.8-kb transcript. A 5.2-kb COX-1 mRNA in cerebral cortex, other regions of the forebrain, heart, and muscle can contain all or part of intron-1 and is the human analog of the COX-3 mRNA in dog (Chandrasekharan et al., 2002). In megakaryocytes, all three transcripts can be induced to different extents by mitogens such as phorbol esters (Plant and Laneuville, 1999). COX-1 in NIH3T3 cells is expressed as two transcripts of 2.8 and >7.0 kb in size (Evett et al., 1993). The 2.8-kb transcript encodes COX-1 and is greater than 10 times the abundance of the >7.0-kb transcript, which has been poorly characterized. At least some of the >7.0-kb transcript contains intron-1 and is analogous to the 5.2-kb intron-1-containing transcript in humans. COX-2 is expressed in many organisms as three alternatively polyadenylated transcripts of 4.2, 3.8, and 2.2 kb in size. The 3.8- and 2.2-kb transcripts arise from polyadenylation at cryptic nonconsensus sites containing the sequence AUUAAA (Evanson, 2002). Noncoordinated expression of these transcripts has been observed (Evanson, 2002). For example, rat spermatogonial cells contain primarily a 2.8-kb COX-2 transcript, and COX-2 in these cells was found to localize primarily within the nucleus (Neeraja et al., 2003). Thus alternative 3'-untranslated regions may serve to direct subcellular locations of COX isoenzymes. In addition to variant COX mRNAs, which potentially produce COX or PCOX proteins with altered or expanded biological function, is the issue of mutations and epigenetic (e.g., CpG methylation; Deng et al., 2002) changes in COX genes or regulatory regions that may be involved in disease states. Numerous COX-1 and COX-2 single nucleotide polymorphisms (SNPs) have been identified, and a more complete discussion of them has been done by Cipollone and Patrono (2002). Silent and nonsilent SNPs have been identified in COX coding regions, and SNPs of unknown function have also been identified in COX introns, untranslated regions, and upstream regulatory regions (Cipollone and Patrono, 2002). Because of the central role that COX-1 and COX-2 play in physiological and pathophysiological

processes such as inflammation and cancer, it is anticipated that SNPs in COX genes may result in altered susceptibility to diseases. Although the genetic/epidemiological data are at present limited, early studies suggest this to be the case. Lin et al. (2002) associated a Val511Ala polymorphism found in some African Americans with a potential decreased susceptibility to colon cancer (odds ratios 0.56 and 0.67 in two separate study populations). Other SNPs found in the COX-2 promoter region and in intron-6 have been associated with a higher prevalence of type 2 diabetes mellitus in Pima Indians (Konheim and Wolford, 2003). Patients heterozygous for two single nucleotide changes in the COX-1 gene (A842G/C50T) demonstrated greater inhibition of platelet COX activity by aspirin. Finally, a SNP (-756 G>C) in the COX-2 gene promoter has been associated with lower promoter activity. Patients carrying this allele had lower C-reactive protein levels 1 to 4 days after coronary artery bypass graft surgery (Papafili et al., 2002). Thus, future studies of COX variants and mutants are likely to yield new and exciting insights into the roles of COX gene products.

1.6 Synthetic Cyclooxygenase Inhibitors—Nonsteroidal Anti-Inflammatory Drugs (NSAIDs)

NSAIDs have been prominent analgesic/anti-inflammatory/antipyretic medications since 1898 when aspirin was first marketed. COX-2-selective drugs were introduced in 1999. All NSAIDs act as inhibitors of the cyclooxygenase active site of COX isozymes. Important mechanistic differences in the actions of individual NSAIDs with the COX active site are complex.

1. Aspirin. Of the NSAIDs in medical use only aspirin is a covalent modifier of COX-1 and COX-2. The crystallographic studies of Garavito and colleagues (Loll et al., 1995) demonstrated why this drug so efficiently acetylates serine 530 of COX-1. Like other NSAIDs, aspirin diffuses into the COX active site through the mouth of the channel and traverses up the channel to the constriction point formed by Arg120, Tyr355, and Glu524. At this point in the channel, the carboxyl of aspirin forms a weak ionic bond with the side chain of Arg 120, positioning aspirin only 5 Å below Ser530 and in the correct orientation for transacetylation (Loll et al., 1995). Because the catalytic pocket of the channel is somewhat larger in COX-2 than in COX-1, orientation of aspirin for attack on Ser530 is not as good, and transacetylation efficiency in COX-2 is reduced. This accounts for the 10- to 100-fold lowered sensitivity to aspirin of COX-2 in comparison to COX-1.

2. Competitively Acting Nonsteroidal Anti-Inflammatory Drugs. Other NSAIDs besides aspirin inhibit COX-1 and COX-2 by competing with AA for binding in the COX active site. However, NSAIDs significantly differ from each other in whether they bind the COX active site in a time-dependent or independent fashion.

a. Time Dependence. NSAIDs differ dramatically with regard to how quickly they productively bind in the COX active site and how quickly they come out of the COX channel (Marnett and Kalgutkar, 1998). Some NSAIDs have very rapid on and off rates, such as ibuprofen (Selinsky et al., 2001). Such drugs do not show time dependence. They inhibit COX activity essentially instantaneously after addition of the NSAID, and they readily wash out of the COX active site when the NSAID is removed from the environment of the enzyme. In contrast, many NSAIDs such as indomethacin and diclofenac are time-dependent. They require typically seconds to minutes to bind the COX active site. Once bound, however, these drugs typically have low off-rates that may require hours for the NSAID to wash out of the active site. Time-dependent NSAIDs compete very poorly with AA in instantaneous assays of COX activity. Time-dependent NSAIDs bind the COX active site first in a loose interaction and then in a productive tight complex. The rate-limiting step in drug binding is the formation of the tight binding conformation of the NSAID within the COX channel. Of particular importance to this second step in NSAID binding is the constriction point created by the hydrogen bonding network of Arg120, Tyr355, and Glu524 and the proposed difficulty for some NSAIDs to traverse it. A plausible scenario is that time-dependent NSAIDs likely require conformational heterogeneity in the constriction site caused by molecular breathing of the polypeptide to enter into the upper portion of the catalytic channel. One open state of the COX-2 enzyme has been identified crystallographically (Luong et al., 1996). An open state of the COX-1 enzyme that allows NSAIDs to pass the constriction point is likely to be transient since crystallographic studies show no difference in COX-1 conformation bound to time-dependent or nondependent NSAIDs (Selinsky et al., 2001). Once having passed through the constriction site into the catalytic pocket, carboxyl-containing NSAIDs form a salt bridge between the carboxylate of the NSAID and the guanidinium moiety of Arg120 in COX-1 (Loll et al., 1995). The ionic bond formed, however, is stronger for competitively acting NSAIDS than for aspirin. Hydrophobic interactions between the aromatic ring(s) of NSAIDs and the hydrophobic amino acids lining the channel further stabilize binding. The sum of these interactions results in tight binding of many NSAIDs at the constriction point of the channel, where they totally block entry of AA. Cocrystallization studies have been performed for flurbiprofen and COX-1 and COX-2 as well as indomethacin and COX-1, which define the precise binding interactions of carboxyl-containing NSAIDs in the COX binding site (Picot et al., 1994).

b. Selective Cyclooxygenase Inhibitors. Celecoxib (Celebrex) and rofecoxib (Vioxx) were marketed in 1999 as the first NSAIDs developed as selective COX-2 inhibitors. Other NSAIDs including meloxicam (Mobic), nimesulide, and etodolac (Lodine), which were marketed earlier

in Europe or the United States as safer NSAIDs, were found after the discovery of COX-2 to be preferential inhibitors of this enzyme (**Fig. 5**). Currently, second generation COX-2 inhibitors, such as valdecoxib (Bextra; Smith and Baird, 2003) and etoricoxib (Hunt et al., 2003) are in use or are coming to market as are other COX-2-selective agents such as lumiracoxib (Ding and Jones, 2002). NS398 is a particularly important COX-2 inhibitor that is not in clinical use but is commercially available and, therefore, is widely used in pharmacology studies. Celecoxib and rofecoxib are diaryl compounds containing a sulfonamide and methylsulfone, respectively, rather than a carboxyl group. Each of these compounds is a weak time-independent inhibitor of COX-1, but a potent time-dependent inhibitor of COX-2. Like time-dependent carboxyl-containing NSAIDs, time dependence for celecoxib and rofecoxib requires these compounds to enter and be stabilized in the catalytic pocket (Gierse et al., 1999). However, because these drugs lack a carboxyl group, stabilization of binding for both of these drugs does not require Arg120. Instead, a sum of hydrophobic and hydrogen bonding interactions stabilizes binding. Of particular importance is penetration of the sulfur-containing phenyl ring into the hydrophobic outpocketing in the COX-2 catalytic pocket shown in (Kurumbail et al., 1996). The structural basis for NS398 selectivity toward COX-2 is unclear, since its sulfonamide moiety is coordinated in the COX active site by ion pairing, just like carboxyl moieties in nonselective NSAIDs (Marnett and Kalgutkar, 1998).

3. Analgesic/Antipyretic Drugs. Acetaminophen (paracetamol in the United Kingdom) and dipyrone (**Fig. 5**) are important pain and fever relievers that lack anti-inflammatory activity. Acetaminophen is used primarily in North America and Western Europe whereas dipyrone is used extensively in Mexico, South America, Eastern Europe, and Africa. Although older than aspirin and used extensively for decades, acetaminophen has no certain mechanism of action. Flower and Vane (1972) proposed a central action for acetaminophen of inhibiting COX activity in brain. Indeed, neither acetaminophen nor dipyrone is acidic and both agents cross the blood-brain barrier well, but acetaminophen is a poor inhibitor of purified COX enzymes (Ouellet and Percival, 2001). Marginal inhibition of COX-1 can be achieved by performing inhibition studies at low arachidonate levels in the presence of low oxidant tone (Ouellet and Percival, 2001). Even under these conditions COX-2 was not inhibited at physiological concentrations. In whole cells, COX inhibition by acetaminophen has been observed in microglia (Greco et al., 2003), platelets, and leukocytes (Sciulli et al., 2003). Oates et al. (Boutaud et al., 2002) showed that in human umbilical vein endothelial cells in culture, acetaminophen inhibits COX-2 with an IC_{50} of 66 μM, well within the therapeutic range in humans. It is unclear what factors may make COX susceptible to inhibition by acetaminophen in these whole cells, although changes in oxidant tone have been proposed (Boutaud et al., 2002). Recently,

Chandrasekharan et al. (2002) identified a COX-1 variant, COX-3, that was sensitive to inhibition by acetaminophen and dipyrone in whole insect cells expressing the protein. The variant was identified in dog brain and may represent a central target of analgesic/antipyretic drugs. Salicylate has analgesic, antipyretic, and anti-inflammatory activity, but unlike aspirin, is a poor inhibitor of COXs *in vitro*. In this regard, it resembles acetaminophen. Mitchell et al. (1997) found that salicylate does inhibit COX activity when substrate concentrations are maintained at low levels, similar to the findings of Ouellet and Percival (2001) for acetaminophen. Recently Oates and Marnett have proposed that acetaminophen and salicylate both inhibit COX by redox mechanisms with sodium acetaminophen acting as a peroxidase cosubstrate and sodium salicylate acting at the cyclooxygenase active site (Aronoff et al., 2003).

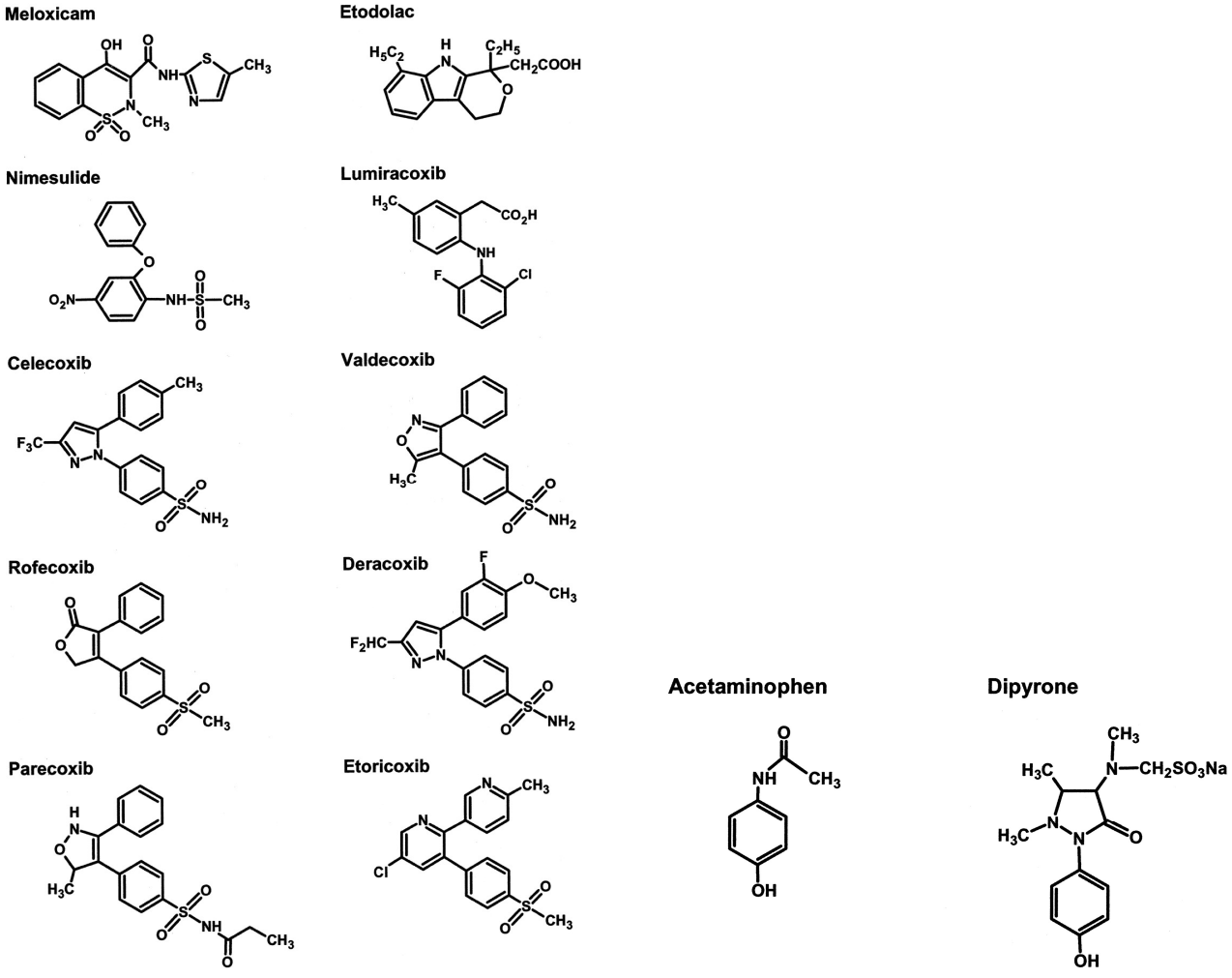


Figure 5. Structural comparison of selected commercially available COX-2 selective inhibitors.

2. Pharmacological Actions of Cyclooxygenase Isozyme-Generated Prostanoids

2.1 Prostaglandin Receptors

Prostaglandin receptors are designated by the letter "P" and a prefix of "D", "E", "F", "I", or "T" to signify preference for prostaglandins D, E, F, I, or thromboxane, respectively. To date, four subtypes of EP receptors have been identified, EP1–EP4. In addition to classical prostanoids that act via plasma membrane-derived G-protein-coupled receptors, several COX products such as PGJ_2, 15-deoxy-^{12,14}-PGJ_2 (15d-PGJ_2) and PGA_2 can activate nuclear receptors of the PPAR class. Although it is not clear whether these classes of compounds are generated under physiological conditions and thus act as physiologically relevant inducers of PPARγ receptors, they are stimulators of this nuclear receptor pathway (Forman et al., 1995). Recent studies show that 15d-PGJ_2 is produced from the COX-2 pathway. 15d-PGJ_2 is found in chronic inflammatory exudates of animal models during the late resolution phase. In this study, the authors showed that treatment with COX-2 inhibitors inhibited the appearance of 15d-PGJ_2, suggesting that it is produced from the COX-2 pathway (Gilroy et al., 1999). Recent studies show that in addition to stimulating the PPARγ receptors, these nuclear-acting prostanoid ligands inhibit the IκB kinase activity and thereby block the NFκB transcription factor pathway (Rossi et al., 2000). Indeed, treatment of vascular endothelial cells and ECV304 bladder cancer cells resulted in cellular apoptosis that requires the PPARγ activity, suggesting that nuclear-acting prostanoids may act to down-regulate angiogenesis. Indeed, PPARγ-activating prostanoids, such as 15d-PGJ_2 induce synoviocyte apoptosis and inhibit the development of adjuvant-induced arthritis in animal models (Kawahito et al., 2000). These data raise the possibility that the COX pathway may induce anti-angiogenic effects by nuclear-acting prostanoids.

2.2 Inflammation

Both PGE_2 and PGI_2 have been found in the synovial fluid from knee joints of arthritic patients (Bombardieri et al., 1981). In the rat model of carrageenan-induced paw edema, PGE_2 is the major PG involved in inflammation and pain, since antibodies to PGE_2 inhibit both symptoms in this rat model (Portanova et al., 1996). Carrageenan-induced hyperalgesia in the rat paw was also reversed by administration of SC58635 (now known as celecoxib), a selective COX-2 inhibitor, demonstrating that PGE_2 synthesis by the COX-2 enzyme is responsible for inflammatory symptoms in this animal model (Zhang et al., 1997). PGI_2 has also been detected in inflammatory lesions, and there may well be species differences, because inflammation is completely suppressed

in mice in which the IP receptor for PGI_2 has been deleted. It is likely, therefore, that both PGE_2 and PGI_2 contribute to the development of inflammatory erythema and pain (Higgs et al., 1978). Unexpectedly, in COX-2 gene-deleted mice the inflammatory response was not affected. However, only an acute inflammatory response was tested, which almost certainly involved COX-1 rather than COX-2. The cyclopentenone PGs may be antiinflammatory since 15-deoxy-PGJ_2 seems to resolve carrageenan-induced pleural inflammation in rats (Rossi et al., 2000). This anti-inflammatory activity may be mediated, at least in part, by inhibition of $I_\kappa B$ kinase (Straus et al., 2000).

2.3 Pain

Prostaglandin E_2 does not cause pain when applied to an unprotected blister base on a human forearm, but greatly potentiates the pain induced by pain-producing mediators such as bradykinin or histamine (Ferreira, 1972). Therefore, Ferreira concluded that the pain-producing action of inflammatory mediators such as bradykinin or histamine was increased when PGs sensitized chemical receptors on primary afferent nerve terminals. PGs are therefore hyperalgesic. To produce its hyperalgesic action, PGE_2 released during the inflammatory response or by other trauma, lowers the activation threshold of tetrodotoxin-resistant sodium channels on sensory neurons. PGI_2 rather than PGE_2 may be involved in short-lasting hyperalgesia since it was more potent than PGE_2 in producing hyperalgesia in the rat and dog models (Ferreira et al., 1978). PGI_2 is mainly responsible for the stretching response to an intraperitoneal (i.p.) injection of zymosan in mice, and IP receptor-deficient mice showed greatly reduced nociceptive responses to i.p. administration of dilute acetic acid (Murata et al., 1997). The stretching response to acetic acid is mediated mainly by COX-1, since it is abolished in COX-$1^{-/-}$ mice (Ballou et al., 2000). Although the major PG involved in the stretching response is PGI_2, stretching responses to acetic acid or phenylbenzoquinone are reduced by 50% in mice with a deleted EP1 receptor. This provides evidence that PGE_2 as well as PGI_2 mediates nociceptive responses to these hyperalgesic agents (Stock et al., 2001). Thus, both PGE_2 and PGI_2 can sensitize nociceptors on sensory nerve terminals to painful stimulation. Several studies, however, suggest that agonists for the IP receptor can activate sensory neurons in the absence of any other nociceptive stimuli. For example, the stable prostacyclin analogs, carbaprostacyclin and iloprost, produce stretching responses when injected i.p. into mice (Akarsu et al., 1989). In addition, PGI_2 and cicaprost increased spontaneous activity and mechanically evoked discharges of articular mechanonociceptors in the rat ankle joint arthritis model. Infusions of iloprost or cicaprost into patients suffering from vascular occlusive disease cause pain at the infusion site and headaches are a frequent side effect of this treatment (Shindo et al., 1991). A high

density of IP receptors have been found on sensory neurons. Binding sites for [^{3}H]iloprost were observed in the rat dorsal root ganglion and the dorsal horn of the spinal cord (Pierce et al., 1995). In the mouse dorsal root ganglion, almost 40% of neurons demonstrated binding for IP receptor mRNA (Oida et al., 1995). IP receptors in sensory neurons are linked to the activation of adenyl cyclase and phospholipase C and can thus modulate the activity of ion channels and neurotransmitter release through activation of protein kinases A and C (Smith et al., 1998).

2.4 Fever

Fever is caused by PGE_2 released by inflammatory mediators from endothelial cells lining the blood vessels of the hypothalamus. Bacterial LPS from infecting organisms, or circulating IL-1, stimulate the expression of COX-2 and of PGE synthase in endothelial cells that constitute the blood-brain barrier (Samad et al., 2001). PGE_2 generated by PGE synthase diffuses out of the endothelial cells into the organum vasculosum lamina terminalis (OVLT) region of the hypothalamus, which is responsible for controlling fever. The pyretic action of PGE_2 is mediated by the EP3 receptor, since mutant mice lacking this receptor do not develop fever after administration of PGE_2, IL-1, or LPS (Ushikubi et al., 1998). Pyrexia-producing PGE_2 is formed by COX-2 for selective COX-2 inhibitors, such as rofecoxib, abolish fever in several species, including humans and LPS fails to raise the core temperature of COX-$2^{-/-}$ mice (Li et al., 1999). Although COX-2 is primarily involved in the fever response to LPS, source components of this response are dependent on COX-1 (Zhang et al., 2003)

2.5 Immune System

Mouse macrophages stimulated with inflammatory mediators to induce COX-2 release PGE_2 and PGI_2, whereas stimulated human monocytes and macrophages secrete large amounts of PGE_2 together with TXA_2 (Fels et al., 1986). Neutrophils make moderate amounts of PGE_2, whereas mast cells produce almost exclusively PGD_2. No prostanoids appear to be made by lymphocytes, although both COX-1 and COX-2 have been detected in these cells (Pablos et al., 1999). Release of PGE_2 by macrophages may act as a negative feedback control mechanism, reducing further activation through increase of cAMP thus resulting in inhibition of immune function. PGE_2 also inhibits IL-2 and interferon γ(IFNγ) production from T lymphocytes (Betz and Fox, 1991) and IL-1 and TNFα release from macrophages; however, immature cells of the immune system are stimulated by PGE_2. For example, PGE_2 induces immature thymocytes and B lymphocytes to differentiate and acquire the functional characteristics of mature cells (Parker, 1986). It has been suggested that PGE_2 produced by tumor cells accounts for the depression of the immune system associated with cancer.

Large amounts of PGs are produced by certain tumor cells, which induce a generalized state of immunodeficiency (Plescia et al., 1975). This immunosuppression was prevented in tumor-bearing mice by inhibitors of PG synthesis such as indomethacin (Pollard and Luckert, 1981). Treatment of rheumatoid arthritis with aspirin-like drugs leads to inhibition of PG formation and thus to removal of the immunosuppressant effect of these eicosanoids. Removal of immunosuppression may be one of the factors responsible for the cancer-inhibiting action of the NSAIDs. Another consequence of removing the suppression of immune processes by PGs may be the enhancement of cartilage breakdown seen with NSAIDs *in vitro* and *in vivo*.

2.6 Gastrointestinal Tract

PG synthesis can be demonstrated to occur in every part of the gastrointestinal tract. In rat tissues, using vortex generation, the rank order of PG synthesis, as determined by bioassay techniques, was greatest in gastric muscle and forestomach, followed by gastric mucosa, colon, rectum, ileum, cecum, duodenum, jejunum, and esophagus (Whittle and Salmon, 1983). PGE_2 contracts gastrointestinal smooth muscle through stimulation of smooth muscle EP1 receptors. Prostanoids are "cytoprotective" in the gastrointestinal tract, as strikingly demonstrated by the finding in rat that gastric damage induced by topical application of strong acids, hypertonic solutions or ethanol, could be reduced by coadministration of various PGs (Miller, 1983). The mechanism of the cytoprotective action is complex and depends on a combination of several mechanisms.

1. Both PGE_2 (acting on the EP3 receptor) and PGI_2 (acting on the IP receptor) reduce secretion of gastric acid, even histamine-stimulated acid secretion, by the parietal cells of the stomach. This action is species-dependent since PGI_2 is more active than PGE_2 in anesthetized rat, conscious dog, and monkey, whereas PGE_2 is a more potent inhibitor of acid secretion in the stomach of the anesthetized dog (Shea-Donohue et al., 1982).

2. Intravenous infusions of PGE_2 or PGI_2 exert a direct vasodilator action on the gastric mucosa. Increase in gastric mucosal blood flow is obviously beneficial in maintaining the functional integrity of the gastric tissue (Whittle et al., 1978).

3. PGE_2 is synthesized by epithelial and smooth muscle cells in the stomach, and intragastric administration of PGE_2 to humans stimulates the release of viscous mucus, which could play a defensive role against mucosal injury (Allen and Garner, 1980) by gastric acid. Other than providing a physical barrier, mucus may act to create an unstirred layer of secreted bicarbonate on the epithelium (Bahari et al., 1982) and hence help to neutralize hydrogen ions diffusing back

from the lumen into the mucosa. PGE_2 stimulates bicarbonate secretion via the EP3 receptor, thus application of acid induces more severe damage to the stomach mucosa in $EP3^{-/-}$ mice than in wild-type animals (Takeuchi et al., 1999).

Most surprising has been the finding that animals without the COX-1 gene did not spontaneously develop stomach ulcers (Langenbach et al., 1995). This has been explained by postulation of an adaptation process whereby increased production of nitric oxide or calcitonin gene-related peptide may have taken over the cytoprotective role of the absent PGs. An alternative explanation is that both COX-1 and COX-2 may be required for gastrointestinal mucosal defense. $COX\text{-}1^{-/-}$ or $COX\text{-}2^{-/-}$ mice were more susceptible to colonic injury with dextran sodium sulfate than wild-type mice, but the administration of a selective COX-2 inhibitor exacerbated the mucosal injury with dextran sodium sulfate in $COX\text{-}1^{-/-}$ mice (Morteau et al., 2000). Similarly, neither the selective COX-1 inhibitor, SC-560, nor the selective COX-2 inhibitor, celecoxib, administered to rats produced gastric damage, even though SC-560 reduced both gastric PGE_2 synthesis and gastric blood flow; however, the combination of SC-560 with celecoxib resulted in gastric erosions in all rats. Celecoxib, but not SC-560, increased leukocyte adherence to the vascular endothelium of the gastrointestinal microcirculation. Thus, it appears that inhibition of the activity of both COX-1 and COX-2 is required to produce gastric damage (Wallace et al., 2000). This work was confirmed by Gretzer et al. (2001) who showed that cotreatment of rats with SC-560 and the COX-2-selective inhibitor, rofecoxib, induced severe gastric lesions.

2.7 Cardiovascular System

Various prostanoids are secreted by vascular cells, including PGI_2, PGE_2, and $PGF_{2\alpha}$, among others. In addition, cells in the vascular wall respond to various prostanoids. The major prostanoid secreted by endothelial cells is PGI_2, as the prostacyclin synthase enzyme is enriched in this cell type. This prostanoid binds to the IP receptors on vascular smooth muscle cells and inhibits vascular contraction (FitzGerald et al., 1983). The IP receptor couples to the G_s protein and increases intracellular cAMP concentrations, thus antagonizing the contractile agonists and inhibiting the mitogen-activated protein kinase pathway (FitzGerald and Patrono, 2001). In platelets, the IP receptor signaling antagonizes the aggregation response and thus inhibits thrombosis. PGI_2 synthesis by the COX pathway is important in normal control of vascular homeostasis and thrombosis. Interestingly, an unexpected role of PGI_2 in the control of the inflammatory process was elucidated by the deletion of the IP receptor in knockout mice (Murata et al., 1997). This is probably related to the ability of PGI_2 to induce vascular relaxation, which is important in the increased blood

flow that occurs during inflammation. PGE_2 and $PGF_{2\alpha}$, in contrast to PGI_2 can induce either vasoconstriction or vasorelaxation, depending on the vascular bed (FitzGerald et al., 1983). These effects are mediated by specific expression of the respective receptor subtypes on the vascular smooth muscle cells. These findings indicate that the products of the COX pathway mediate complex and critical homeostatic interactions in the vessel wall. PGE_2 can also potently relax vascular smooth muscle contributing to the characteristic vasodilatation (via the EP2 receptor) leading to the erythema seen in acute inflammation. This increases blood flow through inflamed tissues and thus augments the extravasation of fluid, facilitating edema formation (Williams and Peck, 1977). EP2 receptors generally mediate arterial dilatation and are also involved in salt-sensitive hypertension. An infusion of PGE_2, normally hypotensive, raised blood pressure in $EP2^{-/-}$ mice, whereas $EP2^{-/-}$ mice fed a high salt diet became hypertensive. There was no change in systolic blood pressure of control animals on a high salt diet (Kennedy et al., 1999). Blood platelets contain only COX-1, which converts AA to the potent pro-aggregatory and vasoconstrictor eicosanoid TXA_2, the major COX product formed by platelets. TXA_2 has a half-life at body pH and temperature of 30 s, degrading to inactive TXB_2. It was proposed in 1976 that PGI_2 and TXA_2 represent the opposite poles of a homeostatic mechanism for regulation of hemostasis *in vivo* (Moncada et al., 1976). Stimulation of the TP receptor on platelets leads to their aggregation and $TP^{-/-}$ mice have greatly prolonged bleeding times, demonstrating the importance of TXA_2 in hemostasis (Murata et al., 1997). TP receptors are coupled through regulatory G proteins to increased intracellular phosphoinositol hydrolysis. Antagonists for the TP receptor on platelets are of interest to inhibit platelet aggregation and prevent further thrombosis after myocardial infarction. TXA_2 also causes contraction of all vascular and airway smooth muscle by stimulating the TP receptor. As expected, blood platelets of mice with a nonfunctional COX-1 gene did not aggregate to AA (Langenbach et al., 1995). Normal endothelial cells and vascular smooth muscle cells express COX-1; however, COX-2 was identified as a shear stress-inducible gene in vascular endothelial cell cultures (Topper et al., 1996). These data suggest that vascular endothelial cells express COX-2 in response to normal blood flow. Indeed, recent studies in human volunteers after administration of COX-2 inhibitors suggest that total body prostacyclin synthesis (as measured by the quantitation of urinary metabolites) is contributed significantly by the COX-2 isoenzyme (McAdam et al., 1999). Immunocytochemical studies, however, suggest that COX-2 expression in normal vessels (both large and small) is negligible to undetectable, whereas that of COX-1 is readily detectable (Crofford et al., 1994). These observations suggest that COX-1 is highly expressed whereas COX-2 is expressed at a much lower level in the normal vascular tissues. In contrast, high levels of COX-2 are detected in activated and proliferating vascular tissues, for example angiogenic microvessels,

atherosclerotic lesions, and inflamed tissues. Normal production of prostacyclin is critical for vessel tone control and inhibition of thrombosis (FitzGerald et al., 1983). This is because PGI_2/TXA_2 balance is critical. After ingestion of aspirin, platelet thromboxane synthesis and vascular prostacyclin synthesis are inhibited rapidly; however, nucleated vascular cells recover their ability to synthesize prostacyclin rapidly, estimated to be ~6 h. In contrast, thromboxane synthesis is inhibited for a much longer period, since platelets lack the ability to resynthesize COX via de novo protein synthesis (Jaffe and Weksler, 1979). Thus, aspirin is a first line of defense against thrombotic and vaso-occlusive vascular diseases. Whether COX-2 inhibition presents a risk for thrombotic events requires further study. Atherosclerotic lesions occur in large vessels like the carotid and the coronary arteries. Plaques are classified as stable and unstable plaques, depending on the degree of lipid accumulation and inflammation in the pathologic tissue. Unstable plaques have high levels of metalloproteinase production, contain more activated foam cells, and have lipid accumulation in the lesion. They are highly prothrombotic and lead to rupture, culminating in the occlusion of the vessel (Lusis, 2000). COX-2 expression was found to be elevated in human atherosclerotic plaques. Monocytic foam cells and vascular endothelial cells, express high levels of COX-2 in these lesions (Schonbeck et al., 1999). It is likely that oxidized low-density lipoprotein components, such as lysophosphatidyl choline, as well as cytokines and growth factors found in the lesions induce the COX-2 gene expression. The role of COX-2 in these lesions in not well understood. Studies with COX-2 null mice and COX-2 inhibitors are required to better define the causal role (if any) of COX-2 in atherosclerosis and other vascular pathologies. Recently there is renewed interest in the role of COX products in the formation of new vessels, a process commonly referred to as angiogenesis (Hla et al., 1993). Early studies by Gullino showed that PGE_2 is a potent inducer of angiogenesis in the corneal models of angiogenesis (Ziche et al., 1982); however, the mechanisms involved are not well understood since PGE_2 does not potently stimulate endothelial cell migration, proliferation, and morphogenesis. In addition, various *in vivo* studies indicate that NSAIDs inhibit angiogenesis in various *in vivo* models. Such studies were inconclusive since the doses of drugs used to inhibit angiogenesis are much higher than the doses required to inhibit COX-1 or -2 activity. It is known that high concentration of NSAIDs have effects independent on COX enzyme activity. Various studies reported that PGE_2 is a potent inducer of vascular endothelial cell growth factor (VEGF) expression in rheumatoid synovial fibroblasts and osteoblasts. These data suggest that PGE_2 can induce angiogenesis indirectly by up-regulating VEGF expression of stromal cells. The receptor subtype involved in VEGF expression is not known, but cAMP increases may be involved, suggesting that EP2 and/or EP4 subtypes may be critical (Ben-Av et al., 1995). The induction of angiogenesis by the COX-derived PGE_2 may be potentially involved in colon cancer . Tsujii et al.

(1998) reported that enhanced COX-2 expression in colon cancer cells modulates the angiogenic behavior of endothelial cells in the coculture system. The authors showed that secretion of angiogenic factors, such as VEGF and fibroblast growth factor, is modulated by COX-2 overexpression. The mechanistic details of how COX-2 regulates angiogenic growth factor expression secretions are not well understood. Furthermore, the DuBois laboratory has recently extended these findings and showed that tumor angiogenesis and growth of explanted tumors are reduced in the COX-2 null mice; suggesting that host COX-2 induction by the tumor cells contributes to tumor angiogenesis and ultimately the growth of the tumors. These observations, coupled with the findings that COX-2 is overexpressed in the angiogenic lesions, suggest that COX-2 inhibitors may possess an antiangiogenic effect in various pathologic conditions such as rheumatoid arthritis and solid tumors; however, effective inhibition of angiogenesis should lead to disease modification in rheumatoid arthritis, an effect that is not observed in chronic clinical use of NSAIDs and COX-2 inhibitors (Crofford, 2000). Thus, the role of COX-2 and its products in angiogenesis in various pathological contexts is unclear at present. The effects of COX-1 and -2 on angiogenesis are assumed to be due to the actions of secreted prostanoids that act in an autocrine and/or paracrine manner; however, the ability of the peroxidase activity to utilize various reducing equivalents may also contribute to those effects (Ohki et al., 1979). Some *in vitro* and *in vivo* studies have raised the possibility that NSAID-treated COX enzymes as well as active site mutants of the COX enzymes that fail to synthesize prostanoids induce effects on transfected cells (Trifan et al., 1999). Although these studies are suggestive, definitive involvement of the peroxidase activity of the COX enzyme in various physiological phenomena dependent on COX-1 or -2 expression is not yet demonstrated. Although the mechanistic studies have yielded a multitude of possibilities of the COX pathway to regulate angiogenesis, unequivocal evidence that COX enzymes regulate angiogenesis is lacking at present. Clearly, angiogenesis during embryo development does not require the COX pathway since the vascular system develops normally in both COX-1- and COX-2-deficient mice. However, embryo implantation defects seen in COX-$2^{-/-}$ female mice may be related to the effect of prostanoid induction of angiogenesis in the uterine implantation site. In this system, the PPARδ nuclear receptor is essential and induction can be restored by carbaprostacyclin, a pharmacological agonist of the PPARδ receptor; however, various NSAIDs inhibit angiogenesis in models of angiogenesis, such as the sponge and corneal model (Majima et al., 1997). Further studies using both selective antagonists, enzyme inhibitors, as well as receptor gene null mice are required to establish the role of the COX enzymes in angiogenesis *in vivo*. In contrast, prostanoids are required for maintenance of an open ductus and for its closure in the postnatal period (Loftin et al., 2001). The transition from maternal to fetal circulation is followed by the closure of the ductus,

which separates the arterial and venous systems and thereby allows for efficient pulmonary blood flow (Loftin et al., 2001). Premature closure of the ductus leads to abnormal pulmonary pressure and lung dysfunction. Clinical studies have shown that indomethacin treatment produces closure of the ductus, suggesting that prostanoids mediate this process. Indeed, COX-1 and -2 double null homozygous mice develop neonatal circulatory failure due to the failure to close the ductus arteriosus. This phenotype was also seen in EP4 null mice, suggesting that PGE_2 signaling via the EP4 receptor is involved; however, the role of other prostanoids such as thromboxane A_2 is also implicated (Loftin et al., 2001). These data indicate the essential nonredundant function of prostanoids in vascular development and remodeling in the neonatal period (Ushikubi et al., 2000). Whether similar mechanisms operate in pathological vascular remodeling is not known.

2.8 Kidney

The cortex of normal kidneys produces mainly PGE_2 and PGI_2 with very small amounts of TXA_2. The renal medulla produces mostly PGE_2 for which it has a synthetic capacity approximately 20 times that of the cortex (Zusman and Keiser, 1977). Urinary PGE_2 levels are generally regarded as reflecting production of PGE_2 by the kidneys. PGE_2 and PGI_2 have vasodilator actions in the kidney, and intrarenal infusions of these PGs increase renal blood flow. PGs are also natriuretic, inhibiting tubular sodium reabsorption, and in the thick ascending limb of the loop of Henle, they reduce chloride transport. Glomerular epithelial and mesangial cells have the synthetic capacity to form both PGI_2 and PGE_2. These prostanoids are therefore uniquely situated to influence renal blood flow, glomerular filtration rate, and the release of renin. PGI_2 and PGE_2 synthesized in the renal cortex are important stimulators of renin release (Osborn et al., 1984). PGI_2 formed by COX-2 in mesangial cells may directly stimulate renin secretion since upregulation of COX-2 has been observed in the macula densa following salt deprivation (Harris et al., 1994). Different nephron segments synthesize a distinctive spectrum of AA metabolites that behave as either modulators or mediators of the actions of hormones on tubular function. Studies on rabbit medullary cells in the thick ascending limb of Henle's loop (mTALH) revealed that the principal pathway of AA metabolism in this segment of the nephron is via cytochrome P450 (P450) and not COX-1 (Schwartzman et al., 1985). Thus, the major P450-derived AA products synthesized by the rabbit mTALH are 19- and 20-hydroxyeicosatetraenoates (HETEs) and 20-COOH HETE, a metabolite of 20-HETE; however, some COX-2 protein is also expressed constitutively in unstimulated mTALH cells and COX-2 expression increases after treatment with TNF or phorbol 12-myristate 13-acetate (Ferreri et al., 1999). In addition, the products of P450 interact with TNF formed by mTALH cells and with angiotensin II to regulate ion transport in cells of the mTALH. Maintenance of kidney

function in animal models of disease states and in patients with congestive heart failure, liver cirrhosis, or renal insufficiency is dependent on vasodilator PGs. These patients are, therefore, at risk of renal ischemia when PG synthesis is reduced by NSAIDs. Synthesis of PGE_2 is mainly by COX-1, although as mentioned above, there are discrete cells in the macula densa that contain constitutive COX-2. Prostacyclin, made by constitutive COX-2 may drive the renin-angiotensin system (Harris, 1996). FitzGerald's group compared the renal effects of the nonselective COX inhibitor indomethacin with those of the selective COX-2 inhibitor rofecoxib and with placebo in healthy older adults over 2 weeks of treatment. Both active regimes were associated with a transient but significant decline in urinary sodium excretion during the first 72 h. The glomerular filtration rate (GFR) was decreased by indomethacin but not changed significantly by rofecoxib. Thus, acute sodium retention by NSAIDs in healthy adults is mediated by inhibition of COX-2, whereas depression of GFR is due to inhibition of COX-1. The urinary excretion of the PGI_2 metabolite, 2,3-dinor-6-keto-$PGF_{1\alpha}$ was decreased by both rofecoxib and indomethacin, but not by placebo (Catella-Lawson et al., 1999). The implication of this is that prostacyclin is synthesized in endothelial cell by COX-2 rather than COX-1. COX-2 is possibly continuously induced by the shear stress on the arterial wall, rather than being present constitutively (Topper et al., 1996). Young male and female COX-2 gene-deficient mice showed arrested development of the kidneys. Rodent kidneys develop fully only after birth, and COX-2 appears to be important in this process. Failure to develop mature kidneys shortened the life span of the COX-2 gene null mice to approximately 8 weeks. This retardation of renal cortical development could be mimicked in mice and rats by chronic administration of a selective COX-2 inhibitor to the mother during pregnancy and to the pups until weaning (Kömhoff et al., 2000). Failure to develop mature kidneys shortens the life span of COX-2 null mice to approximately 2 weeks in some genetic backgrounds; however, it may be possible to overcome this developmental defect by cross-breeding the original COX-$2^{-/-}$ C57BL/6 mice with a DBA/1 strain (Ballou et al., 2000). The animals of this mixed strain live a full life span, and their kidneys appear to develop normally. This suggests the potent effects of modifier genes on COX-2 regulation of kidney development.

2.9 Lungs

PGs have potent actions on bronchiolar tone and on the diameter of the pulmonary blood vessels. The airways of most species, including humans, contract to $PGF_{2\alpha}$, TXA_2, and PGD_2, whereas PGE_2 and PGI_2 are weak bronchodilators. PGD_2 and $PGF_{2\alpha}$ potently constrict the airways in asthmatic patients and potentiate the constrictor responses to other spasmogens (Fuller et al., 1986). The concentrations of PGD_2 and $PGF_{2\alpha}$ in bronchoalveolar lavage fluid of asthmatic subjects were 10-

fold higher than in control nonasthmatic and atopic individuals. Excretion of the stable metabolite of TXA_2 increases after allergen challenge (Sladek et al., 1990). Thus, raised levels of bronchoconstrictor PGs in the lungs may contribute to allergic bronchospasm during asthmatic attacks. Pulmonary blood vessels are constricted by $PGF_{2\alpha}$ and TXA_2, but in some species they dilate to PGE_2. Prostacyclin is a potent vasodilator of the pulmonary circulation in humans and other species. Blood levels of prostacyclin increase 15- to 20-fold in anesthetized patients with artificial ventilation. This endothelium-derived prostacyclin is well placed to function as a local vasodilator and to prevent the formation of microthrombi. PGI_2 may be important in regulating pulmonary vascular tone during chronic hypoxia. Overexpression of prostacyclin synthase (PGIS) in lung epithelium of transgenic mice prevented development of pulmonary hypertension after exposure to hypobaric hypoxia, whereas lungs of patients with severe pulmonary hypertension expressed lower levels of PGIS than those of control subjects (Tuder et al., 1999). Mediators of inflammation such as bradykinin, histamine, and 5-hydroxytryptamine release PGs from lung tissue. Histamine releases $PGF_{2\alpha}$ from human lung fragments by stimulating H1 receptors. Lungs of asthmatics produce more histamine than normal lungs, which correlates with the greater number of mast cells found in asthmatic lungs (Holgate, 1986). Pro-inflammatory cytokines such as IL-1β and TNFα are present in the inflamed airways of asthmatic patients and induce COX-2 expression in lung epithelial cells, airway smooth muscle, pulmonary endothelial cells, and alveolar macrophages (Mitchell et al., 1995). In the carrageenan-induced pleurisy model of inflammation, levels of COX-2 in the cell pellets of pleural exudate increased maximally 2 h after the injection of carrageenan. This was accounted for by induction of COX-2 in 100% of mast cells, in 65% of resident mononuclear leukocytes, and in 8% of extravasated neutrophils present in the exudates (Hatanaka et al., 1996). Inflammatory stimuli cause differential release of PGs from various regions of the lungs. Human cultured pulmonary epithelial cells stimulated with LPS, IL-1β, TNFα, or a mixture of cytokines synthesize mainly PGE_2 together with smaller amounts of $PGF_{2\alpha}$, PGI_2, and TXA_2. This PG production can be suppressed by dexamethasone (Mitchell et al., 1994). Thus, PGE_2 is the main product of COX-2 induced in lung epithelial cells and *in vitro* studies in animals suggest that epithelial PGE_2 may protect against bronchoconstriction induced by bradykinin, tachykinins, and endothelin (Devillier et al., 1991). Moreover, evidence in COX-2 null mice suggests that COX-2-produced PGE_2 prevents chemically induced pulmonary fibrogenesis. Endogenous PGE_2 may therefore be bronchoprotective in asthma and other pulmonary conditions and may act as an endogenous anti-inflammatory factor (Pavord and Tattersfield, 1995). Aspirin-induced asthma may be triggered by increased release of leukotrienes from inflammatory cells caused by removal of the inhibitory influence of PGE_2, a major product of COX-2 in airways. The true role of PGs in asthma

is unclear. The nonselective NSAIDs have very little effect on airway function in most patients with asthma except to make the disease worse in aspirin-sensitive asthmatics. Perhaps the actions of the bronchoconstrictor PGs are counterbalanced by the protective dilator action of PGE_2. It remains to be seen whether selective COX-2 inhibitors will be beneficial in allergic asthma; however, mast cells, by producing PGD_2 in response to an allergic challenge, may have a pathological role in allergic asthma. Disruption of the gene encoding the DP receptor in ovalbumin-sensitized mice prevented the infiltration of cytokine-responsive cells into the lungs. This suggests that PGD_2 released from mast cells stimulates production of cytokines and chemokines by an action on DP receptors, which leads to recruitment of inflammatory cells into the lungs. A physiological role has been proposed for constitutive COX-2 in the lung, for it was found in noninflamed rat lungs. COX-2 is present in vascular smooth muscle cells of normal rat lungs as well as in lung macrophages and mast cells. Arachidonic acid perfused through isolated rat lungs forms TXA_2 and causes vasoconstriction, which is blocked dose dependently with selective COX-2 inhibitors, suggesting a physiological role for COX-2 in the regulation of pulmonary blood flow (Ermert et al., 1998).

2.10 Reproduction

Human seminal fluid contains high concentrations of several PGs including PGE_2, PGE_1, PGE_3, and $PGF_{2\alpha}$ (Samuelsson, 1963), which perhaps function to relax corporeal smooth muscle. These PGs may also facilitate conception by stimulating contractions of the cervix, fallopian tubes, and uterus. Prostaglandin E_1 given by injection into the corpus cavernosum has been used as a treatment for impotence. The EP2 receptor is important for *in vivo* fertilization, as $EP2^{-/-}$ mice ovulated normally but the ova failed to become fertilized (Tilley et al., 1999). Because of these problems with ovulation and fertilization, $EP2^{-/-}$ female mice give birth to unusually small litters, emphasizing the importance of PGE_2 in reproductive processes. PGE_2 is involved in ripening of the cervix prior to labor and can itself induce labor at any stage of pregnancy. It is made by COX-1 and COX-2, as is $PGF_{2\alpha}$, in the pregnant uterus, fetal membranes, and umbilical cord. COX-2 mRNA in the amnion and placenta increases considerably immediately before and after the start of labor (Gibb and Sun, 1996). $PGF_{2\alpha}$ is involved in reproductive processes such as ovulation, luteolysis and parturition. In most species, except primates and humans, $PGF_{2\alpha}$ activity made by the induction of COX-2 is required for commencement of parturition, since mutant mice lacking the gene for the FP receptor, are unable to give birth. The action of $PGF_{2\alpha}$ on the FP receptor of the corpus luteum induces luteolysis, which terminates progesterone production, and hence triggers parturition, for, in the absence of progesterone, the uterus becomes more sensitive to oxytocin (Sugimoto et al., 1997). Analogs of $PGF_{2\alpha}$ are, in fact, used to synchronize estrus and to produce luteolysis in farm animals.

In humans, the induction of COX-2 in the amniotic membranes and uterine wall at parturition leads to the synthesis of $PGF_{2\alpha}$ and PGE_2, which contract the smooth muscle to expel the fetus (Allport and Bennett, 2001). Normally, PGs acting on the EP4 receptor maintain the patency of the ductus arteriosus before birth, yet most $EP4^{-/-}$ mouse neonates die within 72 h after birth. Histological examination of these animals showed that the ductus arteriosus remained open. Presumably, in the absence of the EP4 receptor, PGs are no longer involved in maintaining a patent ductus arteriosus. Other mechanisms have taken over the role of PGs, but no means exist for the termination of their action at birth. Thus, normal function of the EP4 receptor is essential to mediate neonatal adaptation of the cardiovascular system (Segi et al., 1998). Moreover, some fetuses of COX-1 null mice and all neonates born to homozygous COX-1/COX-2 null animals did not survive, most likely because their ductus arteriosus remained patent after birth (Langenbach et al., 1995). Deletion of the COX-2 gene in female mice resulted in infertility because they did not ovulate. Thus, COX-2 appears to be essential for ovulation in mice. Ovulation was restored in these animals by treatment with PGE_2 or IL-1β demonstrating the role of PGE_2 in ovulation. The role of IL-1β in restoring ovulation of $COX\text{-}2^{-/-}$ mice requires further clarification.

2.11 Brain and Spinal Cord

COX-1 is found in neurons throughout the brain but it is most abundant in the forebrain (Breder et al., 1995), where PGs may be involved in complex integrative functions such as control of the autonomic nervous system and in sensory processing. COX-2 mRNA is induced in brain tissue and cultured glial cells by pyrogenic substances such as LPS, IL-1, or TNF. Low levels of COX-2 protein and COX-2 mRNA have been detected in neurons of the forebrain without previous stimulation by pro-inflammatory stimuli (Breder et al., 1995). These "basal" levels of COX-2 are particularly high in neonates and are probably induced by nervous activity. Intense nerve stimulation, leading to seizures, induces COX-2 mRNA in discrete neurons of the hippocampus, whereas acute stress raises levels in the cerebral cortex (Yamagata et al., 1993). COX-2 mRNA is also constitutively expressed in the spinal cord of normal rats and may be involved with processing of nociceptive stimuli by releasing PGE_2 (Yaksh and Svensson, 2001). The antihyperalgesic action of NSAIDs is mediated by inhibition of constitutive spinal COX-2 but not COX-1 (Yaksh and Svensson, 2001). Endogenous fever-producing PGE_2 is thought to originate from COX-2 induced in endothelial cells lining the blood vessels of the hypothalamus by circulating LPS or IL-1. PGE_2 is synthesized in the human brain as well as PGD_2, which has a more limited distribution. Large amounts of PGD_2 are found in the brains of mammals and in mast cells but practically nowhere else. In addition to PGD_2 itself, PGD synthase and 15-hydroxy-PGD_2 dehydrogenase (which metabolizes

PGD_2) have been identified in mammalian brains. In young rodents, PGD synthase is localized in neurons, whereas in adult animals it is mainly restricted to oligodendrocytes (Urade et al., 1987). The reason for this selective distribution and the significance of PGD_2 in the brain is unknown. DP receptors have been found in the brain as well as in some vascular smooth muscle and blood platelets. They are coupled to adenylate cyclase through a G_s protein and stimulation results in formation of cyclic AMP. PGD_2 and PGE_2 have opposing actions in sleep and temperature regulation. Microinjections of PGD_2 into the preoptic area of the rat brain induces normal sleep, whereas PGE_2 infused into the region of the posterior hypothalamus causes wakefulness (Hayaishi, 1991). Similarly, administration of PGD_2 lowers body temperature and PGE_2 has a pyretic action. It is interesting that patients with systemic mastocytosis fall deeply asleep after periods of production of large amounts of PGD_2 by their mast cells (Roberts et al., 1980).

3. Cyclooxygenase Isozymes in Human Disease

3.1 Treatment of Inflammatory Diseases

NSAIDs are currently used as first-line therapeutics in the treatment of osteoarthritis (OA), rheumatoid arthritis (RA), systemic lupus erythematosis, and other inflammatory syndromes. In each case, NSAID treatment is palliative rather than disease modifying. NSAIDs reduce inflammation and pain in these syndromes. In the short period of 8 years after the discovery of COX-2, selective inhibitors of this enzyme were developed for use in RA, OA, and for pain relief. Even before the discovery of COX-2, pharmaceutical companies were searching for anti-inflammatory drugs that would have less damaging effects on the stomach than existing therapies. In the 1980s, these experiments resulted in the development of three drugs with anti-inflammatory activity but with very little inhibitory effect on PG production by the stomach. Nimesulide, etodolac, and meloxicam emerged from preclinical studies as anti-inflammatory compounds with less damaging effects on the stomach than established NSAIDs. After the discovery of COX-2 in 1991 (Xie et al., 1991), these drugs were shown to have a selective inhibitory action on COX-2 compared with COX-1; however, after the cloning of COX-2, inhibitors were designed with an even greater selectivity for COX-2. Selectivity for the inducible isoform was established by comparing inhibitory potency against COX-1 measured as the IC_{50} with inhibition of COX-2 in isolated enzymes, cultured cells, or in the whole blood assay. Slightly different measurements of selectivity were obtained in each system, but the relative values between drugs and their order of potency generally remained the same. The most reproducible estimates of selectivity have been obtained by comparing inhibitory potency on recombinant human enzymes or by measuring the selectivity with the human whole blood assay (Patrignani et al., 1994). The latter is considered to resemble most closely the clinical situation in patients taking NSAIDs. Blood proteins are present so that drug binding to protein is accounted for and endogenous human enzymes are used. The inhibitory potency against COX-1 is measured on platelets in clotting blood, whereas potency for inhibition of COX-2 is estimated in blood monocytes previously incubated with endotoxin to induce COX-2. The modified William Harvey whole blood assay (Warner et al., 1999) uses cultured A549 cells, instead of blood monocytes, incubated with IL-1 and added to human blood for estimation of activity against COX-2.

3.2 Neoplastic Disease

Prostanoids may be involved in the pathogenesis of cancers. Early studies have recognized that growth factors, tumor promoters, and oncogenes induce prostanoid synthesis. It is now recognized

that such effects are due to the induction of COX-2 in various cell types (Dubois et al., 1998). Early studies also pointed out that metabolism of AA via the COX pathway is enhanced in various human tumors, compared with the nontumorigenic counterparts. For example, mammary tumors secrete high levels of PGE_2 compared with the normal adjacent mammary tissue. The functional role of such a finding has not been clear; however, several theories have been proposed on the role of tumor-derived prostanoids. For example, induction of angiogenesis, induction of tumor cell proliferation, suppression of immune response, and inhibition of cell death. Early work on animal models of tumorigenesis, indicated that NSAIDs profoundly inhibit colon and breast tumors induced by carcinogens in rodents (Reddy et al., 1987). These data, although correlative, strongly suggested that products of the COX pathway may participate in carcinogen-induced tumorigenesis. These evidences were further supported by epidemiological studies of humans who use aspirin and other NSAIDs chronically; it was found in various studies that incidence of various cancers, including, colon, intestinal, gastric, breast, and bladder cancers, were reduced up to 40 to 50%. These large-scale epidemiologic studies strongly suggested that the COX pathway is involved in the cancer chemopreventive activity of NSAIDs in the GI tract (Baron, 2003). Aspirin and sulindac are the best studied NSAIDs with regard to chemoprevention and induction of tumor regression in the colon/rectum, respectively; however, all NSAIDs may share these properties. Epidemiologic evidence implicates both COX-1 and COX-2 in the chemopreventive roles of NSAIDs. Aspirin, which is a preferential COX-1 inhibitor, has recently been shown in three randomized placebo-controlled trials to be "moderately effective" in preventing the appearance of sporadic colorectal adenomas in patients with a history of these tumors (Sandler et al., 2003). Significantly, doses of only 80 to 325 mg/per day, which would be expected to inhibit primarily COX-1, were sufficient to prevent adenoma incidence by 45%, although there was some variation in the degree of effect between studies. COX-2 selective drugs celecoxib and rofecoxib have also been shown to reduce adenoma incidence and to evoke tumor regression in patients with familial polyposis. Duodenal adenomas, which are otherwise untreatable, show some reduction by treatment with celecoxib (Phillips et al., 2002). Many studies have examined the expression of COX-1 and -2 in tumor tissues from various cancers. Early studies by Eberhart et al. (1994) and Sano et al. (1995) showed that COX-2 is overexpressed in $\geq$80% of colorectal cancer tissues. Interestingly, epithelial cells as well as inflammatory cells and stromal cells express this enzyme. In contrast, COX-2 expression is detectable but lower in adjacent normal tissues. COX-1 isoenzyme is expressed in both normal and tumor tissue. This finding has been repeatedly confirmed in other tumors such as pancreas, skin, gastric, bladder, lung, head and neck, among others. These studies suggested that COX-2 may play a role in tumor formation and/or maintenance. After the development of the COX-2 inhibitors, they

were employed in animal models of carcinogenesis. They were also shown to be effective in reducing the incidence of carcinogen-induced tumors; however, nonselective inhibitors of COX and derivatives of some NSAIDs that do not inhibit COX enzyme activity (for example, sulindac sulfone) were also effective, raising questions about the requirement of COX enzymes in tumorigenesis (Marx, 2001). Various other lines of evidence also cast a doubt on the causal role of COX enzymes in tumorigenesis. Aspirin and salicylates, albeit at high doses, were shown to inhibit the IκB kinase pathway, suggesting that additional targets exist for the NSAIDs. In addition, sulindac sulfone was shown to inhibit the PPARδ pathway, which was induced by APC gene deletion; however, recent studies indicate that PPARδ is not required for sulindac-induced epithelial cell apoptosis, which occur at very high nonphysiological levels of this drug (Park et al., 2001). In addition, NSAIDs at high doses inhibited oncogene-induced transformation of mouse embryonic fibroblasts derived from COX-1 and -2 double null embryos (Zhang et al., 1999). Furthermore, neither COX-1 nor COX-2 acted as classical oncogenes in cellular models of transformation. Indeed, overexpression of the COX enzymes was associated with cellular growth arrest in many cell types. Together, these studies have suggested that overexpression of COX enzymes in tumors may not be simply acting as oncogenes in tumor development. Oshima et al. (1996) provided definitive evidence that COX-2 is required for intestinal tumorigenesis in the ApcΔ^{715} deletion mouse model neoplasia. This mutation results in the truncation of the APC tumor suppressor gene, which regulates the level and activity of the β-catenin protein. Enhanced levels of β-catenin results in transcriptional activation of various growth regulatory genes, for example, c-myc, cyclin D1 via the TCF/LEF family of transcriptional regulators. Thus, ApcΔ^{715} deletion mice develop intestinal polyps with a very high penetrance. Deletion of the COX-2 gene in these mice resulted in gene dose-dependent reduction in polyps. In addition, COX-2 inhibitor also reduced polyps in these mice. These data strongly suggested that COX-2 expression is required for intestinal tumorigenesis (Oshima et al., 1996); however, the expression of COX-2 was detected in the stromal tissues of the small intestine and not in the epithelial compartment, suggesting that it may function in a paracrine manner to regulate epithelial cell transformation. Therefore, an endocrine role for COX-2-derived prostanoids cannot be ruled out from this study. Thus, these critical experiments provided strong evidence that COX-2 is required for intestinal tumorigenesis. Langenbach's laboratory showed that deletion of the COX-1 gene also attenuated polyp formation. These data further suggest that both COX-1 and -2 may be important in tumorigenesis. In addition, in carcinogen-induced tumor initiation and promotion model in skin papilloma formation also exhibited similar requirement for both COX-1 and -2 gene expression. Although papilloma numbers were decreased in both COX-1 and -2 deleted mice, the phenotype of the skin polyps was distinct. For example, papillomas formed

in COX-2$^{-/-}$ mice showed increased cellular apoptosis, increased differentiation of keratinocytes, and formed small elongated polyps. In contrast, COX-1-deficient animals formed larger dometopped polyps and did not show differences in keratinocyte apoptosis and differentiation (Langenbach et al., 1999). Takeda and colleagues (2003) have recently advanced the postulate that COX-1 expressed in intestinal stromal cells provides basal expression of PGE_2 sufficient for polyps to grow to 1 mm, whereafter COX-2 and microsomal PGE_2 synthase are induced to support further polyp growth and development of tumor vasculature. These studies indicate that both COX-1 and -2 enzymes play complex roles in tissue homeostasis and participate in multiple nodes of the tumorigenesis process. The question of whether COX-2 overexpression is sufficient to induce tumorigenesis was recently addressed (Liu et al., 2001). Liu et al. overexpressed the human COX-2 gene in the mammary glands of transgenic mice using the mouse mammary tumor virus promoter. This promoter is highly selectively expressed in the mammary epithelium and is hormonally induced. Thus, expression of mouse mammary tumor virus-linked transgenes is induced in the mammary glands during pregnancy and lactation. Transgenic mice expressing the human COX-2 gene exhibited high levels of COX-2 mRNA, protein, and enzymatic activity in the mammary glands, particularly during pregnancy and lactation. The expression of exogenous COX-2 gene did not influence the expression of COX-1 gene in the mammary glands of transgenic mice. The COX-2 transgenic mice showed precocious mammary gland differentiation, which was characterized by premature expression of the β-casein gene and premature lobuloalveolar development in the virgin animals. These effects were reversed by the administration of the COX inhibitor indomethacin, suggesting that they are mediated by secreted prostanoids. The COX-2 transgenic mice underwent normal pregnancy and lactation in the first cycle. Mammary gland involution was delayed, which was associated with decreased apoptosis of the mammary epithelial cells. These findings were in concert with decreased apoptosis observed in COX-2 overexpressing epithelial cells. After repeated cycles of pregnancy and lactation, however, the COX-2 transgenic mice developed tumors in the mammary glands (Liu et al., 2001). The histology of the mammary tumors indicated that invasive, metastatic tumors of both alveolar and ductal histotypes were observed. In addition, the tumors were focal in nature, suggesting that COX-2 overexpression as well as other mutagenic events were required to fully transform the mammary epithelium. COX-2-induced tumors continued to express COX-2 and contained lower levels of the apoptotic regulatory proteins Bax and Bcl-X_L as well as containing higher levels of the anti-apoptotic protein Bcl-2. These data suggest that ectopic overexpression of COX-2 gene is sufficient to transform the mammary gland after repeated cycles of pregnancy and lactation (Liu et al., 2001). These data support the notion that unregulated expression of COX-2, perhaps induced by carcinogenic stimuli or other tumor promoters is an important contributor of

tumorigenesis. Recent transgenic overexpression studies in which COX-2 was targeted to the skin of transgenic mice also support this concept. In this study, hyperplasia of the epidermis and abnormal sebaceous gland differentiation was observed, although spontaneous skin tumors did not develop. Recent work from the same group showed that carcinogen-induced skin papilloma formation is enhanced in COX-2TG mice, suggesting that COX-2 acts as a potent tumor progression factor in the skin (Marks et al., 2003). Even though the effect of COX-2 to induce tissue changes that ultimately lead to tumorigenesis is beginning to be appreciated, the mechanisms involved are not well understood. Many *in vitro* studies, however, support the notion that COX-2 overexpression inhibits apoptosis and that tumor angiogenesis is induced as well (Dubois et al., 1998). Tsujii and DuBois showed that overexpression of COX-2 in intestinal epithelial cells resulted in enhanced E-cadherin expression and decreased apoptosis, when cells are induced with butyrate (Tsujii and DuBois, 1995). Later studies from the same laboratory indicated that PGE_2 stimulation of these cells results in cell survival due to the phosphatidylinositol 3-kinase/Akt pathway. In addition, epithelial cell motility and invasive behavior were also induced (Sheng et al., 2001). The ability of COX-2 overexpression to inhibit apoptosis was observed in other epithelial cells and neuronal PC-12 cells but not in ECV-304 bladder carcinoma cells. Coupled with the finding that apoptosis of mammary epithelial cells are altered in COX-2 transgenic mice (Liu et al., 2001), regulation of apoptosis may be an important event in COX-2-induced tissue changes that lead to tumorigenesis. In addition, induction of angiogenesis by the COX-2 (and the COX-1 pathway) may contribute to the development of tumors (Tsujii et al., 1998). Angiogenesis is regulated by a plethora of factors, the balance of which is thought to be critical. As discussed above, COX-2 expression and secretion of prostanoids such as PGE_2 may induce angiogenesis during cancer development; however, the reason why normal tissue that produces PGE_2 abundantly via the COX-1 pathway fails to induce angiogenesis is not understood. The enhanced production of COX-2, together with other changes that occur during cancer development, may contribute to the overall balance of the angiogenic response during tumor development. Further studies are needed to better define the molecular mechanisms involved in COX-2-induced tumorigenesis. Evidence that NSAIDs reduce cancer incidence and evoke tumor regression in the GI tract has been extensively reviewed elsewhere, as has overexpression of COX-2 in epithelial cancers in humans (Dixon, 2003). The antineoplastic activity of NSAIDs is likely multifactorial, but the induction of apoptosis is essential to the ability of these drugs to cause tumor regression and may also be integral to their ability to prevent tumor growth. Induction in experimentally induced tumors in rodents of tumor regression has been observed in several studies (Oshima et al., 1996). Giardello has also reported an increase in apoptosis in the carcinomas of the colon/rectum following 3 months of treatment with sulindac

(Pasricha et al., 1995). Currently the mechanism(s) by which NSAIDs induce apoptosis in tumors of the GI tract, and perhaps elsewhere, is unknown. Cyclooxygenase and noncyclooxygenase pathways that have been proposed to govern NSAID-induced apoptosis have been reviewed elsewhere (Simmons and Wilson, 2001).

3.3 Alzheimer's Disease

Epidemiologic evidence indicates that NSAID use is associated with a lower incidence or risk of AD. An inverse relationship is seen between NSAID use (particularly aspirin) and AD incidence in case-controlled studies of patients who have osteoarthritis, rheumatoid arthritis, or who use NSAIDs for other purposes. A similar inverse correlation between NSAID use and AD was seen in a co-twin control study of 50 elderly twins with AD onset separated by 3 years or more (Breitner et al., 1995). Both decreased risk of AD among NSAID users as well as a decreased risk of AD with increased duration of NSAID use was found in the prospective Baltimore Longitudinal Study of Aging. In this study, 1686 participants were followed for 15 years, and participants 55 and older were assessed for AD. A decrease in cognitive decline was also associated with NSAID use in the 1-year longitudinal Rotterdam Study (Andersen et al., 1995); however, another longitudinal study, The Medical Research Council Treatment Trial of Hypertension in Older Adults, found that increased beneficial cognitive effects among NSAID users compared with controls was not evident in AD patients over 74. A fourth longitudinal study also found no beneficial cognitive effect of aspirin or other NSAIDs in AD patients with a mean age of 80 (Henderson et al., 1997). Together these studies suggest that the window of efficacy for NSAID use may precede the age of 75; however, in a recent case-control study of specific dementias including AD, vascular, and other dementias in patients 75 and older, a strong inverse correlation between NSAID usage and the presence of AD, but not other dementias, was observed. This study suggests that a lack of effect observed in older patients may be due to confounding neurodegenerative conditions in elderly people that are not affected by NSAIDs. A number of excellent reviews have recently explored the complex and, as yet, unclear roles that COX isozymes play in AD (O'Banion, 1999). This disease exhibits a strong inflammatory component initiated and/or exacerbated by fibrillar β-sheet β-amyloid deposits (Halliday et al., 2000). Proinflammatory cytokines, acute phase proteins, prostaglandins, and other mediators of inflammation are elevated in and around the senile plaques present in AD brains. COX-2 has been reported to be increased in the cortex of AD brains; however, it is important to note that COX-2 is also normally expressed in neurons of the neocortex and hippocampus and appears to be preferentially expressed in glutamatergic pyramidal neurons (Ho et al., 2001). Within these neurons it shows a perinuclear subcellular localization typical of other cells but also may be found

accumulated in dendrites and particularly in dendritic. Pasinetti and colleagues (Ho et al., 2001) have recently correlated increased COX-2 expression in the CA2 and CA3 subdivision of the hippocampal pyramidal layer with increasing dementia through mild to severe stages. Increased COX-2 in the CA1 subdivision was evident only in severe stage AD. The normal function of COX-2 in brain neurons is unknown, and it is unclear whether long-term use of COX-2 inhibitors will have a physiological effect through inhibition of this function. COX-2 can be induced in neurons, microglia, and astrocytes by a variety of neurotoxic stimuli including hypoxia and excitotoxins, such as kainic acid. The current debate is whether COX-2 induction after neuronal insult serves to protect against cell death or promote apoptosis in the expressing neuron itself or in neighboring neurons. Data using animal models and *in vitro* systems support both protective and pro-apoptotic roles (Iadecola et al., 2001). Increased neuronal PG synthesis resulting from increased COX-2 may evoke increased levels of other proinflammatory agents produced by astroglial cells and cause neuronal cell death. This is consistent with the finding that NSAIDs result in a decrease in activated microglial cells in AD patients and in the Tg2576 mouse, which is predisposed to an AD-like syndrome. Ibuprofen treatment in these mice decreases concentrations of inflammatory mediators such as IL-1β, slows β-amyloid plaque deposition, and decreases the number of dystrophic neurites (Lim et al., 2000). These findings suggest that COX isozymes influence the rate of β-amyloid secretion or deposition as well as function in any inflammatory process that results from plaque formation; however, NSAIDs used in both human and animal studies described above were nonselective toward COX enzymes or COX-1 preferential and suggests that COX-1 and COX-2 are important in the pathogenesis of AD. Aspirin is a strongly COX-1 preferential NSAID and a small (44 patients), double blind, placebo-controlled clinical trial of the nonselective drug indomethacin showed reduction in the rate of cognitive decline in AD patients. Diclofenac administered with misoprostol showed no statistically significant effects in an even smaller study although a trend to prevention was seen (Scharf et al., 1999). Several lines of evidence suggest that COX-2 is important in AD. COX-2 is clearly induced following various neurotoxic stimuli as mentioned above. Furthermore, elevated COX-2 is also found in other neurodegenerative diseases such as in the spinal cord of patients with sporadic amyotrophic lateral sclerosis and in the spinal cords of transgenic mice that exhibit an amyotrophic lateral sclerosis-like syndrome (Almer et al., 2001). Genetic deletion of COX-2 in laboratory animals decreases susceptibility to ischemic brain injury and *N*-methyl-D-aspartate-mediated neurotoxicity (Iadecola et al., 2001), and COX-2 overexpression in transgenic mice increases susceptibility to β-amyloid-induced neurotoxicity. A COX-2 selective NSAID reduces focal ischemic brain injury in a rat model, suggesting a role for COX-2 in stroke and a possible role of COX-2-selective inhibitors in stroke treatment; however, pretreatment of mice

with NS398 leads to markedly increased neuronal cell death in the hippocampus and increased mortality following kainate treatment. This latter finding suggests that inhibition of COX-2 induced by excitotoxins may be neuroprotective, but that inhibition of constitutive COX-2 expression may be deleterious in the event of a seizure. In addition to determining the specific roles that COX-1 and COX-2 play in AD, other important issues remain to be addressed with regard to the action of these drugs in this disease. First, dose levels that maximally evoke a protective effect in AD need to be determined for NSAIDs. Limited data suggest that low dose NSAID treatment may be as effective as high doses (Broe et al., 2000). If prevention of AD requires inhibition of both COX-1 and COX-2 as present data suggests, appropriate dose levels may be difficult to establish due to NSAID-induced gastric toxicity; however, if low doses are required, the establishment of regimens with acceptable toxicity levels for most people should be feasible. Second, it will be important to determine whether aspirin or competitively acting NSAIDs more effectively reduce risk of AD. Most studies have detected a preventive effect of aspirin in AD, but some studies comparing aspirin with competitively acting NSAIDs have shown the latter to be better at reducing risk of AD (Stewart et al., 1997). Third, the cells directly involved in the NSAID-induced protection mechanism need to be determined. NSAIDs may inhibit the inflammatory component of AD through inhibiting COX isozymes in neurons or glial cells. Alternatively, if low doses of NSAIDs maximally reduce AD, the important target for anti-AD therapy may be cells of the vascular system such as platelets or endothelial cells.

4. COX-2 Regulation

4.1 Molecular aspects of COX-2 regulation

COX-2 was discovered in 1991 as a primary response gene (Kujubu et al., 1991; Hla and Neilson, 1992). COX-2 expression is regulated by both transcriptional and post-transcriptional mechanisms. The human *COX-2* gene maps to chromosome 1 q25.2–q25.38 (Inoue et al., 1995). It is approximately 8.3 kb long and comprises 10 exons (**Fig. 6a**). Three mRNA transcript variants have been reported, they are respectively 2.8, 4,0, and 4.6 kb in length. The largest exon in the *COX-2* gene encodes the entire 3′-untranslated region (3′-UTR). The latter contains several copies of the "ATTTA" RNA instability element. Sequence analysis of the 5′-flanking region reveals several potential transcription regulator elements (**Fig. 6b**). However, in all studied species, only a limited number of elements, namely the cAMP response element (CRE), the C/EBP-NF-IL6 ("CAAT/enhancer binding protein"), NFAT ("nuclear factor of activated T cells") and NFkB ("nuclear factor kappa B cells") sites, and the E-box are known to be involved in the regulation of *COX-2* gene expression.

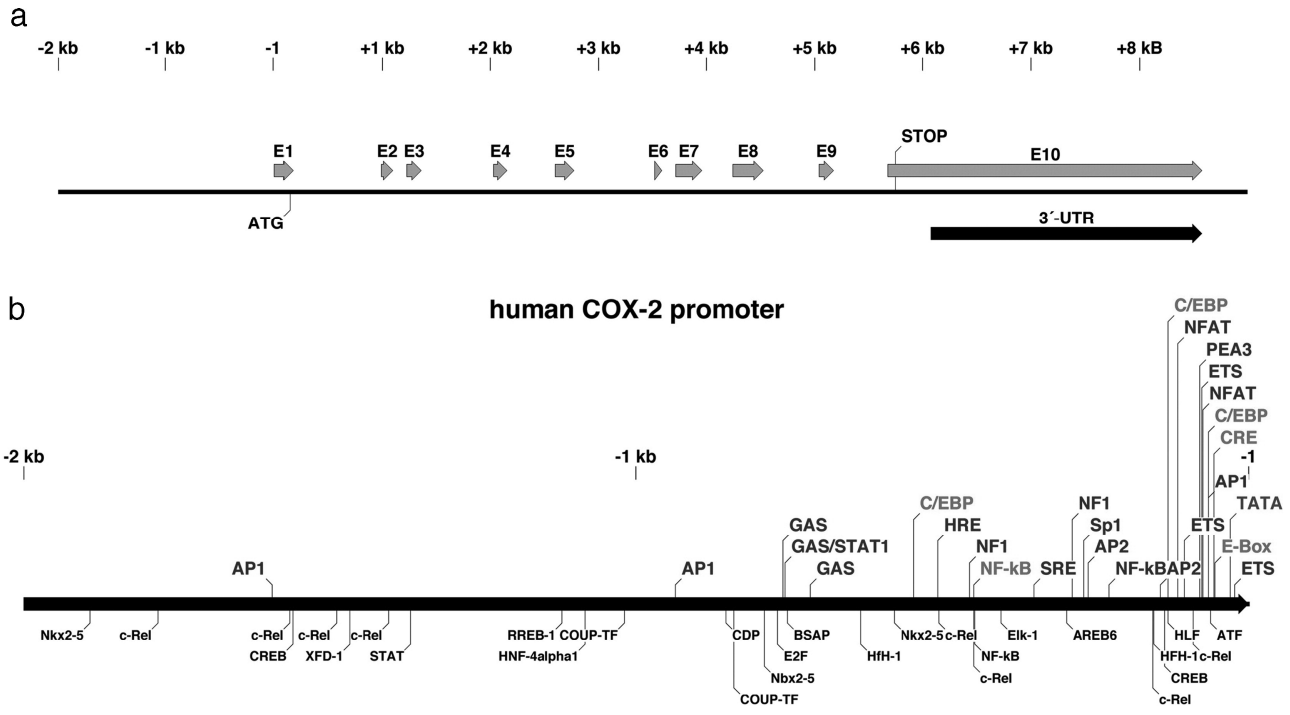


Figure 6. Structure of the human *Cox-2* gene (a). Transcription factor binding sites in the 5′-flanking sequences (promoter) of the human *Cox-2* gene.

4.2 Dynamic Regulation of COX-2 Transcriptional Activation by C/EBPß

The core COX-2 promoter region harbors a canonical TATA element and several functionally important enhancer elements, which are well-conserved between humans and mice (Fletcher et al., 1992; Tazawa et al., 1994) The enhancer elements are localized within a 500-basepair 5'-untranslated region of human COX-2 promoter. Functional analysis of promoter activation in several laboratories including ours has shed light on several salient features regarding COX-2 transcriptional regulation. First, the cAMP response element (CRE) located at –59 to –53 from the transcription start site of human COX-2 is indispensable for basal and induced COX-2 transcriptional activation in human fibroblasts and endothelial cells, and mutation of the CRE element results in a complete collapse of promoter activity. A large number of transactivators including CRE binding protein (CREB), ATF, C/EBP, C-Jun, C-Fos, and USF bind to an overlapped CRE and AP-1 region located at –60 to –40 from the transcription start site (Schroer et al., 2002). Binding of this cluster of transactivators to a small stretch of DNA sequence close to the TATA box is essential for basal and induced COX-2 transcriptional activation. Second, transactivation of COX-2 by proinflammatory mediators requires a concerted upregulation of binding of distinct transactivators to their respective enhancer elements. This point is illustrated by the regulation of transactivator binding in response to stimulation by phorbol 12-myristate 13-acetate (PMA), and tumor necrosis factor-alpha (TNFα). PMA increases binding of CREB-2, C-Jun, and C-Fos to the CRE/AP-1 region and C/EBPß to the C/EBP element at –124 to –132 of human COX-2 (Schroer et al., 2002; Saunders et al., 2001) Mutation of CRE site or C/EBP element completely abolishes the increase in COX-2 expression stimulated by PMA. These results indicate that COX-2 transcriptional activity by PMA depends on upregulation of CREB-2, C-Fos/C-Jun (AP-1), and C/EBPß binding to CRE region and C/EBP site, respectively. In contrast, TNFα induces activation and binding of NF-κB to 2 separate κB enhancer elements on the core promoter region and mutation of either enhancer element greatly reduces the TNFα transcriptional activity (Deng et al., 2003). Thus, COX-2 transcriptional activation by TNFα depends largely on NF-κB activation and binding. Third, DNA-bound transactivators induced by proinflammatory mediators recruit predominantly p300 coactivators to the complex, which interact with the transcription machinery. Furthermore, p300 histone acetyltransferase acetylates histones, thereby opening up chromatin to allow more access to transactivators and acetylates transactivators such as p50 NF-κB subunit to augment NF-κB binding (Deng et al., 2004). CREB binding protein, which is highly homologous to p300, plays a relatively unimportant role because it is expressed in very low abundance in primary cultured human cells such as human umbilical vein endothelial cells and fibroblasts. Lastly, COX-2

transcriptional activation by proinflammatory mediators is regulated by a time-dependent alteration in transactivator levels and switch in transactivator binding. One example is the switch of C/EBP isoforms during PMA-induced COX-2 transactivation (Zhu et al., 2002). C/EBPß has emerged as a key transactivator for COX-2 expression induced by proinflammatory mediators. It has been shown to regulate COX-2 transcriptional activation in murine and human cells induced by diverse proinflammatory mediators (Wadleigh et al., 2000). The C/EBP family proteins comprise 6 members of basic leucine zipper transcription factors (Akira et al., 1997). They are divided into 2 subgroups based on sequence homology: one group comprises C/EBPα, ß, and δ, and the other comprises C/EBPγ, ε , and ζ. Several studies have shown that the ß and δ isoforms are involved in COX-2 transcriptional activation by proinflammatory mediators in murine and human cells (Zhu et al., 2002; Wadleigh et al., 2000). C/EBPß and C/EBPδ bind to C/EBP enhancer elements at –132/–124 and CRE at –59/–53 of human COX-2 promoter in resting fibroblasts or endothelial cells. After treatment with PMA, C/EBPß protein levels are unaltered, whereas C/EBPδ declines in a time-dependent manner. Binding of the ß isoform to C/EBP site is time-dependently increased. Overexpression of C/EBPδ in human fibroblasts by transient transfection results in a marked increase in basal but not PMA-induced COX-2 promoter activity, whereas overexpression of C/EBPß stimulates neither basal nor PMA-induced COX-2 promoter activity. These results suggest that C/EBPδ binds constitutively to CRE and C/EBP enhancer elements and is involved in regulating the basal COX-2 promoter activity. By contrast, C/EBPß is dormant and does not bind to C/EBP site at resting state. Its binding activity is increased by signaling from PMA, and its increased binding to the C/EBP site plays an important role in COX-2 transcriptional activation. It is interesting that C/EBPδ proteins undergo degradation after PMA treatment. It is unclear how C/EBPδ is degraded. Nevertheless, the results suggest that C/EBPδ degradation may be important for making the C/EBP enhancer element unoccupied and accessible to activated C/EBPß. COX-2 transcriptional activation is further regulated by C/EBPß variants. Three variants of C/EBPß are detected in human fibroblasts and endothelial cells: 46-kDa full-length (FL), 41-kDa liver-enriched transcription activating protein (LAP), and 16-kDa liver-enriched transcription inhibitory protein (LIP). LAP and LIP are truncated forms of FL C/EBPß with deletion of the amino-terminal regions of C/EBPß. They are translated from C/EBPß mRNA by using alternative translation start sites because of a leaky ribosomal scanning mechanism. LAP, like FL C/EBPß, activates transcription, whereas LIP suppresses gene transcription (Descombes and Schibler, 1991; Liao et al., 1999). We observed LIP but not LAP binding to C/EBP site of COX-2 promoter in resting cells, and PMA enhanced LIP and induced LAP binding, without altering the cellular LIP or LAP protein levels. LIP is a dominant-negative mutant of LAP, and its overexpression in human cells abrogates PMA-

induced LAP binding and COX-2 promoter activity (Zhu et al., 2002). LIP controls basal and PMA-induced COX-2 transcriptional activation and may play an important role in limiting the extent and duration of COX-2 expression. C/EBPß (FL and LAP) in resting cells does not bind to C/EBP enhancer element because C/EBPß harbors an intramolecular bipartite inhibitory element located between the N-terminal transactivating domain and C-terminal DNA-binding and leucine zipper (dimerization) region. This intramolecular inhibitory element is abolished by phosphorylation of serine or threonine residues adjacent to this element. It has been shown that phosphorylation of C/EBPß at Thr-235 by p42/p44 mitogen-activated protein kinase (ERK1/2), Thr-266 by p90 ribosomal S6 kinase (RSK), Ser-288 by protein kinase A, and Ser-325 by calmodulin-dependent kinase IV enhance C/EBPß binding activity (Wegner et al., 1992; Buck et al., 1999). The signaling pathway via which proinflammatory mediators activate C/EBPß has not been reported. Identification of kinases that activate C/EBPß should be valuable for providing specific target for drug discovery. C/EBPß plays an important role in mediating vascular diseases. It is involved in mediating transcription of COX-2 as well as inducible nitric oxide synthase and cytokines (Matsusaka et al., 1993). The results of a recent study have shown that administration of a C/EBP decoy oligonucleotide into a balloon-injured carotid artery of a rabbit atherosclerosis model reduced intimal hyperplasia and attenuated vascular inflammation accompanied by a complete inhibition of C/EBP protein binding to a consensus C/EBP sequence (Kelkenberg et al., 2002). Taken together, these findings underscore the importance of C/EBPß and C/EBPδ in regulating COX-2 promoter activity induced by proinflammatory stimuli. The extent and duration of COX-2 expression are regulated by several inter-related molecular events, including C/EBPδ degradation, C/EBPß activation and binding, and competitive inhibition of LIP. These events are likely to influence the extent of vascular lesions and are potential targets for developing new therapeutic strategies.

4.3 Other signaling pathways

1. Lipopolysaccharide (LPS). Historically, gram (−) bacterial LPS was the first inducer of COX-2 expression to be identified in macrophages (Lee et al., 1992). It is now known that most pro-inflammatory mediators induce the expression of COX-2. More specifically, LPS and other TLR ligands bind to MyD88-associated receptors and via MEK/ERK induce the transcription factor activator protein 1 (AP1). LPS also activates the TRAF6/NIK/Tpl2/IKK/NFkB pathway, which also leads to induction of COX-2 transcription. Tpl2 signals also lead to ERK1/2 activation, which in turn activates p90RSK and MSK1, which phosphorylate CREB, a central regulator of COX-2 transcription (Eliopoulos et al., 2002). LPS activates C/EBPβ and C/EBPδ via p38MAPK and ERK1/2. It should be noted that C/EBPβ and CREB play a major role during the initial stage of

COX-2 transcriptional activation while C/EBPδ maintains an already induced transcription (Chen et al., 2005). The transcription complex at the COX-2 promoter requires the transcriptional co-activator p300. It now appears that p300 binds on CREB, AP1, C/EBP and NFkB, controlling the initiation of transcription (Deng et al., 2004).

2. Nitric oxide (NO). NO affects COX-2 directly by increasing its catalytic activity and indirectly by triggering several signalling cascades that affect transcription. Thus, NO reacts acutely with superoxide anions to form peroxynitrite anions ($ONOO^-$), which enhance COX-2 catalytic activity in a direct manner. It should be noted here that NO may affect carcinogenesis in two parallel ways: first, via activation of COX-2 catalytic activity and secondly in synergy to COX-2 by inducing VEGF production (Surh et al., 2001). NO and reactive oxygen species (ROs) induce COX-2 mRNA expression via the beta catenin/TCF pathway leading to activation of the polyoma enhancer activator 3 (PEA3) transcription factor. Moreover, NO utilizes cAMP/PKA/CREB and JNK/Jun/ATF2 signalling cascades affecting COX-2 transcription (Liu et al., 2004 and Park et al., 2005).

3. Cytokine. Several pro-inflammatory cytokines such as IL-1 or IFN-γ induce COX-2 expression. The major signalling pathway involved is a cAMP- and PKA-dependent activation of CREB. It is of interest that among the MAPK pathways, p38MAPK appears to be involved while ERK1/2 appears not to participate (Wu et al., 2005). On the contrary, TNF-α-induced COX-2 expression depends heavily on activation of ERK1/2 and NFkB.

4. Growth factor and growth factor receptor. COX-2 expression is induced by several growth factors including IGF, TGFα and EGF. Growth factor-induced COX-2 expression takes place in both normal as well as cancer cells. The IGF-induced signals are mediated by PI3Kinase and Src/ERK but not by p38MAPK while the effect of TGF and EGF is mediated by p38MAPK, ERK1/2 and PI3K (Chun and Surh, 2004).

5. UVB irradiation. UVB induces COX-2 mRNA expression in cancer cells. UVB activates the transcription factors CREB and ATF1 via a cAMP-dependent pathway. Induction of p38MAPK and PI3K but not NFkB appears to be essential for UVB-induced COX-2 expression (Bachelor et al., 2005).

6. *Neuropeptide*. Fine-tuning of COX-2 expression levels can occur by extracellular signals such as G-protein coupled receptor (GPCR) binding proteins. The stress neuropeptides corticotropin

releasing factor (CRF) and urocortins augment LPS-induced COX-2 expression via cAMP/PKA/CREB activation (McEvoy et al., 2004).

7. Ceramide. Accumulation in the cytoplasm of this sphingosine-based lipid-signalling molecule appears to regulate COX-2 expression resulting in inflammation and tumorigenesis. It has been also implicated in the aging process. More specifically, it has been shown that ceramide is the mediator of TNF-α-induced COX-2 expression in fibroblasts and of cannabinoid-induced COX-2 expression in neuroglioma cells (Pettus et al., 2003). It should be noted that the induction of COX-2 expression by ceramide involves NFkB but not AP1 or CREB (Wu et al., 2003).

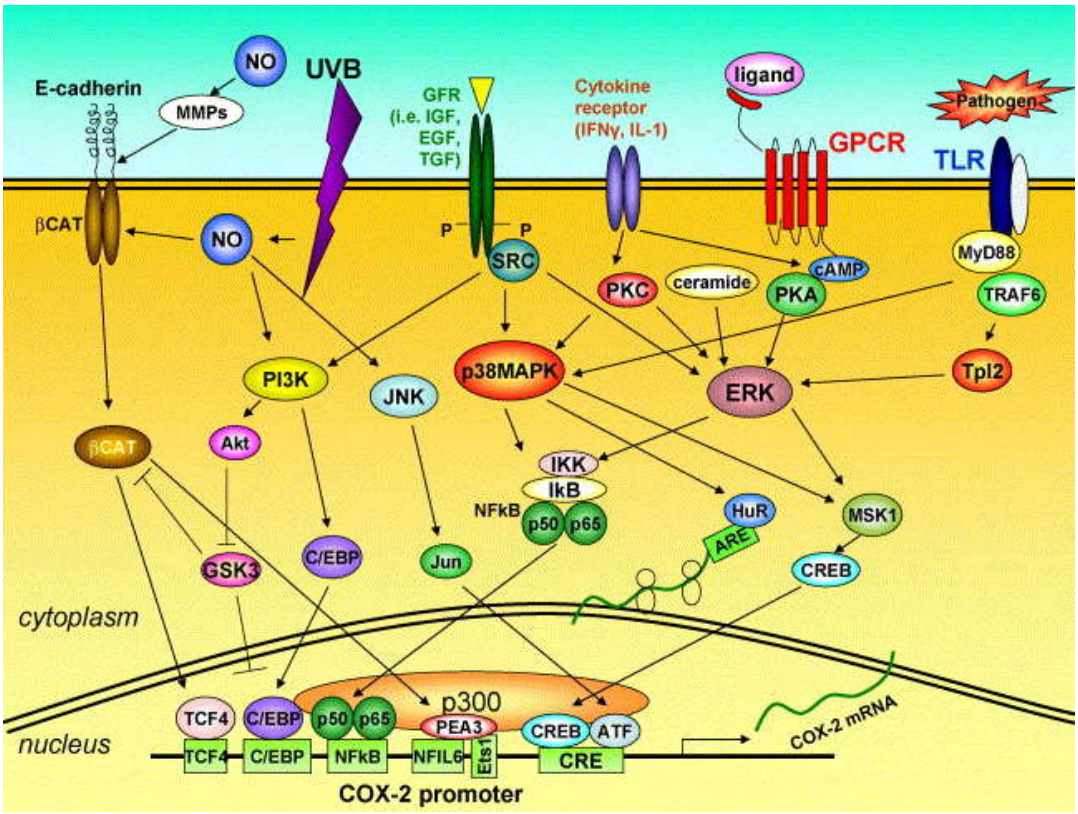


Figure 7. COX-2 transcriptional regulation.

4.4 Post-transcriptional regulation

The mRNA stability has been reported to be crucially involved in the regulation of COX-2 expression (Dixon et al., 2000). Exon 10 of the cyclooxygenase-2 gene encodes the entire 3′-UTR, and contains several copies of the pentameric ATTTA sequence. The AUUUA motif (also called AU-rich element — ARE) is found in the 3′-UTR of many unstable cytokine- and proto-oncogene-encoding mRNAs, and these motifs regulate mRNA stability and translation (Barreau et al., 2005).

Involvement of these ARE in the post-transcriptional regulation of COX-2 expression has been reported (Cok and Morrison, 2001). Interestingly a small fragment of the human COX-2 3′-UTR (CR1; + 1 to + 123 of the 3′-UTR) containing 6 AREs was sufficient to destabilize a reporter mRNA in transfection assays. These AREs have been shown to regulate gene expression by interaction with different RNA-binding proteins. Important RNA-binding proteins shown to bind to COX-2 AREs and to regulate COX-2 expression (mRNA stability or translatability) are AUF1, HuR, TIA-1, β-catenin, CUGBP2, TIAR and TTP (Phillips et al., 2004; Lee and Jeong, 2006). Interestingly, the overexpression of COX-2 in most colon carcinoma cells seems to be promoted by a parallel enhanced expression of the ARE-mRNA stabilizing protein HuR (Dixon et al., 2001). ARES aside, other sequence elements of the human COX-2 mRNA have been shown to be involved in the post-transcriptional regulation of COX-2 protein expression. For instance, transfection experiments using the 3′-UTR of the human COX-2 mRNA with or without the AREs (ΔARE) demonstrated that the AREs play a role in the IGF-1-mediated enhancement of COX-2 mRNA stability. Depending on the cell type analyzed, different signalling pathways (MAPK, AMPK, PKC, PI3K etc., alone or in combination) are involved in regulating COX-2 expression at the post-transcriptional level (Eberhardt et al., 2007). These signalling pathways directly or indirectly regulate the activity, localization or expression of several of the RNA-binding proteins discussed above. Several pro-inflammatory stimuli, which induce *COX-2* gene expression, also stimulate MAPK. In particular, the activation of p38MAPK has been shown to be critically involved in the post-transcriptional regulation of several pro-inflammatory genes. Furthermore, p38MAPK has been shown to regulate the localization and activity of several RNA-binding proteins involved in post-transcriptional control of COX-2 expression. Accordingly, specific inhibitors of p38 block the accumulation of COX-2 mRNA in a variety of cells subjected to pro-inflammatory signals. In Hela cells stimulated with IL-1α and β, and in primary human monocytes stimulated with LPS, inhibition of p38 results in a rapid and specific destabilization of COX-2 mRNA but has little effect on COX-2 transcription. Similar findings of p38 activation on mRNA stability were reported in human monocytes as well as myofibroblasts (Mifflin et al., 2004). In addition to p38MAPK, ERK1/2 and JNK have also been shown to be involved in the post-transcriptional regulation of COX-2 expression in some cell types. The post-transcriptional regulation of COX-2 expression by bile acid and ceramide in rat intestinal epithelial cells and IGF in human ovarian cancer cells has been shown to depend on ERK1/2. However, the exact mechanisms (e.g., RNA-binding proteins involved) have not been determined. In murine J774 macrophages, specific inhibition of JNK by SP600125 resulted in reduction of LPS-induced COX-2 expression by destabilization of the COX-2 mRNA (Nieminen et al., 2006). JNK activity has been shown to modulate Tristetraprolin (TTP) expression

and TTP destabilizes the COX-2 mRNA and (Phillips et al., 2004). However, whether the SP600125-mediated modulation of TTP expression is involved in the SP600125-dependent inhibition of LPS-induced COX-2 expression in J774 macrophages is not known. In addition to MAPK, other signalling pathways seem to be involved in the post-transcriptional regulation of COX-2 expression in different cell types. In human ovarian cancer cells IGF-1-induced stabilization of the COX-2 mRNA depends on the activation of PI3K and PKC in addition to the activation of ERK1/2 and p38MAPK (Dean et al., 1999). Furthermore, in human mesangial cells the ATP-induced stabilization of the COX-2 mRNA is inhibited by PKC inhibitors or specific downregulation of PKC-α by siRNAs. Interestingly, in this cell type ATP incubation induces PKCα-dependent HuR phosphorylation, which results in enhanced nuclear to cytoplasmic shuttling of HuR and in turn in stabilization of COX-2 mRNA (Doller et al., 2007). Activation of the AMP-activated kinase (AMPK) has been shown to reduce COX-2 expression. AMPK has been shown to regulate the nucleo-cytoplasmic shuttling of HuR. Activation of AMPK reduces cytoplasmic HuR levels (Wang et al., 2002). However, whether AMPK-activation reduces COX-2 mRNA stability has not been determined.

5. COX-2 and Colorectal Cancer

With an estimated 147,500 new cases and 57,100 deaths in 2003, colorectal cancer (CRC) is the third most common cancer in the United States and is the third leading cause of cancer-related mortality (Jemal et al. ,2003). Given the relatively high incidence of colorectal cancer and its refractoriness to available anti-cancer therapies, prevention and treatment of this disease is of major public health importance. Interest in cyclooxygenase (COX) as a therapeutic target for CRC derives from epidemiologic studies that have consistently shown a 40–50% reduction in CRC incidence among chronic users of nonsteroidal anti-inflammatory drugs (NSAIDs) (Giovannucci et al., 1995). COX is the best defined molecular target of NSAIDs and regulates the synthesis of prostaglandins (PGs) and related eicosanoids from arachidonic acid. Isoforms of COX include the constitutively expressed COX-1 and the inducible COX-2 enzymes. COX-2 (also known as prostaglandin H synthase-1 or prostaglandin endoperoxidase synthase 2) is up-regulated in response to inflammatory cytokines, growth factors and tumor promoters. COX-2, but not COX-1, has also been shown to be overexpressed in an estimated 40% of human colorectal adenomas and 80% of adenocarcinomas relative to normal epithelial cells. In animal models of colon cancer, COX-2 expression has been detected in intestinal adenomas in azoxymethane (AOM)-treated rodents and in the Apc^{Min} mouse which carries a fully penetrant, dominant mutation in the murine APC gene. In another murine model of FAP, deletion of the murine COX-2 gene in $APC^{\Delta 716}$ mice was found to dramatically reduce the number of intestinal polyps in double knockout mice relative to COX-2 wild-type animals (Oshima et al., 1996). These data provide the best evidence of the association of COX-2 and intestinal tumorigenesis. Potential mechanisms by which COX-2 may contribute to tumorigenesis include stimulation of angiogenesis, inhibition of apoptosis, and increased cell invasiveness. In genetic and in carcinogen-induced colon cancer model systems, treatment with traditional NSAIDs or selective COX-2 inhibitors has been shown to reduce tumor size and multiplicity (Mahmoud et al., 1996). The relevance of these findings to human neoplasia was shown in patients with germline mutations in the APC gene and the syndrome of familial adenomatous polyposis (FAP), where treatment with the NSAID sulindac or the selective COX-2 inhibitor celecoxib has been shown to effectively regress existing colorectal adenomas relative to placebo. Selective COX-2 inhibitors, known as coxibs, were developed recognizing that COX-2 is induced at sites of inflammation and that inhibition of COX-1 is believed to be largely responsible for the adverse effects associated with NSAID treatment. Studies indicate that coxibs are associated with significantly fewer gastrointestinal toxic events relative to traditional NSAIDs (Bombardier et al. ,

2000). While the specific anti-tumor mechanism(s) of NSAIDs and coxibs remain unknown, studies have identified potential COX-dependent and -independent targets.

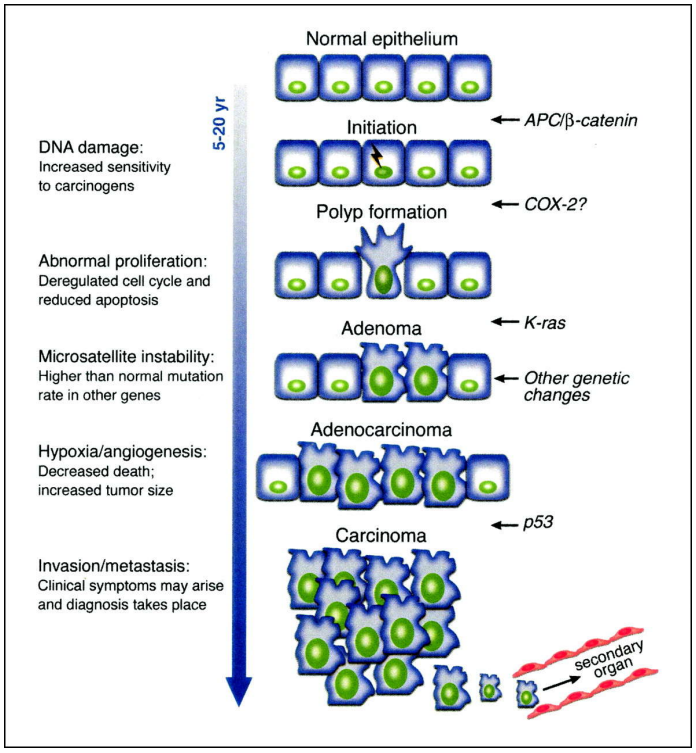


Figure 8. A genetic model for colorectal tumorigenesis

5.1 COX-2 expression in colorectal cancer

COX-2 proteins are overexpressed in approximately 80% of human colorectal cancers and in 40% of colorectal adenomas relative to normal mucosa. By immunohistochemistry, COX-2 localizes predominantly to the cytoplasm of neoplastic epithelial cells although some stromal staining is observed (**Fig. 9**). Normal epithelium, however, is negative for COX-2. Results have been consistent regarding the frequency of COX-2 protein expression in human colorectal cancers (Sinicrope et al., 1999). COX-2 messenger RNA (mRNA) levels have also been shown to be increased in colorectal cancers relative to normal mucosa. Levels of COX-1, however, are equivalent between the normal mucosa and colorectal neoplams consistent with its constitutive expression. Stromal cell expression of COX-2 in macrophages, fibroblasts, and endothelial cells has been detected in these tumors but to a lesser extent than in epithelial cells (Masferrer et al., 2000). COX-2 overexpression in other epithelial cancers including breast, pancreas, esophagus, stomach, and lung is also found primarily in the malignant epithelial cells. Furthermore, intestinal adenomas from APC^{Min} mice (Williams et al., 1996) and colon carcinomas from AOM-treated rats [6,41] show a predominant localization of COX-2 to neoplastic epithelial cells. Some expression of COX-

2 has been seen in stromal cells, specifically in macrophages or fibroblasts within polyps from Apc^{Min} mice [42–44]. In $Apc^{\Delta 716}$ mice, COX-2 appears to be limited to stromal cells in intestinal polyps, shown to be fibroblasts and endothelial cells (Sonoshita et al., 2002).

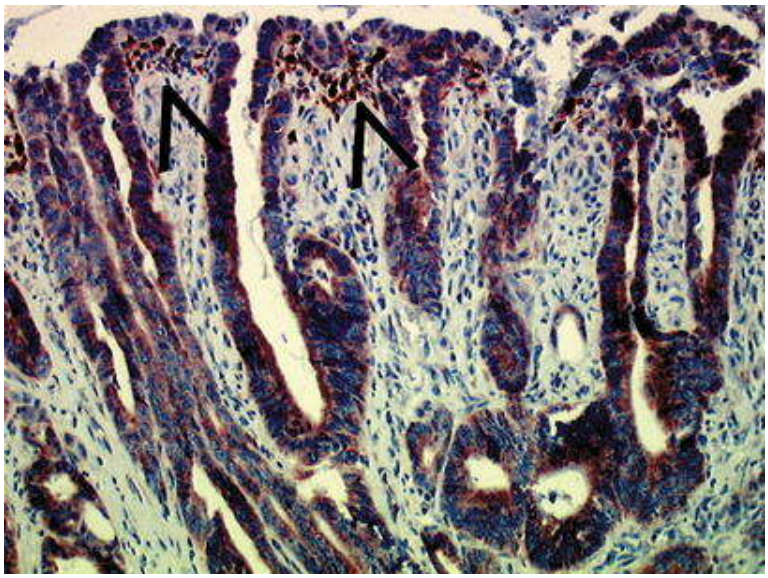


Figure 9. COX-2 expression in tumor colon cells (Immunohistochemistry, x200)

$Apc^{\Delta 716}$ mice carry a truncated APC gene and serve as a model for FAP. The explanation for these differences in COX-2 localization remain unknown, but suggest that COX-2-mediated synthesis of PGs may promote tumor growth by acting on neoplastic epithelial cells, stromal cells, or both. Studies by Karnes et al. (1998) have shown that human colon cancers with defective DNA mismatch repair (MMR), as manifested by a high frequency of microsatellite instability (MSI) and/or germline mutations in MMR genes, have reduced rates of COX-2 expression relative to microsatellite stable (MSS) cases. The lack of COX-2 expression in some of these cases raised the possibility of a specific silencing mechanism for the COX-2 gene. Since aberrant hypermethylation of 50 CpG islands has been implicated in the transcriptional silencing of genes including the hMLH1 gene promoter in sporadic colon cancers with MSI, we examined the methylation status of the COX-2 promoter in a series of colorectal tumors (Toyota et al. , 2000). Aberrant methylation of COX-2 was detected in 12 of 92 (13%) sporadic CRCs and in 7 of 50 (14%) colorectal adenomas. No association was observed between COX-2 methylation and MSI status. COX-2 methylation was significantly associated with the presence of the CpG island methylator phenotype (CIMP) and inversely related to p53 mutation. Although the functional consequences of aberrant methylation of COX-2 in colorectal tumorigenesis remain to be determined, these findings suggest that overproduction of PGs may not be necessary for tumor progression in these tumors. The

chemopreventive efficacy of a COX-2 inhibitor in hMSH2-deficient APC^{Min} mice was studied by Lal et al. (2001). An equivalent decrease in small intestinal polyp number was found in hMSH2-deficient APC^{Min} mice vs. MMR-proficient APC^{Min} mice treated with the COX-2 inhibitor. COX-2 expression in small and large intestinal polyps did not correlate with treatment response and no decrease in colonic polyps was found. These data suggest that coxibs may be effective chemopreventive agents in patients with defective DNA MMR due to germline defects or methylation abnormalities. Finally, COX-2 overexpression may confer prognostic information in human colorectal cancers and in selected other epithelial cancers. In a study of 76 colorectal cancer patients, COX-2 overexpression in primary tumors was associated with more advanced tumor stage, increased tumor size, and positive lymph node status. Moreover, a high level of dichotomized COX-2 expression was associated with reduced patient survival rates (Sheehan et al. , 1999).

5.2 Role of COX-2 in tumorigenesis

Multiple studies have shown that NSAIDs can prevent experimental colon cancer. Specifically, studies in carcinogen-induced colon cancer in rodents demonstrate that NSAIDs and coxibs can prevent aberrant crypt foci, adenomas and carcinomas of the colon (Reddy et al., 2000). In the APC^{Min} mouse model wherein adenomas develop predominantly in the small intestine, suppression of intestinal tumorigenesis has been observed with NSAIDs and coxibs (Jacoby et al. 2000). Administration of coxibs has also been shown to suppress intestinal polyposis in the $APC^{\Delta 716}$ mouse. A critical link between COX-2 and colorectal tumorigenesis was shown in $Apc^{\Delta 716}$ mutant mice mated to COX-2 knockouts, where a dramatic reduction in intestinal polyp number was found in doubly null progeny compared to COX-2 wild-type animals (Oshima et al., 1996). The observation that these animals had incomplete suppression of all polyps suggests that while COX-2 is important, it is not essential for intestinal polyp development. Taken together, these data represent the best evidence of the importance of COX-2 in intestinal tumorigenesis. Data also indicate that COX-2 can induce cellular transformation *in vivo*. In transgenic mice that overexpress the human COX-2 gene in mammary glands using a mammary tumor virus (MMTV) promoter, Liu et al. (2001) found that multiparous but not virgin mice showed significant increases in mammary gland carcinomas compared with age-matched controls. Similarly, constitutive COX-2 expression in basal keratinocytes of transgenic mice resulted in epidermal hyperplasia and dysplasia. Thus, the relationship between COX-2 and tumorigenesis has been clearly demonstrated. While the specific mechanism underlying this relationship remains unknown, studies indicate that COX-2 may

potentially mediate tumor development and progression by effects on apoptosis, angiogenesis, and tumor invasiveness.

1. Apoptosis resistance. Epithelial tissue homeostasis is maintained by a balance between cell proliferation and cell death. It is believed that disruption of this homeostatic balance may give rise to tumors. Bedi et al. (1995) found a progressive decrease in apoptotic rates during colorectal tumorigenesis. Furthermore, studies by Moss et al. (1996) have shown that adenomas are characterized by an inverted apoptotic gradient relative to normal mucosa. *In vitro* studies have addressed the consequences of forced COX-2 overexpression. Transfection of rat intestinal epithelial cells with the COX-2 cDNA resulted in increased adherence to extracellular matrix, resistance to butyrate-induced apoptosis, and up-regulation of Bcl-2 expression; these alterations were reversed by sulindac sulfide. Forced COX-2 expression in HCT-15 human colon cancer cells could inhibit apoptosis induction by anti-neoplastic drugs. Specifically, COX-2 overexpression was associated with inhibition of the mitochondrial apoptotic pathway characterized by reduced cytochrome c release, attenuated caspase-9 and -3 activation, and up-regulation of Bcl-2 in HCT-15 cells (Sun et al., 2002). Additionally, ectopic COX-2 expression in HCT-15 cells also suppressed the membrane death receptor (DR) apoptotic pathway via transcriptional repression of DR5 expression. Treatment of stable COX-2 transfected cells with the DR5 ligand, recombinant human TNF-related apoptosis-inducing ligand (TRAIL also known as Apo2L), resulted in decreased apoptosis compared to vector alone transfectants. Interestingly, the NSAID sulindac sulfide was shown to up-regulate DR5 expression in HCT-15 cells and in other colon cancer cells. Furthermore, the combination of TRAIL and sulindac sulfide reduced cell survival to a greater extent than did either drug alone (Tang et al., 2002). COX-2 overexpression is also associated with apoptotic resistance *in vivo*. In mammary glands, decreased apoptotic indices were found in mammary epithelial cells from transgenic mice that overexpress COX-2 (Liu et al., 2001). Furthermore, increased anti-apoptotic Bcl-2 and Bcl-X_L proteins and reduced pro-apoptotic BAX expression were found in these cells. Collectively, these data suggest that COX-2 can reduce apoptotic susceptibility which may contribute to tumor development and/or progression.

2. Increased invasiveness and angiogenesis. COX-2 expression has been shown to increase cellular adhesion and invasiveness which may influence tumor metastasis. COX-2 and the matrix metalloproteinase (MMP) matrilysin, were shown to be co-expressed in 80% of human colorectal cancers. Matrilysin was strongly expressed at the invading tumor edge whereas COX-2 was expressed throughout the neoplastic epithelium (Shattuck-Brandt et al., 1999) . In the Caco-2 colon

cancer cell line, forced COX-2 expression resulted in increased invasiveness, activation of matrix metalloproteinase-2 (MMP-2), and increased RNA levels for the membrane-type metalloproteinase (Tsujii et al., 1997). Interestingly, these effects were reversed by sulindac sulfide. Similarly, forced COX-2 expression in a transitional cell carcinoma cell line was associated with increased MMP-2 activation and angiogenesis. Levels of MMP-2 and MMP-9 were reduced by NS398, a selective COX-2 inhibitor, in the human prostate tumor cell line DU-145 (Attiga et al., 2000). In the 1980s, a link between PGs and angiogenesis was shown wherein NSAIDs inhibited vascularization in tumor xenografts. In an *in vitro* angiogenesis model, Jones et al. (1999) found that nonselective and COX-2 selective NSAIDs inhibit angiogenesis through direct effects on endothelial cells and have PG-dependent and PG-independent components. Ectopic expression of COX-2 in Caco-2 and HCA-7 cells was associated with production of high levels of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), transforming growth factor β-1 (TGFβ-1), platelet-derived growth factor and endothelin-1 which stimulate endothelial migration and endothelial tube formation (Tsujii et al., 1998) (**Fig. 10**).

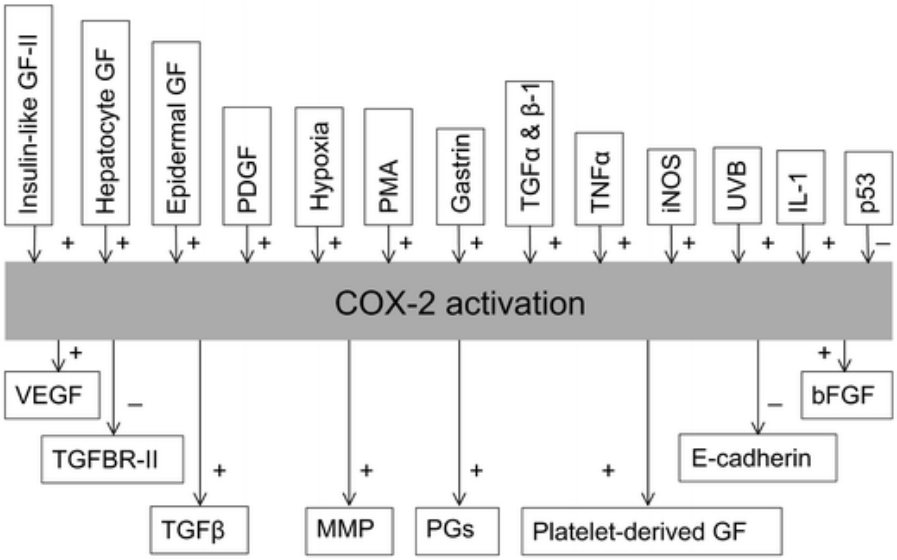


Figure 10. COX-2 activation pathways in tumor angiogenesis.

COX-2 expression enhanced bFGF-induced angiogenesis by PG-mediated expression of VEGF in rat sponge implants. A significant correlation between COX-2 and VEGF expression was found in human colorectal cancers and both were correlated with increased microvessel density. When Lewis lung carcinoma cell xenografts were implanted into COX-2 null mice, a decrease in vascular density was observed relative to those in wild-type mice. More recent data have also shown that selective COX-2 inhibitors can reduce angiogenesis *in vitro* and *in vivo* (Masferrer et al., 2000).

Aspirin and NS398 were shown to inhibit angiogenesis and the production of angiogenic factors in colon cancer cells with COX-2 overexpression. In a rat model of angiogenesis, celecoxib blocked corneal blood vessel formation while SC-560, a specific COX-1 inhibitor, had no effect. In this same assay, Daniel et al. (1999) reported that endothelial migration and angiogenesis can be inhibited by a TxA2 receptor antagonist, suggesting that COX-2 derived TxA2 may be an important activator of angiogenesis.

5.3 Anti-tumor effects of nonsteroidal anti-inflammatory drugs (NSAIDs)

Numerous epidemiological studies indicate that regular and prolonged intake of NSAIDs, particularly aspirin, is associated with a 40–50% reduction in CRC incidence [2–4]. Anti-tumor effects of NSAIDs have been shown in the Apc^{Min} mouse, the AOM-treated rat, and in tumor xenografts. Colorectal adenoma regression has also been observed for the NSAID sulindac and celecoxib compared to placebo in patients with FAP (Steinbach et al., 2000). To date, the exact mechanism(s) by which NSAIDs exert their anti-tumor effects are incompletely understood. While COX is the best defined molecular target of NSAIDs, evidence indicates that there are both COX-dependent and COX-independent effects of NSAIDs. Importantly, at lower doses of NSAIDs, COX-dependent mechanisms appear to the most relevant to the human disease. The chemopreventive properties of NSAIDs have traditionally been attributed to COX inhibition and a reduction in prostaglandin levels. Inhibition of the COX-2 enzyme by genetic or pharmacologic approaches has been shown to be sufficient to inhibit tumorigenesis. Studies in animal models show equivalent or greater efficacy for the prevention of intestinal neoplasms with coxibs compared to traditional NSAIDs. Treatment of $APC^{\Delta 716}$ mutant mice or COX-2 wild-type mice with a novel COX-2 inhibitor or sulindac was shown to reduce polyp number (Oshima et al., 1996). In a recent study using this mouse model, the selective COX-2 inhibitor rofecoxib was shown to significantly reduce the number and size of small intestinal and colon polyps at clinically achievable concentrations. Studies have also attempted to address the role of COX-1. Chulada et al. (2000) showed an equivalent reduction in tumor multiplicity in COX-1 and COX-2 knockout Apc^{Min} mice when compared to wild-type controls. In contrast, using Lewis lung carcinoma cell xenografts in C57BL/6 mice, tumor growth was markedly attenuated in COX-2 null, but not COX-1 null or wild-type Apc^{Min} mice. The explanation for these disparate results is unknown and the contribution of COX-1 inhibition to the anti-tumor effects of NSAIDs awaits further study. Inhibition of COX and PG synthesis by NSAIDs cannot entirely explain many of the experimental results obtained. Both nonselective COX and selective COX-2 inhibitors have been shown to inhibit cell proliferation and

to induce apoptosis of colon and several cultured tumor cell lines (Piazza et al., 1995). However, the doses of NSAIDs found to exert these effects *in vitro* have generally been well in excess of doses that can be achievable *in vivo*, thereby casting suspicion as to the clinical relevance of these findings. Yet, in intestinal tissues from animals and humans treated with NSAIDs, modulation of apoptotic rates has been found in association with tumor inhibition and regression (Samaha et al., 1997). Specifically, NSAID treatment is associated with increased rates of apoptosis in the intestinal epithelium of animal models of colon cancer as well as in FAP patients (Sinicrope et al., 2003). To date, the molecular and biochemical pathways responsible for the pro-apoptotic effects of NSAIDs remain poorly understood. Sulindac sulfide triggers the caspase-dependent mitochondrial apoptotic pathway in colon cancer cells. Furthermore, this drug can also modulate the levels of membrane death receptor DR5 which regulates the apoptotic response to its ligand TRAIL (He et al., 2002). A role for the pro-apoptotic BAX gene in NSAID-induced apoptosis in colon cancer cell lines has been demonstrated (Zhang et al., 2000). In this study, NSAIDs decreased levels of the antiapoptotic gene BCL-X_L, thereby increasing the cellular ratio of BAX: BCL-X_L; BAX(-/-) cells were resistant to NSAID-induced apoptosis (**Fig. 11**). Additional evidence for COX-independent effects include the observation that (1) NSAIDs can inhibit the growth of cancer lines devoid of COX-2 expression as they do for those producing COX-2 (Hanif et al., 1996); (2) murine embryonic fibroblasts with homologous knockout of COX-1 and COX-2 alleles remain sensitive to the antiproliferative and pro-apoptotic effects of NSAIDs (Zhang et al., 1999); (3) suppression of growth factor-stimulated mitogenesis in HCA-7 colon cancer cell lines by the COX-2 inhibitors NS983 and SC-58125 did not correlate with a reduction in PG levels, and exogenous PGs (PGE2 and PGF2) failed to restore mitogenesis (Coffey et al., 1997). The addition of PGE2 to NSAID-treated colon cancer cells has been shown to reverse their growth inhibitory and pro-apoptotic effects in some (Tsujii et al., 1995) but not other (Elder et al., 2000) reports. (4) The NSAID sulindac is a pro-drug that is metabolized to its sulfide and sulfone derivatives (Piazza et al., 1995). While the sulfide derivative is a potent inhibitor of COX, the sulfone metabolite lacks COX-inhibitory activity. However, sulindac sulfone has been shown to exert anti-proliferative and pro-apoptotic effects *in vitro* and is an effective chemopreventive agent in an animal model of colon cancer, offering yet further evidence of COX-independent anti-tumor effects. Other biochemical targets may contribute to the COX-independent effects of NSAIDs. High doses of aspirin were found to inhibit signaling by the transcription factor NF-kB (Kopp et al., 1994). This effect appears to be mediated by inhibition of the IkB kinase enzyme (Yamamoto et al., 1999) which is responsible for activation of the NF-kB pathway by phosphorylating the inhibitory subunit of NF-kB and targeting it for destruction (**Fig. 11**). Aspirin and sulindac can inhibit the activity of IkB

kinase *in vitro*. Peroxisome proliferator-activated receptors or PPARs have also been proposed as important targets for NSAIDs (He et al., 1999). PPARs are a family of ligand-activated transcription factors that are members of the nuclear-hormone receptor superfamily. In a study by He et al. (1999), PPARδ expression was repressed by the APC tumor suppressor gene in CRC cells. This repression was mediated by beta-catenin/Tcf-4 responsive elements within the PPARδ promoter. Furthermore, the NSAID sulindac was shown to bind to and inhibit the DNA-binding activity of PPARδ (**Fig. 11**). Overexpression of PPARδ was also found to suppress the induction of apoptosis by sulindac. In a subsequent study, however, PPARδ null cell lines grown as xenografts in nude mice displayed similar sensitivity to sulindac-induced apoptosis when compared to PPARδ ± and wild-type controls (Park et al., 2001). Taken together, multiple lines of evidence suggest that cellular targets of NSAIDs other than inhibition of PG biosynthesis may be important for their antitumor effects.

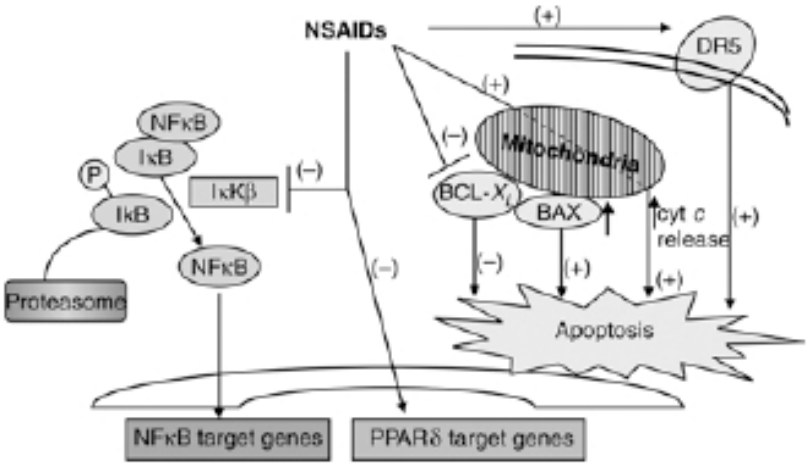


Fig. 11. Anti-tumor effects of NSAIDs

5.4 COX-2 and colorectal cancer: regulation by Wnt pathway

Direct genetic evidence that COX-2 plays a key role in colorectal tumorigenesis was provided by Oshima *et al.* (1996), who showed that knocking out the *COX-2* gene caused a marked reduction in the number and size of intestinal polyps in *Apc* knockout mice, a murine model of familial adenomatous polyposis. *APC* gene inactivation plays a critical role at an early stage in the development of both inherited and sporadic forms of colorectal cancer (Kinzler and Vogelstein, 1996), also, mutant APC DNA has been detected in feces from patients with this cancer. APC is a member of the Wnt signal transduction pathway (Bienz and Clevers, 2000) and interacts with a

variety of cytoplasmic proteins including glycogen synthase kinase-3β and axin family proteins, and β-catenin. Wild-type APC can bind to β-catenin and direct its intracellular degradation. However, mutated APC does not bind to β-catenin, resulting in its nuclear translocation (Behrens et al., 1996). Moreover, upon the activation of EP2 receptors by PGE_2, the α subunit of G_s binds the RGS domain of Axin, thereby promoting the release of GSK-3ß from its complex with Axin. Concomitantly, free ßγ subunits stimulate the PI3K-PDK1-AKT signaling route, which causes the phosphorylation and inactivation of GSK-3ß. β-catenin is a major component of adherence junctions linking the actin cytoskeleton to members of the cadherin family of transmembrane cell-cell adhesion receptors. PGE_2 can also activate EGFR indirectly through the stimulation of c-Src and the activation of matrix metalloproteinases (*MMP*) that convert latent EGFR ligands (such as heparin-binding EGF-like growth factor and TGFα) into their active forms, thereby stimulating the EGFR-initiated signaling network. β-Catenin translocates into the nucleus, where it complexes with Tcf-4 transcription factors and regulates the expression of specific genes, *e.g., c-myc, cyclin D1,* and *PPAR*δ (Tetsu and McCormick, 1999). Wnt family members are critical in developmental processes and have been shown to promote carcinogenesis. The Wnt-signaling pathway inactivates GSK-3β, which results in subsequent stabilization of β-catenin and stimulates Tcf-4-mediated gene transcription. Although the APC status affects COX-2 expression, and COX-2 has been proposed to be a downstream target of the Wnt-signaling pathway (Howe et al., 1999), this hypothesis has not been fully investigated.

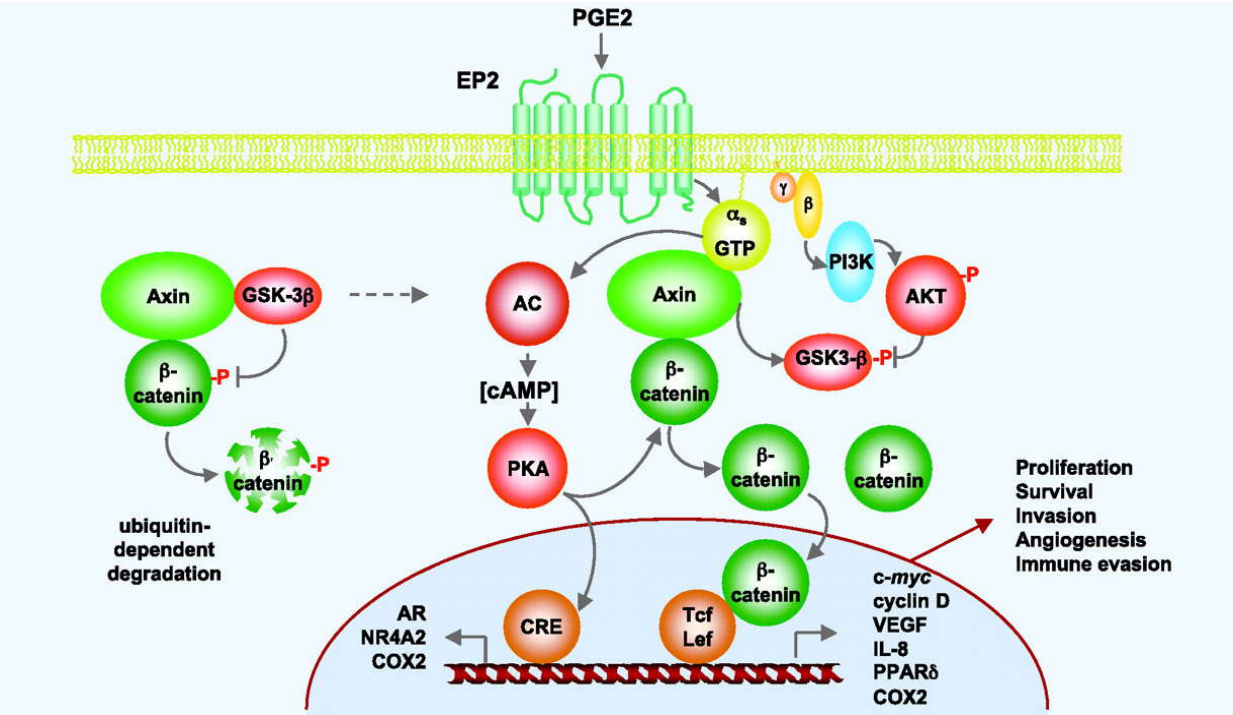


Figure 12. The Wnt signalling pathway and COX-2 regulation.

5.5 COX-2 and colo-rectal cancer: regulation by the hypoxic pathway

Adaptation to hypoxia is critical for tumor cell growth and survival and is achieved largely by transcriptional activation of genes that facilitate short- and long-term adaptive responses (Semenza, 2002). Hypoxia induces COX-2 in colorectal tumor cells and this up-regulation is mediated directly by hypoxia-inducible factor 1 (HIF-1). In addition, COX-2 up-regulation by hypoxia represents a critical adaptive mechanism that promotes colorectal tumor cell survival and angiogenesis under hypoxic conditions. It is of interest to note that, in response to hypoxia, COX-2 expression is enhanced in both colorectal adenoma and carcinoma cells, suggesting that hypoxia may contribute to COX-2 overexpression at early stages of colorectal tumorigenesis. Additionally, hypoxia up-regulates COX-2 expression in colorectal tumor cells with different basal levels of COX-2, suggesting that hypoxia may act synergistically with other pathways implicated in COX-2 up-regulation. COX-2 up-regulation by hypoxia has been described previously in human umbilical vascular endothelial (Schmedtje et al., 1996) and corneal epithelial cells (Bonazzi et al., 2000) to be mediated by NF-κB and peroxisome proliferator-activated receptors, respectively. In addition, Csiki et al. reported that COX-2 is up-regulated in hypoxic lung cancer cells in an HIF-1-dependent manner (Csiki et al., 2006). Kaidi et al. (2006) showed that HIF-1 directly binds a specific HRE located at –506 on the *COX-2* promoter, enhancing COX-2 protein expression and PGE_2 production. This could explain, at least in part, how colorectal tumor cells maintain their growth and survival under hypoxic conditions. Until now, great emphasis has been placed on the role of COX-2/PGE_2 in tumor cell growth and survival under normoxic conditions, mediated by activating phosphatidylinositol 3-kinase/MAPK pathways (Sheng et al., 2001) as well as up-regulating the prosurvival protein Bcl-2. The increase in COX-2 and PGE_2 levels in hypoxic colorectal tumor cells represents a short-term adaptive response that allows cell survival during hypoxia, which could have important implications for colorectal tumorigenesis. Although the mechanisms by which PGE_2 promotes cell survival in hypoxia are not completely elucidated here, data suggest that it is likely to be occurring through the activation of MAPK (Wang et al., 2005; Kaidi et al. 2006). In addition to mediating short-term survival and metabolic responses in tumors, hypoxia also induces long-term responses, mediated chiefly by the secretion of VEGF. For colorectal tumors, in particular, COX-2 plays a critical role in VEGF induction and stimulation of angiogenesis (Tsujii et al., 1998). Infact, PGE_2 has important role in increasing VEGF levels in colorectal cancer cells in normoxia and also during hypoxia. This potentiation is achieved by the effect of PGE_2 in enhancing the transcriptional activity of HIF-1 through the activation of MAPK pathway, consistent with previous reports that described the involvement of MAPK pathways in the modulation of HIF-1 transcriptional activity (Sang et al., 2003). The ability of PGE_2 to potentiate HIF-1 transcriptional activity is particularly

interesting because HIF-1 is involved in the regulation of several other pathways implicated in tumorigenesis (Semenza, 2003). Therefore, PGE_2 up-regulation in colorectal tumor cells during hypoxia may modulate the expression of several other HIF-1-target genes, which could have implications for tumor cell survival, angiogenesis, invasion and metastasis, and subsequently tumor progression. The link between hypoxia and COX-2 suggests possible overlapping functions that collectively drive the progression of colorectal and potentially other solid tumors where hypoxia is commonly observed. Recently, PGE_2 has been shown to amplify the expression of COX-2 during colorectal tumorigenesis through a positive feedback loop involving the constitutive activation of Ras-MAPK. Increased levels of PGE_2 during hypoxia, resulting from COX-2 up-regulation, potentiate HIF-1 transcriptional activity, which results in further up-regulation of COX-2 because *COX-2* is a HIF-1 target gene. This positive feedback loop may be important in maintaining COX-2 overexpression in hypoxic colorectal tumor cells (**Fig. 13**).

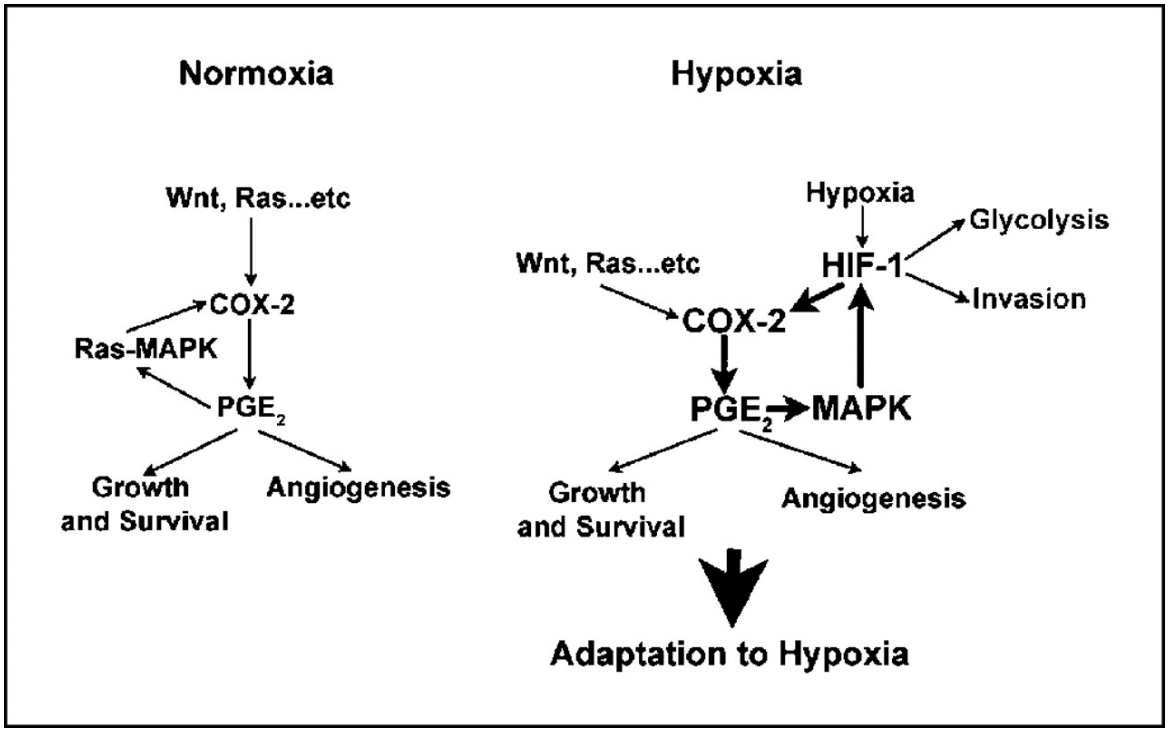


Figure 13. The hypoxic survival pathway in colo-rectal cancer.

Chapter II
The Mechanism of RNA Interferenc e (RNAi)

1. Origins of RNA Interference

RNA Interference (RNAi) has revolutionized studies to determine the role of a gene. The advent of massive genome sequencing projects has highlighted the marked need for a means of elucidating gene function. Loss of function studies using antisense and homologous recombination are cumbersome and variably successful. RNAi now provides a rapid means of depleting mRNAs by introducing double-stranded RNA homologous to a particular message leading to its sequence-specific degradation. As with many great discoveries, the history of RNAi is a tale of scientists able to interpret unexpected results in a novel and imaginative way. Napoli and Jorgensen were the first to report an RNAi type of phenomenon in 1990 (Napoli et al., 1990). The goal of their studies was to determine whether chalcone synthase (CHS), a key enzyme in flavonoid biosynthesis, was the rate-limiting enzyme in anthocyanin biosynthesis. The anthocyanin biosynthesis pathway is responsible for the deep violet coloration in petunias. In an attempt to generate violet petunias, Napoli and Jorgensen overexpressed chalcone synthase in petunias, which unexpectedly resulted in white petunias. The levels of endogenous as well as introduced CHS were 50-fold lower than in wild-type petunias, which led them to hypothesize that the introduced transgene was "cosuppressing" the endogenous CHS gene. In 1992, Romano and Macino reported a similar phenomenon in *Neurospora crassa* (Romano and Mancino, 1992), noting that introduction of homologous RNA sequences caused "quelling" of the endogenous gene. RNA silencing was first documented in animals by Guo and Kemphues, who observed that the introduction of sense or antisense RNA to par-1 mRNA resulted in degradation of the par-1 message in *Caenorhabditis elegans* (Guo and Kemphues, 1995). At that time, antisense was one of the most attractive means of eliminating gene expression. Antisense was thought to function by hybridization with endogenous mRNAs resulting in double-stranded RNA (dsRNA), which either inhibited translation or was targeted for destruction by cellular ribonucleases. Surprisingly, when Guo and colleagues performed control experiments using only the sense par-1 RNA, which would not hybridize with the endogenous par-1 transcript, the par-1 message was still targeted for degradation. This finding caused investigators to rethink the current dogma. In 1998, Fire and Mello published a seminal paper that provided an explanation for the previously reported silencing of endogenous genes by "cosuppression", quelling and sense mRNA" (Fire et al., 1998). Working with *C. elegans*, they

tested the hypothesis that the trigger for gene silencing was not single-stranded RNA (ssRNA) but double-stranded RNA (dsRNA). They reasoned that the seemingly paradoxical finding of Guo and Kemphues showing that introduction of sense RNA leads to gene silencing could have been due to the contamination of preparations of ssRNA by dsRNA resulting from the activity of bacteriophage RNA polymerases. To address this possibility, Fire and Mello extensively purified the sense and antisense ssRNA preparations, then directly compared their effects to dsRNA on the unc-22 gene. The purified ssRNAs (sense or antisense) were consistently found to be 10- to 100-fold less effective than dsRNA targeting the same mRNA. Indeed, ssRNA was found to be effective only if the sense strand was injected into the animals, followed by the antisense strand or *vice versa*, suggesting that hybridization of the ssRNA to form dsRNA occurred *in vivo*. Thus, Fire and Mello provided the first explanation for previous observations, implicating integrated transgenes in the production of dsRNA in plants and fungi, and contamination of sense RNA by dsRNA in worms. While this work established an entirely new conceptual framework for the effects of RNA on gene silencing by highlighting a role for dsRNA, a plethora of questions remained regarding the mechanism by which dsRNA could cause the degradation of endogenous mRNA. When dsRNA was injected into one region of a worm or plant, it caused systemic silencing, which led to the hypothesis that the RNAi effect was mediated by a stable silencing intermediate. This hypothesis was further supported by the observation that gene silencing could be passed from parent to progeny in *C. elegans* (Voinnet and Baulcombe, 1997; Grishok et al., 2000). The existence of stable intermediates was first demonstrated by plant virologists Hamilton and Baulcombe (1999). Although it was generally thought that the dsRNA had to unwind in order for the antisense strand to bind to the mRNA, the full-length antisense strand was never detected. This led Hamilton and Baulcombe to search for shorter forms of the antisense RNA derived from the dsRNA. They hypothesized that antisense RNA could serve as a guide, binding to the mRNA and causing its degradation. When Hamilton and Baulcombe detected antisense RNA that had an estimated length of 25 nucleotides (nt), they suggested that this length was necessary for RNAi specificity. The following year, two independent teams of biochemists used extracts from *Drosophila* cells to identify the silencing intermediate (Hammond et al., 2000; Zamore et al., 2000) . Upon fractionation, both groups found that 21–23 nt RNA always copurified with RNAi, suggesting that dsRNA was converted to shorter intermediates, small interfering RNAs (siRNAs) capable of binding to their homologous target mRNAs, leading to cleavage of the transcript. To determine definitively that the 21–23 nt dsRNAs are the effector molecules of the RNAi pathway, Tuschl and colleagues incubated Drosophila cell extracts with chemically synthesized 21–22 nt dsRNAs targeting a firefly luciferase transcript (Elbashir et al., 2001b). The siRNAs were able to act as

guides to mediate cleavage of the target mRNA. siRNAs with 2–3 nt overhangs on their 3' ends were more efficient in reducing the amount of target mRNA than siRNAs with blunt ends. The target mRNA was found to be cleaved near the center of the region encompassed by the 21–22 nt RNAs 11 or 12 nt downstream of the first base pair between the siRNA and target mRNA (Elbashir et al., 2001b). These short chemically synthesized 21–22 nt siRNAs were capable of silencing not only heterologous but also endogenous genes in mammalian cells (Elbashir et al., 2001a). Up to this point, the use of RNAi was limited to flies, worms, and plants, as the introduction of long dsRNA into mammalian cells elicits an interferon response that causes a general inhibition of translation abrogating the specificity of RNAi. The finding that short dsRNA could silence genes heralded the use of RNAi in mammalian cells.

2. Mechanism of action of RNAi

2.1 Small interfering RNAs (siRNAs)

The initial descriptions of RNAi focused on the post-transcriptional suppression of target genes mediated by the introduction of homologous dsRNA [over ≈100 nucleotides (nts)] into model organisms. Subsequently, these dsRNAs were found to be processed into smaller 21–23 nt dsRNAs, termed small interfering RNAs (siRNAs), with 3' dinucleotide overhangs generated by the RNase III endoribonuclease Dicer (Bernstein et al., 2001; Elbashir et al., 2001b). siRNAs were found to be active independent of processing from larger dsRNAs. As the immune response precludes the use of long dsRNAs in mammalian cells, it was not until this discovery that RNAi could be identified in these systems (Elbashir et al., 2001a). siRNAs direct the cleavage of targeted mRNAs. Cleavage is mediated by a single strand of the siRNA duplex, termed the guide strand, after incorporation into a ribonucleoprotein complex known as the RNA-induced silencing complex (RISC). RISC contains Argonaute proteins. This family of proteins is highly diverse, but all members are characterized by the presence of two domains, the Piwi-Argonaute-Zwille (PAZ) and PIWI domains (Parker and Barford, 2006). The PAZ domain specifically recognizes the characteristic 3' termini of processed effectors and the PIWI domain adopts an RNase H-like structure that can catalyze the enzymatic cleavage of RNA. There are eight known Argonaute proteins in humans, but of these only Argonaute 2 (Ago2) has been found to generate cleavage-competent RISC (Liu et al., 2004a). In addition to target cleavage, Ago2 is also responsible for guide strand selection. This occurs through

the asymmetric unwinding of duplex RNAs, whereby the guide strand is preferentially retained within RISC and the other strand, termed the passenger strand, is degraded (Matranga et al., 2005).

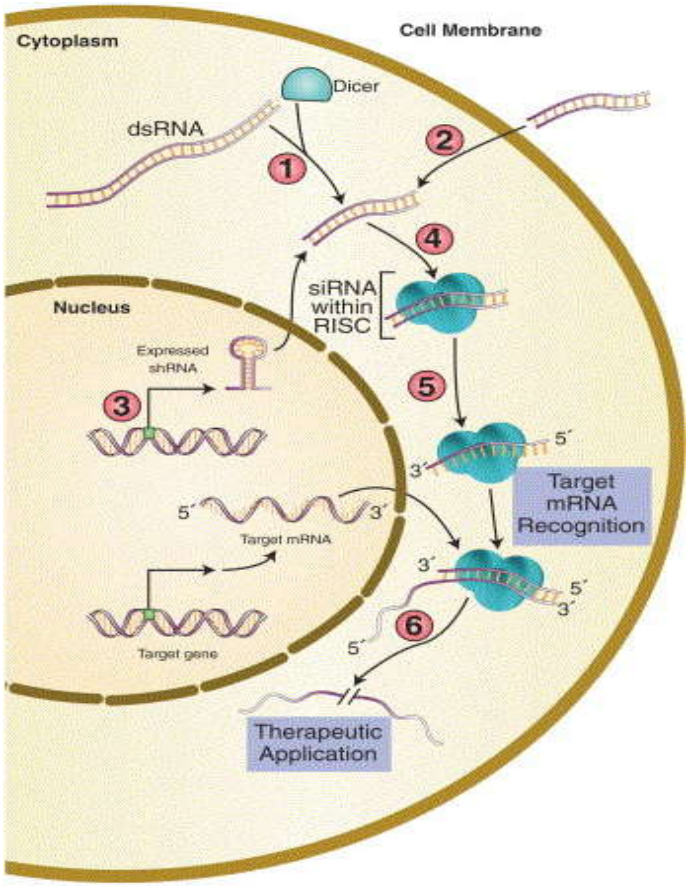


Figure 14. Mechanism of action of siRNAs.

2.2 MicroRNAs (miRNAs)

Although the introduction of exogenous siRNAs results in the RISC-dependent cleavage of target transcripts, the documented occurrence of endogenous cleavage complexes is not common in mammalian cells. Rather, it is another species of small RNA, termed microRNAs (miRNAs), that uses the innate RNAi machinery. miRNAs interact with transcripts possessing partial complementarity, primarily within target 3' untranslated regions (UTRs). miRNAs were originally identified as a species of small RNA (≈22 nt) that regulates genes required for development in the nematode *C. elegans*. Known as small temporal RNAs, these were the first examples of a large number of small endogenous RNAs that can regulate gene expression. miRNAs are generated through the processing of genomically encoded primary miRNA transcripts (pri-miRNAs) by a multisubunit complex that at its core consists of the RNase III endoribonuclease Drosha and, in

mammalian cells, the DGCR8 protein. The processing of primary miRNA transcripts yields hairpin structures known as precursor miRNAs (pre-miRNAs). Following export to the cytoplasm via Exportin5 (Exp5), pre-miRNAs are processed by Dicer to produce mature miRNAs that incorporate into miRNA ribonucleoprotein complexes (miRNPs). These complexes are similar, if not identical, to RISC. The precise mechanism by which individual miRNAs recognize their target sites on the mRNAs has not yet been completely unraveled but some general patterns have been determined (**Figs. 15** and **16**). The sequence motif bound by the miRNA is situated in the 3'-untranslated region (3'-UTR) of the transcript, i.e. between the protein-coding region of the mRNA and its poly(A) tail (Stark et al., 2005). By sequence comparison of miRNAs and their cognate mRNA target sequences it has been found that nucleotides 2 to 8 of the miRNA constitute a "seed region" that in most cases binds to a perfectly complementary recognition sequence on the mRNA. The central part of the miRNA usually lacks complementarity to the mRNA (typically nucleotides 10 and 11), whereas the 3'-region of the miRNA binds more or less specifically to the mRNA and contributes partly to the specificity and affinity of the miRNA:mRNA complex (Brennecke et al., 2005). In a few instances, the seed region does not show complete complementarity to the target sequence and in these cases a strong binding of the miRNA 3'-region to the mRNA is required to stabilize the RNA duplex (as seen in the interaction of miR-10 with the *sex combs reduced (scr)* transcript of *Drosophila melanogaster* (Enright et al., 2003). miRNAs that rely mainly on their seed sequence for binding may exert a function on the mRNA by themselves, whereas those that bind less strongly due to a weaker seed sequence often have to act in concert with other miRNAs binding to the same mRNA to cause an effect. Based on these binding requirements, computational calculations indicate that each miRNA on average recognizes about 100 different mRNA targets (Brennecke et al., 2005). Depending on the mode of base pairing between the miRNA and mRNA, one of two regulatory pathways is employed—the short interfering RNA (siRNA) or the miRNA pathway. If perfect base complementarity exists between the miRNA and mRNA, the mRNA will be processed through the siRNA pathway and cleaved in a miRNA-directed manner. An endonucleolytic cleavage of the mRNA is catalyzed by the ribonuclease in RISC (Ago2 in humans) in the center of the region binding the miRNA and as a result the mRNA is degraded (Bagga et al., 2005). This mode of gene silencing is common in plants, whereas only a few examples of mRNA silencing in this manner have been reported for animals (one example is the miR-196-directed cleavage of the HOXB8 transcript during mouse embryogenesis (Yekta et al., 2004). However, this mechanism has proven to be invaluable as an experimental tool for specific inactivation of target mRNAs in cells through RNA interference (RNAi), where binding of a synthetic miRNA with perfect match to the target mRNA causes degradation of the latter through the siRNA pathway.

Despite the existence of the siRNA pathway in human cells, most human miRNAs regulate mRNA function through the miRNA pathway. Three modes of action have been unraveled, which result in translational repression and partial mRNA decay: Repression of the initiation step of translation, repression of the elongation phase of translation, and a general destabilization of the transcript as a result of poly(A)-tail shortening. miRNA-directed inhibition of translational initiation was supported by the finding of an effect of let-7 on the translation of reporter mRNAs in HeLa cells (Pillai et al., 2005). Here, the distribution of polysomes of let-7-repressed mRNAs was shifted towards the lighter fractions of a sucrose gradient in a manner similar to that observed when using known inhibitors of translational initiation. Analogously, the cationic amino acid transporter 1 (CAT-1) mRNA, which is repressed by miR-122 in hepatocarcinoma cells under regular growth conditions, was found in the light polysomal fraction. If, however, the cells were starved, translational derepression of CAT-1 mRNA occurred and was accompanied by a shift in polysomal distribution towards the heavier fractions (Bhattacharyya et al., 2006). The mechanism of miRNA-directed repression requires a mRNA with a 5'-terminal m^7G cap structure—as demonstrated by the lack of inhibition by let-7 and CXCR4 miRNA of mRNAs, which are made to use a different mechanism of translational initiation, namely internal ribosomal entry sites (IRES). Protein production may also be inhibited by miRNAs during the process of translation. In *Caenorhabditis elegans*, repression of the lin-14 mRNA by the miRNA lin-4 does not involve a change in polysome distribution, indicating that repression occurs after initiation of translation. A number of reports demonstrate an association between miRNAs and actively translating polyribosomes also in human cells. The exact mechanism of repression has not been clarified yet but is probably due to either a decrease in the elongation rate and/or degradation of the nascent protein (Nottrott et al., 2006). Besides translational inhibition, an accelerated decay of the transcripts may also be observed following their interaction with miRNAs by a mechanism distinct from the siRNA-type mRNA cleavage. This mechanism of degradation relies on the recruitment of deadenylating and decapping enzymes by the miRNAs with a subsequent degradation of the cognate transcript as result. Mounting data indicate that mRNAs silenced by miRNA accumulate in cytoplasmic compartments known as processing bodies (P-bodies) (Sen et al., 2005). The mRNAs found in these locations are devoid of ribosomes and other translation factors. The P-bodies are rich in enzymes involved in mRNA deadenylation, decapping, and degradation, and are believed to cause decay of the miRNA-inhibited mRNAs (Sheth and Parker, 2003). In some instances, however, the mRNAs instead appear to be stored in an inactive form in the P-body with the potential to re-enter the cytoplasm and re-engage in translation (Brengues et al., 2005). One example of this is the miR-122-directed

repression of CAT-1 in hepatocarcinoma cells during normal growth, which is relieved by starvation and results in re-translation of the CAT-1 mRNA (Bhattacharyya et al., 2006).

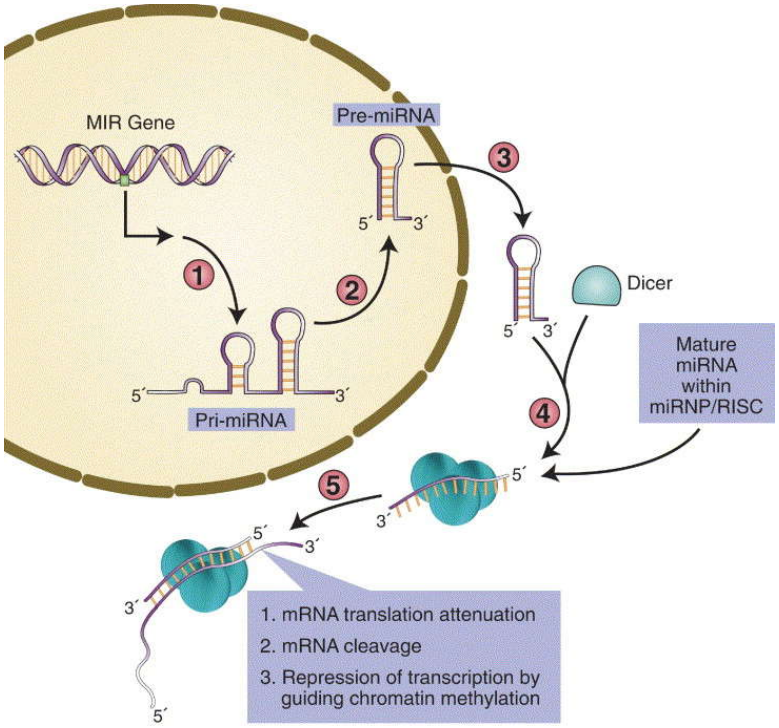


Figure 15. Mechanism of miRNAs formation.

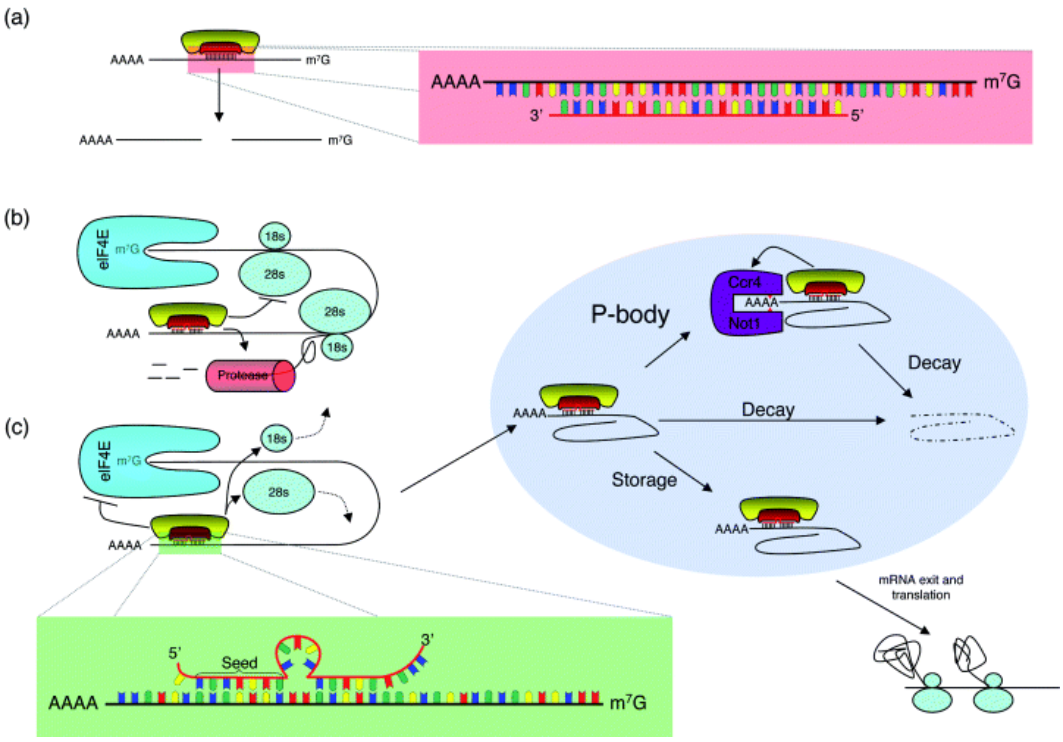


Figure 16. Possible mechanisms of miRNA-mediated repression of target mRNAs in animals.

3. Applications of RNAi in mammalian systems

In most cases, the aim of RNAi-based experiments is the sequence-dependent cleavage and reduction of protein-encoding mRNAs. Although most studies have focused on the RNAi analysis of these targets, any RNA species can be targeted (for example, noncoding RNA transcripts or viral RNAs). Only a limited number of mammalian cell types can tolerate RNAi induced by large, exogenous dsRNAs (e.g., embryonic stem cells). Thus, it is usually necessary to use one of two broad categories of RNAi effector molecules in mammalian systems. These include siRNA duplexes, formed through the annealing of two independent RNA strands, or single-stranded RNA molecules that contain a dsRNA domain, termed short-hairpin RNAs (shRNAs). In both cases, RNAi effectors are designed to possess full complementarity with target transcripts, thereby resulting in their cleavage.

3.1 RNAi effectors used for biological analysis in mammalian cells

siRNAs can be generated through the annealing of synthetic oligonucleotides. Most synthetic siRNAs consist of 19 perfectly matched complementary ribonucleotides and 3' dinucleotide overhangs that, for ease of synthesis, often consist of deoxyribonucleotides. Synthetic siRNAs are available from a number of commercial vendors. More rarely, siRNAs are generated by a number of other methods including *in vitro* transcription, plasmid-based tandem or convergent expression cassettes, polymerase chain reaction (PCR) or the endonuclease digestion of large dsRNAs that produce pools of siRNAs. The introduction of synthetic siRNAs into cultured mammalian cells usually uses standard physico-chemical transfection methods, such as those based on cationic lipids, cationic polymers, or electroporation. Empirical testing is required to determine the most efficacious transfection conditions for any given cell system. Once well-optimized, transfected siRNAs can yield a substantial decrease in the steady-state levels of target mRNAs for 24–120 h. As opposed to direct transfection, shRNAs are usually expressed from plasmids or viral-based expression vectors. shRNAs are designed to mimic miRNA precursors. Consequently, they are processed by the endogenous RNAi machinery and loaded into RISC. A number of different shRNA expression systems have been described. Variations include differences in promoter-terminator combinations, linker sequences, flanking sequences, duplex length, and regulatory elements that can be used for spatial and/or temporal-specific expression. Additionally, selection markers, used to generate stable cell lines, and unique sequence elements, used to identify active shRNAs among larger populations (discussed below), have been employed. As opposed to siRNAs, the stable expression of shRNAs allows for a nontransient reduction of targeted mRNAs. Thus, the

choice of RNAi effector (siRNA or shRNA) depends on the question under investigation. No matter the choice, it is always important to include negative control effector molecules in any RNAi-based experiment. These controls, which are commercially available, incorporate sequences with minimal complementarity to any endogenous transcript. In addition to improving conditions for their cellular introduction and/or expression and subsequent processing, many studies have been directed toward maximizing activity as a function of effector sequence (Schwarz et al., 2003). For example, an understanding of any bias related to guide strand selection has obvious implications for design. Analysis has revealed that the strand most easily unwound from its 5' end is preferentially incorporated into RISC (Reynolds et al., 2004). Thus, effector design incorporates such bias to encourage selection of intended guide strands. Studies have indicated other positional biases. For example, high thermodynamic stability is preferred between nucleotides 5–10 of the guide strand (Reynolds et al., 2004). Furthermore, empirical comparisons between large sets of effective and ineffective siRNAs have led to the development of algorithms that assist in the generation of active siRNAs. These types of design tools are incorporated into the production of commercial siRNAs and are also publicly available. Sequences generated by these tools merely have an increased probability of mediating RNAi. Only experimentation will establish the activity of any given RNAi effector. Of note, an increasing number of validated sequences are available from commercial sources and are being characterized and collated by the scientific community. Considerations of the target are also important for maximizing RNAi. As RNAi effectors are designed according to reported reference sequences, any discrepancies between those and the actual target sequences within systems under study, for example as a consequence of single nucleotide polymorphisms (SNPs), may prevent efficient RNAi (Martin and Caplen, 2006). However, the influence of sequence discrepancies may be less than predicted owing to the fact that RISC can sometimes tolerate mismatches within targets, especially those distal from the cleavage site (Martin et al., 2007). Despite possibly interfering with RNAi, sequence aberrations can potentially be used to selectively target mutated transcripts associated with disease. This approach has been applied in a number of contexts including the targeting of cancer-specific mutations, the targeting of a single-base mutation associated with the dominant genetic disorder spinocerebellar ataxia, and, most recently, for the silencing of mutant β-globin as an approach toward treating sickle cell anemia (Miller et al., 2003). In addition to potential sequence discrepancies, one should also ensure that RNAi effectors target all known transcript variants of genes under study. Inevitably, even with an increased understanding of RNAi, effectors invariably exhibit a spectrum of activity. Thus, it may be prudent to obtain more than one effector against targets under investigation.

3.2 Off-target effects

The ability of RNAi effectors to elicit specific downregulation of intended targets while minimizing or controlling for unintended effects, termed off-target effects, is critical for the meaningful application of RNAi. Off-target effects are known to arise from a variety of mechanisms, which include both sequence-independent and sequence-dependent processes. Sequence-independent effects, or nonspecific effects, generally involve those relating to transfection conditions (e.g., lipid transfection reagents), inhibition of endogenous miRNA activity, or stimulation of pathways associated with the immune response. Sequence-dependent effects primarily concern the unintentional silencing of targets sharing partial complementarity with RNAi effector molecules through miRNA-like interactions, but also include receptor-mediated immune stimulation through the recognition of certain nucleotide motifs. As discussed below, there are a number of approaches toward controlling for both types of off-target effects.

1. Nonspecific Effects. Nonspecific effects resulting from the inhibition of endogenous miRNA activity appear to depend on saturation of Exp5. For example, the shRNA-mediated inhibition of miRNA activity is mitigated by the overexpression of Exp5. Also consistent with Exp5 as a saturatable component of RNAi, its overexpression, but not the overexpression of other RNAi components, enhances the activity of both miRNAs and shRNAs (Yi et al., 2005). As siRNAs do not require export from the nucleus, their activity would not be expected to depend on Exp5. However, some studies have found that siRNA-mediated RNAi is dependent on Exp5, where Exp5 prevents entry, and subsequent dilution, of siRNAs into non-nucleolar areas of the nucleus (Ohrt et al., 2006). This is still controversial, as other studies have found no relationship between Exp5 and siRNA activity (Yi et al., 2005). At the very least, it is clear that the activity of endogenous miRNAs can be disrupted by the overexpression of shRNAs. The consequences of this not only manifest in cell culture, but also *in vivo*, as Grimm and colleagues have shown that a high percentage of shRNAs can cause lethality in mice regardless of shRNA target, or even the presence of a target. Moreover, this toxicity correlated with high shRNA expression (Grimm et al., 2006). Findings that RNAi effectors can saturate the endogenous machinery emphasize the importance of using RNAi effectors at the lowest possible effective concentrations. Additionally, the use of negative control siRNAs or shRNAs is paramount for the proper interpretation of results. In the case of shRNAs, it does not seem adequate to simply use an empty vector control, as this does not control for shRNA-mediated inhibition of the endogenous miRNA machinery.

Despite early perceptions that siRNAs of less than 30 nts would avoid the immuno-stimulatory activity exhibited by larger RNA molecules (Elbashir et al., 2001), it has since been shown that siRNAs can activate the immune response in a sequence-independent, concentration-dependent manner. For example, 21-nt siRNAs have been shown to induce an interferon response in human glioblastoma T98G cells through a process dependent on the activation of the dsRNA-dependent protein kinase (PKR), and at least partially dependent on siRNA concentration (Sledz et al., 2003). Similarly, both externally delivered siRNAs and shRNAs were found to induce an interferon response in HEK293 and HaCaT keratinocyte cell lines (Kariko et al., 2004). Additional studies in HEK293 cells found this response to be primarily dependent on Toll-like receptor 3 (TLR3) (Kariko et al., 2004). Importantly, the induction of an interferon response is cell-line dependent, with long siRNAs of 27 nt unable to activate a response in certain cell lines, including HeLa cells (Reynolds et al., 2006). The expression of shRNAs can also induce an interferon response. As with siRNAs, shRNA-mediated activation also appears to be concentration dependent (Bridge et al., 2003). Thus, similar to saturation of the endogenous RNAi machinery, the use of lowest effective concentrations and negative control RNAi effectors are necessary to control for stimulation of interferon-type responses. Additionally, chemical modifications that help prevent the activation of PKR have been described.

2. Sequence-Dependent Effects. siRNAs can also induce an immune response through sequence-dependent effects, particularly when it is part of a lipid or polycation complex *in vivo*. More specifically, certain nucleotide motifs, especially GU-rich sequences, can induce interferon-α (IFN-α), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α), probably through activation of TLRs. For example, a subset of liposome-encapsulated siRNAs was found to induce a substantial, dose-dependent IFN-α response in mice. These siRNAs also stimulated an immune response in human peripheral blood mononuclear cells and isolated plasmacytoid dendritic cells. The stimulatory siRNAs were found to share UGUGU motifs that were presumably recognized by endosomal TLR7 and/or TLR8 (Judge et al., 2005). Similarly, siRNAs were found to stimulate IFN-α production in human plasmacytoid dendritic cells through a GUCCUUCAA motif. In this case, experiments confirmed that stimulation was dependent on recognition by TLR7. Because nonimmune cells do not express detectable TLR7 or TLR8, sequence-dependent immune stimulation is not thought to influence experiments conducted in commonly used cell lines (Marques et al., 2006), but sequence-dependent stimulation is clearly an important issue regarding *in vivo* applications. Although specific siRNA nucleotide motifs can activate an immune response, the primary source of sequence-dependent off-target effects originates from partial complementarity between RNAi effectors and off-target transcripts. Such interactions are similar to those exhibited by endogenous miRNAs,

which usually share complementarity between nucleotides within their 5' ends and regions within target 3' UTRs (Lewis et al., 2005). In fact, much like miRNA targets, off-targeted transcripts are enriched in those containing complementarity between their 3' UTRs and hexamer (nts 2–7) and heptamer (nts 2–8) sequences within 5' ends of RNAi effectors (Jackson et al., 2006). Some studies have found these effects to be nontitratable, with dose responses mirroring that of on-target transcripts (Jackson et al., 2003). Others have found these effects to be concentration-dependent, whereby the use of low siRNA concentrations can significantly mitigate off-target interactions (Semizarov et al., 2003). Importantly, most detailed studies of off-target effects are conducted using gene expression analysis. However, since miRNAs can impede translation in a manner disproportionate with alterations in target mRNA levels, the magnitude of off-target effects may be underestimated. Sequence-dependent off-target effects can have functional consequences. For example, different siRNAs targeting the same gene can exhibit varying effects on the mRNA and protein levels of key cellular genes, independent of on-target silencing (Scacheri et al., 2004). Accordingly, a high percentage of siRNAs can induce a toxic phenotype. For example, 51 of 176 randomly selected siRNAs directed against either firefly luciferase or human *DBI* reduced the viability of HeLa cells by more than 25%, a trend that was reproducible in different cell lines. From a practical perspective, off-target effects can have a profound effect on experimental results. For example, Lin and colleagues determined that the top three "hits" from a siRNA-based screen for targets affecting the hypoxia-related HIF-1 pathway resulted from off-target effects (Lin et al., 2005). For two of these three "hits," activity could be traced to interactions within the 3' UTR of *HIF-1A* itself. Of note, off-target effects not only affect experiments conducted in mammalian systems, but can also influence studies in *Drosophila* (Ma et al., 2006). There are a number of ways to control for, and help minimize, sequence-dependent off-target effects. Many of these relate to siRNA design features. For example, the use of asymmetric design, which helps to minimize the loading of passenger strands into RISC, thereby reducing associated off-target effects, and the use of siRNAs designed to avoid homology with untargeted transcripts. Both of these considerations are typically incorporated into the design of commercially available siRNAs. Increased stringency may be gained through the development of new algorithms that include emphasis on avoiding complementarity between siRNAs and untargeted 3' UTRs. Chemical modifications that reduce sequence-dependent off-target effects have also been described. For example, the incorporation of 2'-O-methyl groups within the first two 5' nucleotides of siRNA passenger strands reduces passenger strand-mediated activity. Similarly, a 2'-O-methyl ribosyl substitution at position 2 of the guide strand can significantly reduce sequence-dependent off-target effects (Jackson et al. , 2006). Modified siRNAs exhibiting reduced off-target effects are commercially available. As with

sequence-independent effects, experimental conditions should be optimized to use the lowest effective dose of the RNAi effector. Despite all of these considerations, the occurrence of sequence-dependent off-target effects may be unavoidable. Consequently, efforts should be made to help validate hits. All RNAi-derived phenotypes should be confirmed with additional RNAi effectors against the same target. Moreover, the downregulation of target mRNA and protein levels should be characterized and correlated with the observed effects. For example, the inactivity of a follow-up siRNA does not necessarily imply that the activity of the first resulted from off-target effects, especially if the second siRNA is unable to downregulate target levels. Conversely, a phenotype induced by only a fraction of siRNAs directed against the same target, despite equivalent silencing by all siRNAs, would be suspicious. Overall, it is difficult to prescribe the number of independent RNAi effectors necessary for target validation, but it would certainly require at least two. No matter how many independent RNAi effectors are tested, it could be possible that observed phenotypes result from cooperative effects between target-specific downregulation and nonspecific effects. Even a rescue experiment using a target construct resistant to RNAi could not control for such scenarios. Thus, confirmation of phenotypes under different experimental conditions (e.g., the use of a different lipid reagent or the use of an siRNA to confirm an shRNA-derived phenotype) may help to eliminate some of these possibilities. Additionally, RNAi-independent methods, such as the chemical inhibition of identified targets, should be used to corroborate phenotypes where possible.

3.3 Application of RNAi-based technologies

RNAi has enormous potential for the treatment of many genetic and acquired diseases. For example, RNAi could potentially be used to reduce the levels of toxic gain-of-function proteins, trigger cytotoxicity within tumors, or block viral replication. The use of RNAi-based therapeutics is especially appealing as RNAi can be used to modulate the expression of proteins not normally accessible by more traditional pharmaceutical approaches. For example, nondruggable targets lacking ligand-binding domains or proteins sharing high degrees of structural homology that are difficult to target as individuals are all accessible by RNAi. The *in vivo* application of RNAi was described within a year of the first cell culture experiments, with reports describing the transient inhibition of transgenes within the livers of mice. This was accomplished through high-pressure tail vein injection of both siRNAs and shRNAs (McCaffrey et al., 2002). Subsequent *in vivo* studies have focused on the improved delivery and efficacy of RNAi effectors. These efforts have used the experience gained through two decades of developing ribozyme and antisense-based therapeutics and the gene therapy field as a whole. Currently, most *in vivo* studies using synthetic siRNAs use

lipid-based carriers with or without modification of the siRNA itself, whereas most shRNA-based studies employ the standard viral vector expression systems used in traditional gene therapy.

1. In Vivo Application of Synthetic siRNAs. The *in vivo* delivery of synthetic siRNAs must account for the need to ensure resistance to exonuclease digestion, the maintenance of duplex stability, good pharmacokinetics, and the minimization of nonspecific immunological responses. Accordingly, a number of siRNA chemical modifications that address these issues have been examined. Many of these modifications are analogous to those incorporated in RNase H-dependent antisense oligonucleotides. A common modification to improve stability is the use of a partial phosphorothioate backbone, particularly within the 3' overhangs of both siRNA strands. Furthermore, the inclusion of 2'-O-methyl dinucleotides at the 3' end of the antisense strand has also been shown to improve stability. As mentioned, avoiding immune stimulation is also critical. The selection of sequences that avoid GU-rich sequences and/or modification with 2'-O-Me nucleotides or locked nucleic acids (LNAs) have all been shown to inhibit stimulation of the immune system without concomitant loss of efficacy (Judge et al., 2006). Chemical modifications have also been engineered to improve cellular uptake. For example, cholesterol-conjugated siRNAs, corresponding to the *ApoB* gene, have been delivered into the livers of mice as a potential strategy for the treatment of familial cholesterolemia and, possibly, for the broader treatment of atherosclerosis (Soutschek et al., 2004). These conjugates were found to induce a significant decrease in both liver *ApoB* mRNA and plasma ApoB protein levels, as well as downstream lipoprotein and cholesterol levels. These effects were much greater than those observed with nonconjugated analogs. Unfortunately, the quantity of material necessary for efficient silencing was incompatible with scale-up to larger preclinical models, thus follow-up studies in nonhuman primates used a different delivery strategy (Zimmerman et al., 2006). In addition to directly modifying siRNAs for improved characteristics, carrier molecules also have the potential to protect siRNAs from the extracellular environment and improve intracellular delivery. A wide variety of polymer- or lipid-based delivery systems have been described. For example, cationic polyethylenimines have been used for siRNA transfection *in vivo*, including delivery to lung and xenografts following subcutaneous, intraperitoneal, and intrathecal administration (Thomas et al., 2005). A large number of different liposome-based carriers have also been developed for the *in vivo* delivery of siRNAs. One such system that has been relatively well characterized uses lipid-polyethylene glycol (PEG) mixtures to encapsulate siRNAs. This delivery system has been used for the systemic delivery of *APOB*-targeted siRNAs into the livers of nonhuman primates, causing a significant reduction in both *APOB* mRNA and protein levels. Furthermore, a relatively sustained (11-day) reduction in low-density lipoprotein was observed in animals receiving the highest dose of siRNA lipid (2.5 mg/kg).

Similarly, this lipid-encapsulated siRNA system has been used in studies directed toward inhibiting viral infections (Mousses et al., 2003).

2. In Vivo Application of shRNAs. The first studies applying shRNAs *in vivo* used plasmid DNA (Lewis et al., 2002); however, most subsequent studies have focused on the use of viral vectors. The choice of viral delivery system usually depends on the cell type under investigation and on the need for short- or long-term shRNA expression. For example, adenoviral (AV) and herpes simplex viral vector systems have been primarily used for short-term expression, while adeno-associated viral (AAV) vectors and the integrating viral vector systems based on retroviruses (RVs) and lentiviruses (LVs) have usually been used for long-term expression or for applications in nondividing cells. An important adaptation of RNAi has come from the ability to stably express shRNAs in blastocytes or embryonic stem cells, from which transgenic animals can be generated. Initial "proof of concept" experiments used shRNAs to target overexpressed marker genes (e.g., green fluorescence protein) in transgenic animals. These studies used either direct injection or lentiviral transduction of early embryos (Hasuwa et al., 2002). Subsequent studies have demonstrated the feasibility of targeting endogenous genes within embryonic stem (ES) cells (Rubinson et al., 2003). These models broadly mimic the phenotype of traditional knockout mice. Consequently, the constitutive expression of an shRNA for the generation of an RNAi-based transgenic is only compatible with genes that do not compromise animal viability. To circumvent embryonic lethality, shRNA-based conditional expression systems have been developed. These include Cre-Lox-based systems whereby the shRNA is flanked by LoxP sites that prevent shRNA expression. Tissue-specific or temporal-specific shRNA expression can then be achieved by crossing shRNA transgenic mice with *Cre* recombinase expressing mice (Ventura et al., 2004). Although Cre-lox RNAi-based systems are irreversible, reversible expression, predominantly using doxycycline-based control systems, has been described. RNAi transgenics have also been used in animals not normally amenable to traditional homologous recombination techniques, including rat and goat. In the case of goat, a RNAi transgenic was generated through somatic cell nuclear transfer from a LV-transduced goat fibroblast stably expressing an shRNA corresponding to the prion protein (Golding et al., 2006). While the development of RNAi transgenics was initially hailed with great excitement, its broader use has not been adapted as quickly as may have been anticipated. This may be due to difficulties in obtaining efficient lentiviral transfection of embryos or ES cells, difficulties in generating ES clones that stably express shRNAs or problems associated with variations in knockdown efficiency. The recent adaptation of the more conventional pronuclear injection procedure may enable wider use of RNAi in the development of transgenics. shRNAs have also been used in xenograft tumor models, particularly in mice. One of the first examples of this was targeting an activated mutant of *K-RAS*

found in the pancreatic carcinoma cell line CAPAN-1 using a mutant-specific shRNA expressed from a retroviral vector (Brummelkamp et al., 2002). In contrast to control cells, the *KRAS*-targeted cells failed to form tumors in athymic mice, demonstrating the ability of RNAi-mediated silencing to suppress tumor formation *in vivo*. Another interesting example involves targeting the tumor suppressor *TP53* in mouse *Eμ-Myc* hematopoietic stem cells. By using shRNAs that mediated different degrees of *TP53* message reduction, the percentage of mice developing lymphoma could be varied as a function of TP53 protein levels. More recent variants of this method include the use of an inducible expression system that can be activated upon xenograft tumor formation, potentially generating a better clinical model for the identification and validation of anticancer molecular targets (Li et al., 2005).

3.4 "Trans-kingdom" RNAi

Several years ago, it was demonstrated that systemic gene silencing could be attained in the nematode *Caenorhabditis elegans* when it ingested *E. coli* engineered to produce interfering RNAs, suggesting that RNAi-mediated information transfer between species or kingdoms might be possible (Timmons and Fire, 1998; May and Plasterk, 2005). Bacteria engineered to produce a short hairpin RNA (shRNA) can induce trans-kingdom RNAi *in vitro* and *in vivo* also in mammalian systems. Nonpathogenic *Escherichia coli* can be engineered to transcribe shRNAs from a plasmid containing the invasin gene *Inv* and the listeriolysin O gene *HlyA*, which encode two bacterial factors needed for successful transfer of the shRNAs into mammalian cells. Upon oral or intravenous administration, *E. coli* encoding shRNA induce significant gene silencing in the intestinal epithelium and in human colon cancer xenografts in mice (Xiang et al., 2006). These results provide an example of trans-kingdom RNAi in higher organisms and suggest the potential of bacteria-mediated RNAi for functional genomics, therapeutic target validation and development of clinically compatible RNAi-based therapies.

3.5 Prediction of microRNA targets (Mazière and Enright, 2007. review)

Currently, there are 474 confirmed microRNAs (miRNAs) in humans, although there might be many more. miRNAs are expected to have multiple targets; however, few have been confirmed experimentally (only 66 of potentially thousands so far). In the absence of high-throughput experimental techniques to determine the targets of miRNAs, it is vital that computational techniques are developed to unravel their regulatory effects and implications for diseases and

diagnostics. Indeed, recent studies have already implicated miRNAs in numerous human diseases, such as colorectal cancer, chronic lymphocytic leukaemia and fragile X syndrome. Hence, both the miRNA itself and its regulatory targets are potentially druggable. The prediction of miRNA targets has been ongoing since the 3′ untranslated regions (3′UTRs) of transcripts were determined to contain binding sites for them. The efficacy of computational approaches to locate and rank potential genomic binding sites is supported by the relatively high degree of miRNA complementarity to experimentally determined binding sites. Despite the later identification of hundreds of miRNAs in a variety of species, through large-scale and sequencing projects, only a handful of targets had been identified experimentally, for an even smaller number of miRNAs. Given the laborious nature of experimental validation of targets, and despite the limited data available, it was imperative that computational approaches be developed that could produce reliable and testable predictions.

1. miRNA size. The apparent complementarity between miRNA and target could have been seen as an advantage for computational analysis. However, other features of miRNA–UTR associations make matters more complicated. Conventional sequence alignment algorithms assume longer sequences than the 20–23 nucleotides of miRNAs. This short length makes ranking and scoring of targets difficult because statistical techniques for sequence matching (such as Karlin–Altschul statistics) require longer sequences. Binding sites actually consist of regions of complementarity, bulges and mismatches. Because standard sequence analysis tools were designed for sequences with longer stretches of matches and fewer gaps, they are much less useful for miRNA target prediction. Recently, position 2–7 of miRNAs, the so-called ‘seed’ region, has been described as a key specificity determinant of binding, and requires perfect complementarity and. If one ignores GC content and performs an order of magnitude calculation, then a perfect match for a six-nucleotide seed region of a miRNA should occur approximately once in every 1.3 kb in a genome – in other words, on average, almost once in every human 3′UTR. However, it would not seem realistic for a single miRNA to regulate more than a few hundred targets. Effective regulation of transcript translation requires that miRNAs and their targets are located in the same cellular compartments. Hence, most of these theoretical targets correspond to false positives.

2. Identification of 3′UTRs. To identify miRNA targets in a given species, knowledge of the set of 3′UTRs for this species is a vital step. Despite the accumulation of genome sequences for many species, the location, extent or splice variation of 3′UTRs is still poorly characterized for many mammals. Some species-specific projects, such as the Berkeley *Drosophila* Genome Project (BDGP), produce high-quality transcript information that makes possible the accurate determination

of a 3′UTR, from stop codon to polyadenylation site. For other species, such as *Homo sapiens*, some transcripts are well defined, whereas others remain poor in their description. The Ensembl database uses alignment of cDNAs and expressed sequence tags to genomic sequences to extract 3′UTR regions, and so far, evidence is available for human, mouse and zebrafish genomes. However, ~30% of human genes lack definitive 3′UTR boundaries. These regions can be estimated by selecting a downstream flanking sequence of the stop codon, corresponding to the length of an average human 3′UTR (e.g. 1 kb). Experimental techniques, such as tiling arrays, and ditag or cage tagging, seem to be promising approaches for the generation of high-quality 3′UTR datasets. Attaining reliably annotated and verified 3′UTR datasets will potentially benefit target prediction more than making small improvements to existing prediction methods. In the context of drug discovery, both 3′UTRs and miRNA genes represent drug target candidates through either the generation of synthetic miRNAs or the repression or overexpression of existing miRNAs.

3. Conservation analysis. Solutions to reduce the number of false positives in target predictions include filtering out those binding sites that do not seem to be conserved across species. The use of predicted binding sites conserved across orthologous 3′UTRs in multiple species are considered more likely to reduce the number of false positives. However, recently evolved miRNAs, such as miR-430 in zebrafish, might not have conserved targets in the scope of the currently available set of fish genomes. One caveat of conservation analysis concerns the set of species that are compared: looking for conserved targets between humans and chimpanzees will not be helpful, given that at least 99% of the entire transcript will be conserved. Other species might seem more relevant for comparing with human transcripts (e.g. mouse, rat, or dog), but the fact is that genomes are not sequenced according to their evolutionary distances. As a result, the number of false positives can effectively be greatly reduced but this is at the expense of increased false negatives.

4. Computational target-prediction approaches. Different methods have been developed for computational target prediction (**Tab. 1**). These might or might not be made available as functional packages but the results are always available, at least as a precomputed set of transcripts, through online resources.

Methods and resources for miRNA target prediction					
Method	**Type of method**	**Refs**	**Method availability**	**Data availability**	**Resource**
Stark *et al.*	Complementarity	[21]	Online search	Yes	http://www.russell.embl.de/miRNAs/
miRanda	Complementarity	[22]	Download	Yes	http://www.microrna.org/
miRanda miRBase	Complementarity	[1]	Online search	Yes	http://microrna.sanger.ac.uk/
TargetScan	Seed complementarity	[18]	Online search	Yes	http://www.targetscan.org/
TargetScanS	Seed complementarity	[17]	Online search	Yes	http://www.targetscan.org/
DIANA microT	Thermodynamics	[24]	Download	Yes	http://diana.pcbi.upenn.edu/
PicTar	Thermodynamics	[33]		Yes	http://pictar.bio.nyu.edu/
RNAHybrid	Thermodynamics and statistical model	[25]	Download		http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/
miTarget	SVMe	[37]	Online Search		http://cbit.snu.ac.kr/~miTarget/
TarBase	Experimentally validated targets		N/A	Yes	http://diana.pcbi.upenn.edu/tarbase.html

Table 1. Methods and resources for miRNA target prediction.

5. Algorithms for miRNA target prediction. The challenge of predicting miRNA targets has resulted in the development of several methods, which fall into different categories. We can distinguish three types of target sites: 5′-dominant canonical, 5′-dominant seed only and 3′-compensatory (**Fig. 17**). These differ in the level of complementarity of miRNA sequences to the site sequences. Therefore, the main approaches look for sequence complementarity and/or for favourable miRNA–target duplex thermodynamics. To increase the signal-to-noise ratio, some methods require strict complementarity between the seed region of the miRNA and the predicted target. Conservation of binding sites is also often used as a metric to improve the raw results.

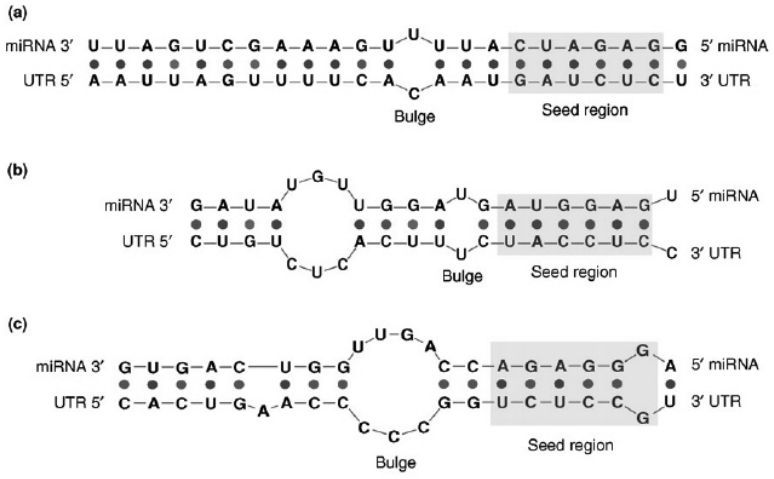


Figure 17. Approximate secondary structures of the three main types of target site duplex mRNA-miRNA.

6. Complementarity searching. The first algorithms did not develop statistical background models to evaluate the significance of each detected hit; rather, they were oriented towards recovery of known targets and the detection of further targets for experimental validation, so that our knowledge of miRNA binding dynamics might be improved. In most cases, they used complementarity initially to identify potential targets, followed by iterative rounds of filtering based on thermodynamics, binding site structure and conservation. After these filtering steps, a score is typically applied to each detected target; this score can be useful for target ranking. Initial attempts at false-positive rate estimation usually relied on comparing detection methods for real miRNAs and shuffled control miRNAs.

7. The method of Stark and co-workers. Large-scale prediction of miRNA targets was first successfully published for *Drosophila melanogaster*, for which well-annotated and accurate 3′UTRs could be obtained from the BDGP. The sequence search tool HMMer was used to identify the reverse complement miRNA sequences. Similar profiles were built to enable G:U wobble matches. Following the prediction algorithm, the resulting 3′UTRs were filtered for conservation in *Drosophila pseudoobscura* and *Anopheles gambiae*. The detected target sites were scored and used as an input for the MFold algorithm, to evaluate the thermodynamic stability of the miRNA–target association. Despite a statistical model based on the normal distribution rather than the extreme value distribution, the method predicted previously validated *D. melanogaster* miRNA binding sites. Many previously unknown binding sites were also predicted, six of which were experimentally validated.

8. miRanda. The miRanda algorithm was the second method to be published. As with the method by Stark et al., miRanda identifies potential binding sites by looking for high-complementarity regions on the 3′UTRs. The scoring matrix used by the algorithm is built so that complementary bases at the 5′ end of the miRNA are rewarded more than those at the 3′ end. Hence, the binding sites exhibiting a perfect or almost-perfect match at the seed region of miRNAs display a better score. The resulting binding sites are then evaluated thermodynamically, using the Vienna RNA folding package. This first version of miRanda successfully predicted many known targets in D. melanogaster. The BDGP 3′UTRs dataset was used, and the results were filtered, as described above, to limit predictions to targets conserved in *D. pseudoobscura*. When classified according to gene ontology terms, the miRanda-predicted targets were shown to display specific functional patterns for each miRNA. Expression data analysis confirmed this property by suggesting that many individual miRNAs have highly specific roles in particular tissues, processes and pathways. When basic parameter settings are used, the approximated false-positive rate was between 24% and 39%.

These values are significantly decreased when multiple sites are considered. Newer miRanda versions implement a strict model for the binding sites that requires almost-perfect complementarity in the seed region with only a single wobble pairing. Other variations of these algorithms are currently under development for a version 3.0 of miRanda. These incorporate a statistical model equivalent to that used by RNAHybrid, thereby they efficiently reduce the rate of false-positive predictions. Despite their similar methods and identical input datasets, the scoring and ranking strategies devised by Stark et al. and miRanda are different: only 40% of the respective top-ten miRNA targets predicted by both methods overlap. It seems that even small differences in the criteria used for ranking and scoring lead to large differences in the set of predicted targets. The multiplicity of miRNA binding sites on the same 3′UTRs drastically improves their statistical significance in both methods. This is confirmed by experimental evidence showing that multiple sites enhance the silencing effect of miRNAs. However, many miRNAs still seem to operate at a single site on their targets. One given explanation implies that a miRNA exhibiting high complementarity to its single-site target could have the same regulatory effect as a miRNA with a lower level of complementarity but a multiple-site target.

9. TargetScan and TargetScanS. Although the previously mentioned methods attempt to find all potentially complementary sites and then filter them according to different criteria, TargetScan uses a different approach. This method requires perfect complementarity to the seed region of a miRNA and then extends these regions to unravel complementarity outside the region. This aims at filtering many false positives from the beginning of the prediction process. For the same purpose, the conservation criteria, based on the presence of the seed region in an island of conservation, are introduced early in the process by using groups of orthologous 3′UTRs as input data. The following step is common to the other methods: the predicted binding sites are tested for their thermodynamic stability, in this case with RNAFold from the Vienna Package. TargetScan was the first method to be applied for human miRNA target prediction, using mouse, rat and fish genomes for conservation analysis. Shuffled sequences, with maintained dinucleotide compositions that mimic real 3′UTRs, were used to determine the significance of binding sites. The estimated false-positive rate varies between 22% and 31%. The method was shown to predict not only known miRNA binding sites but also novel sites. Luciferase reporter constructs validated 11 of the 15 tested sites. TargetScanS simplified the TargetScan method and improved the target prediction fidelity. The miRNA complementarity is now limited to a six-nucleotide seed, followed by an additional 3′ match of an adenosine anchor at position 1. No other criteria are required once the previous conditions are met; contrary to previous algorithms, single-site 3′UTRs are sufficient for a reliable prediction. TargetScan and TargetScanS feature an efficient reduction in the false-positive rate but, because of

the required strict complementarity in the seed region, loosely conserved targets and those containing wobble pairings are more likely to be missed, including 3′-compensatory sites.

10. PicTar. The PicTar algorithm uses a group of orthologous 3′UTRs from multiple species as the input dataset. The algorithm scans the alignments of 3′UTRs for those displaying seed matches to miRNAs. The retained alignments are then filtered according to their thermodynamic stability. Each predicted target is scored by using a Hidden Markov Model (HMM) maximum-likelihood fit approach. PicTar is the first method that uses the criteria of co-expression in space and time of miRNAs and their targets. The experimental validation of seven out of 13 tested predicted targets, as well as the confirmation of eight of nine previously known targets, demonstrates the efficiency of the algorithm.

11. DIANA-microT. The DIANA-microT method uses a 38-nucleotide window that is progressively moved across a 3′UTR sequence. Using dynamic programming, the free energy (ΔG kcal/mol) of the potential binding site is calculated at each step and compared with the results obtained from shuffled sequences with the same dinucleotide composition as real 3′UTRs. Contrary to sequence complementarity-based methods, DIANA-microT demands 3′ complementarity to the miRNA and does not bother with site multiplicity. Using this technique, all currently known C. elegans miRNA binding sites were predicted successfully, with false-positive rates similar to those found in previously described methods.

12. RNAHybrid. The lack of strong statistical models is one of the main criticisms that can be levelled at the methods previously described here. RNAHybrid was the first method to address this issue by developing a model as robust as those used for large-scale sequence comparison. Contrary to tools such as MFold and Vienna, which are designed for single-sequence folding and therefore require an artificial linker between the miRNA and its potential binding site, RNAHybrid identifies regions in the 3′UTRs that have the potential to form a thermodynamically favourable duplex with a specific miRNA. The maximum free energy of a miRNA is calculated for every 3′UTRs of a set of shuffled 3′UTR sequences with maintained dinucleotide frequencies. Normalisation for both 3′UTR and miRNA length using $S_{norm} = \log(S/mn)$ is applied to these energies. Random energies derived in this manner should exhibit an extreme value distribution (EVD). Subsequently, the parameters of the EVD that best describe the data for a given miRNA are empirically calculated using the derived distribution from shuffled sequences. Each hit to any 3′UTR for this miRNA is then assigned a P value calculated directly from these parameters. Hence, at the scanning stage, miRNAs are scanned against a database of real 3′UTRs, and each hit is compared with the expected distribution and assigned a P value. Moreover, the statistical model implemented in RNAHybrid takes into account

multiple sites and conserved sites, by respectively combining individual P values using Poisson statistics and calculating conservative P values for conserved sites. A statistical fitting approach corrects for highly conserved 3′UTRs by evaluating the overall conservation in the group of sequences compared with the conservation at the site. The resulting statistics cover individual site quality, quantity of sites, whether they are conserved and how significant this conservation is, given the input sequences. The method was successfully tested to predict known targets in D. melanogaster, with a low false-positive rate. The association of P values with predicted targets is an appreciable asset for directly comparing predicted binding sites.

4. MicroRNAs and Cancer

4.1 Disruption of miRNA-directed regulation

As each miRNA is expected to regulate the translation of up to 100 mRNAs (Lim et al., 2005) it is clear that disturbances of the miRNA expression level, processing of the miRNA precursors, or mutations in the sequence of the miRNA, its precursor, or its target mRNA, may have detrimental effects on cell physiology. A number of such aberrations have been associated with cancer, as described in detail below, and can in short be categorized as the following types of lesions:

a) *Alterations of the miRNA expression level.* This may occur by gross genomic alterations such as deletions, insertions, inversions, and translocations (Calin et al., 2004), by epigenetic changes of the miRNA gene (Saito et al., 2006), by insertion of a viral element near the miRNA gene that disrupts normal transcriptional regulation (Wang et al., 2006), or minor genetic changes such as a point mutation in a promoter element of the miRNA gene or in the coding region for a transcription factor that is crucial for miRNA transcription (Xi et al., 2006).

b) *Alterations affecting miRNA processing*. Changes in the expression level of a large number of miRNAs may be a consequence of a disruption of the miRNA-processing apparatus (Sugito et al. ,2006). Changes affecting translation of only a few mRNAs can be caused by an alteration in the primary sequence of the pri-miRNA, which affects its downstream processing efficiency (Diederichs et al., 2006).

c) *Mutations in the miRNA:mRNA interacting sequences.* Alterations in the translation efficiency of a mRNA may also be a result of a base change in the mature miRNA or in the target sequence of the mRNA, which weakens the interaction between the miRNA and the mRNA (Iwai and Naraba, 2005). This interaction is especially sensitive to mutations in the seed region.

There is now ample evidence that the expression of miRNAs is altered in cancer, and that certain changes may be directly implicated in the carcinogenic process. A number of miRNAs have been shown to promote cell proliferation and survival, while others diminish cell proliferation and survival. These two classes of miRNAs may play a central role in cancer development as novel oncogenes and tumor suppressors, respectively. In general, the majority of miRNAs are downregulated in cancer specimens (Lu et al., 2005). In normal tissues, some of these miRNAs have been documented to inhibit the translation of proto-oncogenes by targeting the 3'ends of their

mRNAs. Such miRNAs are therefore considered as "tumor suppressor miRNAs" (TS-miRs) since their normal function is to control the expression of an oncogene. Conversely, certain miRNAs seem to be upregulated in cancer and may act as "oncomiRs" since they can enable the downregulation of a tumor suppressor. Since miRNAs have several potential targets that may be the mRNAs of both oncogenes and tumor suppressors, the actual function of a particular miRNA as either TS-miR or onco-miR may depend on the cellular context. The mechanisms by which miRNA expression is altered in cancer are multifaceted. The function of miRNAs in cancer seems to be disrupted by the same mechanisms as those that affect the expression of protein-encoding genes, i.e. amplifications, translocations, deletions, and point mutations of the pri-miRNA-encoding DNA sequence, and by epigenetic disruption of miRNA transcription (**Fig. 18**).

4.2 MiRNAs as tumor suppressors and oncogenes

1. Tumor suppressor miRNAs. One of the first indications of a direct involvement of miRNAs in cancer was the linking of the miR-15- and miR-16-encoding sequences to a critical region of deletion of only 30 kb at 13q14, which is lost in more than half the cases of chronic lymphocytic leukemia (CLL) (Calin et al., 2002). Previous comprehensive analyses for candidate tumor suppressor genes within this region had turned out to be unsuccessful. However, the targeting of *miR-15* and *miR-16* to the minimal region of LOH (loss of heterozygosity) in CLL combined with the recent findings of germline mutations of *pri-miRs-15* and *-16* in a case of familial CLL indicated that they might represent the long sought CLL-associated tumor suppressor located in this region (Calin et al., 2005). Furthermore, expression analyses have shown that as many as 68% of all CLLs show downregulation of miRs-15 and -16. Both miRNAs were shown to act as tumor suppressors by targeting translation of the anti-apoptotic BCL-2 mRNA (Calin et al., 2002), an oncogene that frequently is found to be overexpressed in CLL. Downregulation of miR-15 and -16 has been shown to correlate with overexpression of the BCL-2 protein, and transfection with either of the two miRNAs completely abolished protein expression and re-established apoptosis in a leukemia model. Another early and well-documented finding was the downregulation of oncogenic Ras by the let-7 family members of miRNAs in lung cancer (Johnson et al., 2005). It was observed that low Let-7 expression correlated with a shortened post-operative survival in lung cancer patients who had undergone potentially curative operative procedures. Since then, a large number of miRNAs have been shown to be downregulated in various cancers, including the downregulation of let-7 (Akao et al., 2006a), miR-143, and miR-145 in colorectal cancer (Akao et al., 2006b), miR-145 in breast cancers (Iorio et al., 2005), and miR-29b in CLL (Pekarsky et al., 2006) and AML (Garzon et al., 2007) (**Tab. 2**).

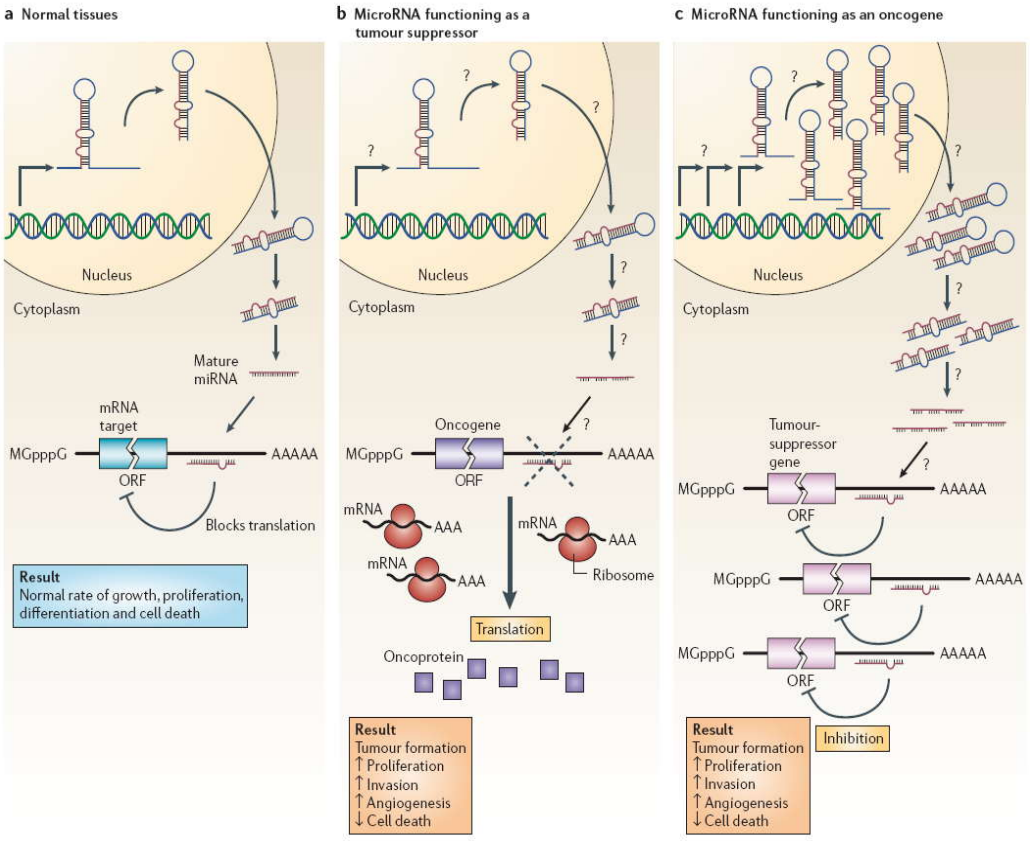


Figure 18. Mechanism of action of tumor suppressor and oncogenic miRNAs

2. Oncogenic miRNAs. In contrast to TS-miRs, oncomiRs are frequently upregulated in cancers and show proliferative and/or anti-apoptotic activity. One of the first oncomiRs to be identified was miR-155, which is co-expressed with the non-protein-coding gene *BIC*. The exact target mRNAs of miR-155 remain to be established, but early observations showed that high expression of this miRNA led to an increase in leukemia and lymphoma formation in chicken. More recent studies showed overexpression of miR-155 in diffuse large B-cell lymphoma (DLBCL), Hodgkin's disease, and primary mediastinal DLBCL (Eis et al., 2005). Initially, high expression was also reported in pediatric Burkitt's lymphoma; however, recent observations show disruption of miR-155 processing in Burkitt lymphoma cell lines (Kluiver et al., 2006). In DLBCL and lung adenocarcinomas, high expression of miR-155 has been associated with aggressive variants of tumors and poor survival (Yanaihara et al., 2006). The members of the *miR-17-92* cluster represent another intensely studied group of potential oncomiRs that are frequently upregulated in lymphomas. This cluster consists of seven individual miRs: miR-17-5p, 17-3p, -18, -19a,-19b1, -20, and 92, which are all encoded from a frequently amplified locus at 13q31.3 (Ota et al., 2004). It was shown in Eμ-Myc transgenic mice that the miR-17-92 cluster, but not the individual miRNAs, could enhance tumorigenesis by inhibiting apoptosis in the c-Myc-overexpressing tumor. Further studies in human cell lines showed

that transcription of the miR-17-92 cluster was directly regulated by c-Myc, and that the individual miRs -17-5p and -20 regulate the translation of E2F1, a transcription factor with both pro-apoptotic and pro-proliferative activity. Thus, co-expression of c-Myc and miR-17 is believed to fine tune E2F1 activity so that proliferation is enhanced and apoptosis is inhibited (O'Donnell et al., 2005). Anti-apoptotic activity has also been documented to be a feature of miR-21, which is highly expressed in glioblastoma. Knockdown of miR-21 in breast- and glioblastoma cell lines led to inhibition of BCL-2 activity, caspase reactivation, and increased apoptotic cell death (Chan et al., 2005). At present, the list of recognized and potential TS- and oncomiRs is rapidly growing. An overview of some of the most well analyzed miRNAs and their targets is given in **Tab. 2**.

miRNA no.	Suggested tumor suppressor activity	Suggested oncogenic activity	Chromosomal localization	Mechanism(s) of alteration in cancer	Target	Type of cancer
Let-7 family	+		Multiple loci	Downregulation	Ras PRDM1	Lung Colon
9		+	1q22	Overexpression	PRDM1	Hodgkin disease
10b	+		2q31.1	Downregulation	NA	Breast
15a	+		13q14.3	Deletion, mutation (rare)	BCL-2	B-CLL, Pituitary adenomas
16–1	+		13q14.3	Deletion, mutation (rare)	BCL-2	B-CLL Pituitary adenomas
17–92 cluster		+	13q31.3	Amplification/ overexpression	Tsp1 CTGF	Lymphoma Colon CML
17–5p	+		13q31.3	Downregulation	AIB1 E2F1	Breast
20a	+		13q31.3	Downregulation	E2F1	
21		+	17q22	Overexpression	Apoptosis related genes BCL-2	Glioblastoma Breast Cholangio-carcinoma Pancreatic
29b	+		1q32.2/7q32.3	Downregulation	MCL-1 TCL-1	AML Aggressive CLL, 11q del
124a	+		8p23.1	Methylation	CDK6	Colon Breast Lung Leukemia Lymphoma
125b	+		11q24.1/21q21.1	Downregulation	NA	Breast cancer
127	+		14q32.31	Methylation	BCL-6	Bladder Burkitt lymphoma
142		+	17	Translocation to Myc	NA	Aggressive B-cell lymphoma
143	+		5q32–33	Downregulation	NA	Colon
145	+		5q32–33	Downregulation	NA	Colon, breast
146b		+	10q24.32	Overexpression	Kit	Thyroid carcinoma
155-bic		+	21q21	Overexpression	NA	Hodgkin DLBCL Primary mediastinal Lymphoma Breast
181b	+		1q31.3	Downregulation	Tcl-1	Aggressive CLL, 11qdel
221		+	Xp11.3	Overexpression	Kit	Glioblastoma Leukemia Thyroid
222		+	Xp11.3	Overexpression	Kit	Leukemia Thyroid
372		+	19q13.41	Overexpression	LATS2	Testicular
373		+	19q13.41	Overexpression Hypomethylation?	LATS2	Testicular

Table 2. Examples of oncogenic and tumor suppressors miRNAs and their targets.

4.3 Genetics and epigenetics changes associated with altered miRNA expression

Early reports stated that more than half of all miRNA-encoding genes are located at chromosomal regions showing frequent genomic instability in cancer, i.e. at sites of minimal deletions/LOH and amplifications, and in relation to common chromosomal breakpoint regions (Calin et al., 2004). This led to the assumption that the miRNAs may play an important role in cancer, and the possible connection between miRNA genes within such regions and cancer has been—and is being—intensively investigated.

1. Mutations in miRNAs. As for miR-15 and -16 in CLL, many miRNAs are encoded from chromosomal regions showing LOH in cancer, and some miRNA genes may be the tumor suppressors targeted in these regions. However, as with other tumor suppressor genes, the most compelling evidence of a miRNA's contribution to cancer is a targeted inactivation of the particular miRNA by an acquired mutation in the cancer cells. The initial observations demonstrated that the frequency of mutations in the pri-miRNA-encoding DNA sequences is high and may have an important influence on mature miRNA formation (Calin et al., 2005). A germ-line mutation was observed in the *miR-16-1-miR-15a* pri-miRNA, and the presence of this mutation correlated with low miRNA expression and deletion of the second allele. The exact mechanism whereby this is mediated is unclear; however, it was suggested that the mutations affect the miRNA hairpin formation. This mutation was found in 2 of 75 CLL patients, and, in total, germline or somatic sequence variations were found in 5 of 42 miRNAs in 11 of the 75 CLL patients, but in none of 160 controls. Many (73%) of the patients with mutant miRNAs had a family history of CLL or other cancers. From this, it was predicted that miRNA gene mutations might play a major role in cancer. However, recent analyses of a large panel of cancer cell lines revealed that although such mutations are predicted to dramatically affect the folding and cleavage of pri-miRNAs, functional studies documented that the processing of pre-miRNA and mature miRNA formation was unaffected (Diederichs et al., 2006). Thus, at present, the role of targeted miRNA mutations in cancer is uncertain and for the majority of TS-miRNAs their association with cancer relies solely on the location of the miRNA gene in a region of minimal chromosomal deletion or their downregulation in cancer. Accordingly, the tumor suppressor properties of these miRNAs need to be further confirmed in functional studies and mouse models.

2. Epigenetic regulation of miRNA expression. It is now well documented that tumor suppressor genes can be silenced by methylation changes of the promoter cytosines and histones. Approximately 40% of miRNAs are encoded within the introns of known genes and are

coordinately expressed with the host gene (Rodriguez et al., 2004). However, other miRNAs are transcribed in the direction opposite to their host gene or located separately from the protein-encoding regions of the DNA. Such miRNAs may be transcribed from their own promoter and can be expected to be regulated similarly to protein-encoding genes, e.g. by epigenetic mechanisms. Therefore, another way to inactivate a TS-miRNA may be by hypermethylation and histone deacetylation of the miRNA promoter region. Recent studies now indicate that this mechanism works to regulate miRNA expression in at least some types of cancer. One of the first studies to show epigenetic regulation of miRNAs demonstrated that treatment of the breast cancer cell line SKBr3 with the histone deacetylase (HDAC) I/II inhibitor LAQ824 resulted in massive changes in miRNA expression. Five miRNAs showed significant upregulation, while most miRNAs, including miR-27, were downregulated. Several potential target mRNAs of miR-27 were upregulated, including the pro-apoptotic protein RYBP and the Sp1 repressor ZBTB10, and it was demonstrated that antisense treatment of miR-27 resulted in upregulation of the same mRNAs (Scott et al., 2006). Another study systematically documented the involvement of epigenetic regulation of miRNAs in cancer. It was shown that a small but significant proportion of miRNA becomes upregulated upon treatment by a combination of an HDAC inhibitor (HDACi), phenyl butyric acid, and the demethylating agent 5-aza-2-deoxycytidine (Saito et al., 2006). In particular, miR-127 was epigenetically downregulated in various cancer cell lines derived from bladder-, breast-, cervix-, pancreas-, lung-, and colon cancer, and Burkitts' lymphoma. In normal human fibroblasts, the miR-127 is transcribed as part of a cluster, whose expression was downregulated in the cancer cell lines. However, by treatment with a DNA methyltransferase inhibitor (DNMTi) and an HDACi the transcription of miR-127 could be upregulated from its own promoter, which is methylated in both normal and cancer cells. The miRNA was next demonstrated to be a specific inhibitor of translation of the proto-oncogene BCL-6, which is upregulated in a large proportion of B-cell lymphomas, where it acts as a transcriptional suppressor of TP53 and downstream effectors of TP53. By reactivation of miRNA-127 by epigenetic therapy it was possible to downregulate BCL-6 at the protein level while leaving the mRNA unaffected, and transfection of miR-127 into non-expressing cells confirmed its ability to downregulate BCL-6 (Saito et al., 2006). A recent study shows that a number of miRNAs are upregulated in the DNMT1/DNMT3b double knockout (DKO) cells, when compared to the wild-type colon cancer cell line HCT-116 (Lujambio et al., 2007). The miR-124a was selected from this panel, and further studies showed that it was inactivated by promoter methylation in a variety of tumors, including colon-, breast-, lung-, and hematopoietic cancers. One oncogenic target of this miRNA was the cell cycle regulator cyclin-dependent kinase 6 (CDK6), which accelerates cell cycle progression through the G1/S checkpoint by phosphorylating Rb.

Indeed, hypermethylation of miR-124a correlated with CDK6 activation and Rb phosphorylation in this study (Lujambio et al., 2007). Although the above studies strongly indicated that epigenetic therapy may at least in part exert its anti-cancer activity by reactivating epigenetically silenced miRNAs, a recent study showed no effect of DNMTi and HDACi on miRNA expression in lung cancer. Since many miRNAs are downregulated in this malignancy this may be mediated via different regulatory mechanisms (Diederichs et al., 2006). Indeed, further studies are required to fully demonstrate the importance and the extent to which epigenetic regulation of miRNAs contributes to cancer. Since DNMTi and HDACi are now used for treating cancer patients it will be interesting to see whether treatment with these drugs will influence the miRNA expression and the translational regulation of oncogenes *in vivo*.

3. miRNAs at translocation breakpoints. Several observations document the presence of miRNAs at chromosomal breakpoints, suggesting their role as translocation partners. The classical example is the translocation of the *miR-142* to the *MYC* oncogene in the t(8;17). In analogy to the translocations of *MYC* to the immunoglobulin gene locus, this translocation brings *MYC* under the control of the *miR-142* gene promoter, which leads to its upregulation in aggressive B-cell lymphoma (Lagos-Quintana et al., 2002). Other indications of a role for miRNAs at translocations include the observation that the *pri-miR-122a* gene is located at chromosome 18 near the *MALT1* gene, which is involved in translocations of the majority of mucosa-associated lymphoid tissue type lymphoma (Calin et al., 2004). Furthermore, insertion of the *miR-125b*, which is a homologue of lin-4, into the rearranged immunoglobulin heavy (IgH) chain gene locus, has been demonstrated in a case of precursor B-cell acute lymphoblastic leukemia. Since most translocations in B-cell tumors involve the IgH-locus, this observation may further support a role for miRNAs at translocation breakpoints (Sonoki et al., 2005).

4. Changes in the miRNA processing apparatus. A number of recent studies have focused on changes in the expression levels of the miRNA processing RNase III enzymes. Pri-miRNAs are cleaved in the nucleus by RNASEN (Drosha) and DGCB8 (Pasha) and the resulting pre-miRNAs are subsequently cleaved by Dicer in the cytoplasm. In esophagous cancer it was shown that high expression of RNASEN correlated with a significantly shortened post-operative survival, and it was suggested that RNASEN might be involved in tumor invasion since particularly strong expression of this enzyme was noticed at the periphery of tumors. The underlying mechanism for RNASEN upregulation was not investigated; however, the gene localizes to 5q13.3, a region that is often amplified in this type of cancer (Sugito et al., 2006). In another study it was shown that low expression of Dicer was associated with a shorter post-operative survival in lung cancer patients.

The potential mechanisms of Dicer downregulation were investigated in this case but although the *DICER1* promoter contains a CpG-island, no methylation was seen in the cases with low expression levels. Studies of allelic loss were not performed; however, in lung cancer, deletions of 14q, which contains the Dicer-encoding gene, are common, and it was suggested that haploinsufficiency may play a role in its downregulation (Karube et al., 2005). Although these studies show a potential role for the expression levels of the miRNA-regulating enzymes in the prognostication of cancer, more studies are needed to establish whether these alterations are causative, i.e. related to specific enzyme-encoding gene disruption, or merely reflect changes in enzyme expression that are secondary to other carcinogenic events.

5. Sequence variations in the miRNA-mRNA binding sites. Inappropriate base pairing due to variations in the 3'-UTR sequence of the target mRNAs or in the mature miRNA sequence may be another mechanism of translational disruption in cancer. Tumors may evade growth inhibition by TS-miRs if they do not bind properly to their target oncogenic mRNA sequence, or alternatively, tumor growth may be enhanced by sequence variations that promote the binding properties of oncomiRs to tumor suppressor mRNAs. Loss of the KIT protein in thyroid cancers has been associated with high expression of the miRs -221, -222, and -146b, and polymorphic changes in 3'-UTR of the KIT-mRNA were demonstrated in half of these cases (He at al., 2005). Owing to the high incidence of familial thyroid cancer, it was speculated whether these polymorphisms might predispose to this disease. Polymorphic changes in the mature miRNA sequences have also been observed. For example, a sequence variation that may alter the target selection has been identified in the mature miR-30c-2, which is overexpressed in many solid tumors (Iwai et al. ,2005).

4.4 miRNAs as new targets for therapy

1. Epigenetic therapy. As described above, miRNAs are downregulated by epigenetic mechanisms in at least some types of cancer, and re-activation of miRNAs by epigenetic therapy has been demonstrated in cancer cell lines (**Fig. 16**). In what to our knowledge is the first *in vivo* study of the effect of epigenetic therapy on miRNA transcription, it was shown that the malignant cells from CLL patients had a significantly altered miRNA expression profile after treatment with the DNMTi 5-azacytidine (Yu et al., 2006). This study holds promise that epigenetic therapy may also have an effect on miRNA re-activation *in vivo*. Whether there is also a correlation with miRNA re-activation and treatment response remains to be established.

2. Anta- and AgomiRs. In mouse models it has been possible to effectively silence endogenously expressed miRNAs. It was demonstrated that miR-122, which is abundant in liver cells, could be specifically silenced by an antagomiR. It was subsequently shown that cholesterol levels were downregulated by this treatment. This study has raised hopes that similar treatment strategies can be used against oncomiRs, which in this way may be silenced before they reach their target mRNAs (**Fig. 19**). Conversely, therapy may also be directed against oncogenic mRNAs. In a study of pancreatic cell lines it was demonstrated that a synthetically designed miRNA (a so-called AgomiR), which targets the oncogenic Gli-1 mRNAs' 3'- UTR, could inhibit cell proliferation (Tsuda et al., 2006). Many research institutions and biotechnological companies are currently working on therapies that directly target miRNAs. However, the *in vivo* function of miRNAs and the possibilities of manipulating their expression levels in patients are still largely unknown and will present a major challenge for future translational research.

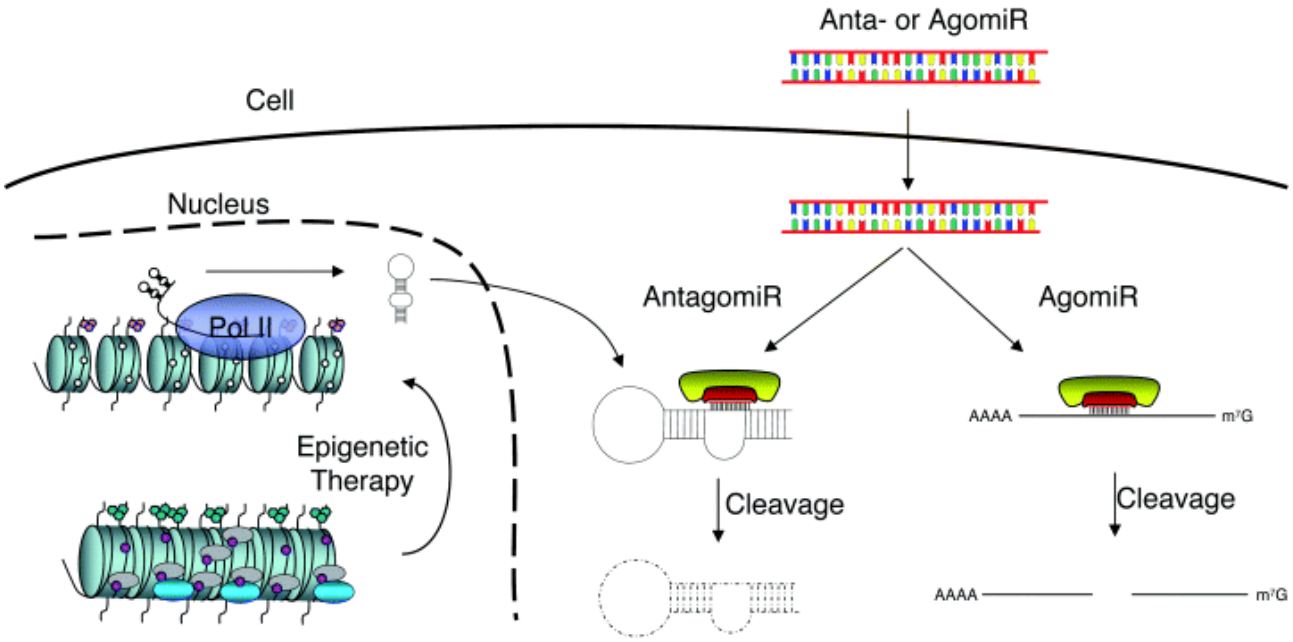


Figure 19. miRNAs as tools for therapy

4.5 Altered miRNAs expression in colorectal cancer

Components of the miRNA/RNAi pathway may be altered during the progressive development of neoplasia. Michael et al., (2003) firstly demonstrated a reduced accumulation of miR-143 and miR-145 in colorectal neoplasia. They supposed that altered accumulation of these mature miRNAs may reflects early changes in the cellular composition of tumors, compared with normal mucosae. The identification of miRNAs that consistently display reduced steady-state levels in tumors raises the possibility that they, or their targets, may be directly involved in the processes that lead to neoplasia. The public miRNA database contains 321 human miRNA sequences, 234 of which have been experimentally verified. To explore the possibility that additional miRNAs are present in the human genome, Cummings and collaborators (Cummings et al., 2006) have developed an experimental approach called miRNA serial analysis of gene expression (miRAGE) and used it to perform the largest experimental analysis of human miRNAs to date. Sequence analysis of 273,966 small RNA tags from human colorectal cells allowed us to identify 200 known mature miRNAs, 133 novel miRNA candidates, and 112 previously uncharacterized miRNA forms. To aid in the evaluation of candidate miRNAs, they disrupted the *Dicer* locus in three human colorectal cancer cell lines and examined known and novel miRNAs in these cells. These studies suggest that the human genome contains many more miRNAs than currently identified and provide an approach for the large-scale experimental cloning of novel human miRNAs in human tissues. Xi et al., (2006) explored the potential relationship between the transcription factor function of p53 and miRNA expression in a colon cancer–related context, as *p53* is one of the most frequently altered tumor suppressor genes in colon cancer due to mutations and deletions. The human HCT-116 (wt-p53) and HCT-116 (null-p53) colon cancer cell lines were chosen as model systems to investigate the role of p53 on the expression of miRNAs. Since the functional miRNAs are localized in the actively translated polyribosome complexes, they have investigated the effect of wt-p53 on miRNAs and their translationally regulated mRNA targets by isolating both actively translated mRNA transcripts and miRNAs from polyribosome complexes from these two colon cell lines. The effect of p53 on miRNA expression and on the expression levels of both steady-state and actively translated mRNA transcripts were analyzed. Their study indicated that the expression levels of a number of miRNAs were affected by wt-p53. Down-regulation of wt-p53 via siRNA abolished the effect of wt-p53 in regulating miRNAs in HCT-116 (wt-p53) cells. Global sequence analysis revealed that >46% of the 326 miRNA putative promoters contain potential p53-binding sites, suggesting that some of these miRNAs were potentially regulated directly by wt-p53. Nearly 200 cellular mRNA transcripts were regulated at the posttranscriptional level, and sequence analysis revealed that

some of these mRNAs may be potential targets of miRNAs. Nakajima et al. (2006) profiled 5 mature miRNAs (*hsa-let-7g, hsa-miR-143, hsa-miR-145, hsa-miR-181b and hsa-miR-200c*) on 46 formalin-fixed paraffin-embedded (FFPE) colorectal cancer patient samples that were treated with S-1, fourth-generation of 5-Fluorouracil (5-FU)-based drugs that has been one of the main anti-neoplastic drugs for treating various solid tumors for nearly a half century. The expression levels of *hsa-miR-143* and *hsa-miR-145* were not significantly different in tumor samples compared to their corresponding normal samples. This was a bit surprising since it had been reported by Michael *et al.* that the expression of *hsa-miR-143* and *hsa-miR-145* in human colorectal cancers decreased compared to normal samples (Michael et al., 2003). The expression level of *hsa-miR-200c* and *hsa-let-7g* was significantly over-expressed in the colorectal tumor samples compared to the corresponding normal. Based on the bioinformatics analysis, *hsa-let-7g* can potentially interact with more than 200 mRNA targets. This includes several critical cell cycle control genes such as *RAS, cyclin D, c-myc* and *E2F* family members. It's known that *E2F* family proteins are key transcription factors for regulating the expression of enzymes involved in DNA synthesis, such as TS and TK. This study firstly demonstrated that *hsa-let-7g* is associated with chemosensitivity to S-1 based chemotherapy. The expression of *hsa-miR-181b* was also strongly associated with patient response to S-1 and many genes, such as cytochrome *C, ECIP-1, MAPPKKK1, TEM6, E2F5, GATA6, PP2B* and *eIF5A*, are predicted to be regulated by *hsa-miR-181b*. Lanza et al. (2007) investigated colon cancer samples (23 characterized by microsatellite stability, MSS, and 16 by high microsatellite instability, MSI-H) for genome-wide expression of microRNA (miRNA) and mRNA. Based on combined miRNA and mRNA gene expression, a molecular signature consisting of twenty seven differentially expressed genes, inclusive of 8 miRNAs, could correctly distinguish MSI-H versus MSS colon cancer samples. Among the differentially expressed miRNAs, various members of the oncogenic miR-17-92 family were significantly up-regulated in MSS cancers. The majority of protein coding genes were also up-regulated in MSS cancers. Their functional classification revealed that they were most frequently associated with cell cycle, DNA replication, recombination, repair, gastrointestinal disease and immune response. *miR-34a* was found to be highly up-regulated in a human colon cancer cell line, HCT 116, treated with a DNA-damaging agent, adriamycin (Tazawa et al., 2007). Transient introduction of *miR-34a* into two human colon cancer cell lines, HCT 116 and RKO, caused complete suppression of cell proliferation and induced senescence-like phenotypes. Moreover, *miR-34a* also suppressed *in vivo* growth of HCT 116 and RKO cells in tumors in mice when complexed and administered with atelocollagen for drug delivery. Gene-expression microarray and immunoblot analyses revealed down-regulation of the E2F pathway by *miR-34a*

introduction. Up-regulation of the p53 pathway was also observed. Furthermore, 9 of 25 human colon cancers (36%) showed decreased expression of *miR-34a* compared with counterpart normal tissues. These results provide evidence that *miR-34a* functions as a potent suppressor of cell proliferation through modulation of the E2F signaling pathway. Abrogation of *miR-34a* function could contribute to aberrant cell proliferation, leading to colon cancer development. Schetter et al. (2008) performed a largest study to date analyzing microRNA profiles in colon cancer tissues using 2 independent cohorts. 5 microRNAs showed a differentiate expression between tumor and nontumorous tissue suggesting that predictable and systematic changes of microRNA expression patterns may occur during tumorigenesis and may be representative of sporadic colon adenocarcinomas. *miR-20a*, *miR-21*, *miR-106a*, *miR-181b*, and *miR-203* were all found to be expressed at higher levels in colon tumors, although it is uncertain whether these changes in microRNA expression patterns are merely associated with colon cancer or causal to the histological progression to cancer. Our data are consistent with published studies that provide evidence for changes in microRNA expression promoting tumor formation, especially for *miR-20a* and *miR-21*. *miR-20a* is part of the *miR-17-92* polycistronic microRNA cluster (Tanzer and Stadler, 2004). Overexpression of this cluster enhances cell proliferation *in vitro* and accelerates tumor formation in animal models. Enforced expression of the *miR-17-92* cluster causes increased tumor size and tumor vascularization in mice by negatively regulating the anti-angiogenic thrombospondin 1 (Tsp1) protein (Dews et al., 2006) Experimental evidence also suggests that increased *miR-21* expression promotes tumor development. *miR-21* is expressed at high levels in most solid tumors (Iorio et al., 2005). Overexpression of *miR-21* acts as an antiapoptotic factor in human glioblastoma cells (Chan et al., 2005). Inhibition of *miR-21* inhibits cell growth *in vitro* and inhibits tumor growth in xenograft mouse models through an indirect down-regulation of the antiapoptotic factor, B-cell lymphoma 2 (Bcl-2) (Si et al., 2007). Studies in human cell lines have shown *miR-21* can also target the tumor suppressor genes, phosphatase and tensin homolog (*PTEN*) and tropomyosin 1 (*TPM1*) (Zhu et al., 2007). These data, taken together, support a causal role for altered microRNA expression during tumorigenesis. Adenomas represent a precursor stage of adenocarcinoma. Adenomas express high levels of *miR-21*. If increased *miR-21* expression promotes colon tumor progression, increased expression in adenomas may be an early cellular event in the progression to cancer. Inhibiting *miR-21* activity may help prevent tumor promotion in populations at high risk of colon cancer, such as individuals with familial adenomatous polyposis. Finally, a recent study reports that expression of *hsa-miR-342*, a microRNA encoded in an intron of the gene *EVL*, is commonly suppressed in human colorectal cancer (Grady et al., 2008). The expression of *hsa-miR-342* is coordinated with

that of *EVL* and these results indicate that the mechanism of silencing is CpG island methylation upstream of *EVL*. The methylation at the *EVL/hsa-miR-342* locus in 86% of colorectal adenocarcinomas and in 67% of adenomas, indicating that it is an early event in colorectal carcinogenesis. In addition, there is a higher frequency of methylation (56%) in histologically normal colorectal mucosa from individuals with concurrent cancer compared to mucosa from individuals without colorectal cancer (12%), suggesting the existence of a 'field defect' involving methylated *EVL/hsa-miR-342*. Furthermore, reconstitution of *hsa-miR-342* in the colorectal cancer cell line HT-29 induces apoptosis, suggesting that this microRNA could function as a proapoptotic tumor suppressor.

4.6 COX-2 and miRNAs

The implantation process is complex, requiring reciprocal interactions between implantation-competent blastocysts and the receptive uterus. Because microRNAs (miRNAs) have major roles in regulating gene expression, Chakrabarty et al. (2007) speculated that they participate in directing the highly regulated spatiotemporally expressed genetic network during implantation. They showed that two miRNAs, mmu-miR-101a and mmu-miR-199a*, are spatiotemporally expressed in the mouse uterus during implantation coincident with expression of cyclooxygenase-2, critical for implantation. More interestingly, the *in vitro* gain- and loss-of-function experiments show that cyclooxygenase-2 expression is posttranscriptionally regulated by these two miRNAs.

Chapter III

The Research Project

1. Preliminary remarks

Despite new methods and combined strategies, conventional cancer chemotherapy still lacks specificity and induces drug resistance. Thus, gene therapy offers the potential to obtain the success in the clinical treatment of cancer. This can be achieved by replacing mutated tumour suppressor genes, inhibiting gene transcription, introducing new genes encoding for therapeutic products, or specifically silencing any given target gene. Concerning gene silencing, the antisense approach presented many practical constraints that have limited its application to cancer therapy. Nevertheless, attention has now shifted onto a more recent discovery in gene silencing, the RNA interference (RNAi). RNAi is a physiological, post-transcriptional mechanism that can effect gene silencing through chromatin remodelling, blocking protein synthesis and, in particular, cleaving specifically targeted mRNA. The effectors of RNAi are short RNA molecules, *small interfering RNAs* (siRNAs) and *microRNAs* (miRNAs), that are fully o partially homologous to the mRNA of the genes being suppressed, respectively. On one hand, synthetic siRNAs appear as an important research tool to understand the function of a gene and the prospect of using siRNAs as potent and specific inhibitors of any target gene provides a new therapeutical approach for many untreatable diseases, particularly cancer. On the other hand, the discovery of the gene regulatory pathways mediated by miRNAs, offered to the research community new important perspectives for the comprehension of the physiological and, above all, the pathological mechanisms underlying the gene regulation. Indeed, changes in miRNAs expression have been identified in several types of neoplasia and it has also been proposed that the overexpression of genes in cancer cells may be due to the disruption of a control network in which relevant miRNA are implicated. For these reasons, I focused my research on a possible link between RNAi and the enzyme cyclooxygenase-2 (COX-2) in the field of colorectal cancer (CRC), since it has been established that the transition adenoma-adenocarcinoma and the progression of CRC depend on aberrant constitutive expression of COX-2 gene. Overexpressed COX-2 is involved in the block of apoptosis, the stimulation of tumor-angiogenesis and promotes cell invasion, tumour growth and metastatization.

2. Objectives

2.1 COX-2 silencing in colon cancer cells

On the basis of data reported in the literature, the first aim of my research was to develop an innovative and effective tool, based on the RNAi mechanism, able to silence strongly and specifically COX-2 expression in human colorectal cancer cell lines, in order to: 1) better comprehend the role of COX-2 overexpression in CRC; 2) provide a new technology to suppress the malignancy of tumor cells, COX-2 mediated, in the hope of a possible *in vivo* application in therapy. The steps of this research line were:

1) Characterization of synthetic siRNA sequences capable to downregulate COX-2 expression effectively and specifically, in a model based on human umbilical vein endothelial cells (HUVEC).
2) Constitutive silencing of COX-2 gene in HT-29 colon cancer cell line, through the use of a retroviral vector system (pSUPER.retro) able to permanently transduct in tumor cells an expression cassette for a short hairpin RNA (shRNA) anti-COX-2.
3) Modification of the pSUPER.retro system, in order to improve COX-2 silencing mediated by shRNA and gain its tumor-specificity in colon cancer cell lines. In particular, the aim was to put the expression of shRNA and then siRNA anti-COX-2 (shCOX2 and siCOX2) under control of specific molecular pathway that result particularly activated in tumor cells (e.g. Wnt/β-catenin signalling pathway).
4) Development of a better RNAi-mediated silencing system, more suitable for a possible *in vivo* application. The starting point was the discovery of "trans-kingdom RNAi" (tkRNAi) by Xiang and collaborators (2006). They found that recombinant *E. Coli* strains (expressing *Inv* and *HlyA* genes) are able to transfer active siRNA in human colon cancer cells either *in vitro* or *in vivo*. My objective was to link the phenomenon of tkRNAi with the improved pSUPER.retro technology, to potently and specifically silence COX-2 enzyme in colon cancer cells.

2.2 COX-2 regulation analysis

Flanking the studies addressed to the setting-up of a RNAi-mediated therapeutical strategy, I proposed to get ahead with the comprehension of new molecular basis of human colorectal cancer. In particular, my second research line was based on two hypothesis regarding the causes that let colon cancer be and develop: 1) overexpression of COX-2 in tumor cells could be due to a misregulation of some microRNAs (miRNAs) important on controlling COX-2 signalling pathways; 2) hypoxia-related pathway could represent a driving force to develop an aggressive cancer phenotype in CRC. The steps of this second objective were:

1) Selection of miRNAs able to regulate COX-2 mRNA, based on computational analyses and data available from literature.

2) Analysis of the expression of selected miRNAs in different colon cancer cell lines, tumor tissues and metastases derived from CRC patients, in order to find a correlation between COX-2 and miRNA expression.

3) Validation of the miRNA-mediated COX-2 regulation by performing *in vitro* assays (e.g. miRNA transfections, luciferase assay).

4) Elucidation of possible molecular pathways that underlie the miRNA-mediated COX-2 regulation.

5) Analysis of hypoxia-induced survival in colon cancer cells, in order to find out a rational molecular model in which COX-2, hypoxia and tumor growth are connected.

Chapter IV

Results Part 1: COX-2 silencing RNAi-mediated

1. COX-2 specific knockdown by siRNAs in HUVE cells and evaluation of 6-keto-PGF1α production. Considering the relevance of endothelial COX-2 in the angiogenic process, I used an *in vitro* angiogenesis experimental model, based on primary human endothelial cells (HUVEC), to detect whether siRNA molecules were capable of downregulating COX-2 expression and inhibiting COX-2-dependent angiogenesis. Four different siRNAs, directed against COX-2 mRNA (Figure 1A), were transfected at 200 pM concentration, by using the Oligofectamine reagent, in HUVEC treated with PMA to enhance COX-2 expression. As shown in Figure 1 (B and C), only two siRNAs (sequences B and C) were capable of reducing COX-2 protein levels by more than 50%, whereas a scrambled siRNA, used as a negative control, was found to be completely devoid of effects. Moreover, I demonstrated that the transient knockdown mediated by siRNAs in HUVEC was highly specific since COX-1 expression resulted unaffected (Figure 1B). In samples in which COX-2 was downregulated, also PGI2 production, evaluated by ELISA assay, significantly decreased up to more than 40% (Figure 1C).

2. siRNA-B inhibition of PMA-induced angiogenesis on 3-D collagen gel. Thus, I chose siRNA sequence-B to perform an *in vitro* angiogenesis test (Figure 2). HUVE cells were able to organize into capillary-like tubular structures when seeded on 3-D collagen gel and stimulated with PMA (compare PMA-stimulated cells in panel B to control cells in panel A). I observed that transfection of siRNA-B in HUVEC strongly affected their ability to organize in tubular structures (panel C), with a significant reduction of vessels number after PMA stimulation (as shown in panel E). Cells transfected with scrambled siRNA (panel D) were still able to differentiate in tubular structures with the same efficiency of PMA-stimulated control cells (as shown in panel E), allowing to exclude toxicity and non-specific effects of siRNA-B on angiogenesis. These results demonstrate that siRNAs are capable to affect the *in vitro* angiogenic process by downregulating COX-2 expression in a strong specific manner.

3. siRNA-B activates the interferon-signalling cascade in HUVEC only at high concentration. I also evaluated whether the transfection of synthetic siRNAs in HUVE cells may activate the interferon-mediated Jak-STAT pathway, as previously reported for other siRNAs molecules. Western Blot analysis of phospho-STAT-1[Tyr701] (active form) levels, normalized against

p85/p91 STAT-1 total protein levels, showed that only an high concentration (200 nM) of transfected siRNA-B is able to trigger the interferon system response, while a lower but effective dose of siRNA (200 pM) does not have any effect on STAT-1 phosphorylation (Figure 3A). PMA-treated samples were used as positive controls. Data from immunofluorescence analysis were in high agreement with these findings (Figure 4B). STAT-1 phosphorylation, followed by nuclear translocation, was strongly increased in samples transfected with siRNA 200 nM, while no relevant differences were detected between samples transfected with siRNA 200 pM and controls.

4. Stable knockdown of COX-2 gene by RNAi in HT-29 cells. A specific sequence for anti-COX-2 short hairpin-RNA, corresponding to siRNA-B, was cloned into pSUPER.retro vector in order to achieve a stable down-regulation of COX-2 in cancer cells. The transcription of this sequence is regulated by H1 promoter for RNA pol-III. Green Fluorescent Protein (GFP) gene expression provides a rapid test for infection efficiency and the gene for puromycin resistance is necessary to select clones expressing shRNAs against COX-2 mRNA. All cassettes are included into retroviral 5'-3' LTRs to allow provirus integration in host cells genome. 3' LTR is inactivated by deletion to avoid virus replication inside infected cells (Figure 4A). The recombinant vector was transfected into Phoenix packaging cells to produce retroviral ecotropic supernatant, used to infect HT29 cells. Infected cells were selected by using standard puromycin treatment (1 μg/ml) for 48 h. Selected HT29 cells [HT29 pSUPER(+)] were analyzed by Western Blot for COX-2 expression. As shown in Figure 4B, COX-2 levels were found to be significantly decreased (more than 70%) in HT29 pSUPER(+) when compared to control cells. The inhibition was still effective when the COX-2 gene expression was stimulated by PMA treatment. COX-2 mRNA levels were analyzed in HT29 pSUPER(+) by Real-Time PCR. Results were in strict accordance with data obtained by Western Blot, confirming the specific COX-2 mRNA degradation by RNAi. In fact, I obtained an 80% reduction of COX-2 mRNA levels either in the absence or in the presence of PMA stimulation (Figure 4C). As a further demonstration of the efficiency of the COX-2 knockdown mediated by RNA Interference, I also found a significant decrease of PGE2 production in HT29 pSUPER(+) cells (Figure 4D).

5. Effect of anti-COX-2 shRNA expression on the interferon-signalling cascade in HT29 pSUPER(+) cells. Since I found, as mentioned above, that the transfection of exogenous synthetic siRNAs is capable to activate the interferon system at high concentrations in HUVE cells, the following aim was to demonstrate whether an endogenous and constitutive production of shRNA in the HT29 pSUPER(+) model had a different effect. Surprisingly, I found that shRNAs, that strongly downregulate COX-2 expression in HT29 pSUPER(+) cells, did not trigger the interferon system

response in the absence of PMA treatment, compared to the control. Both Western Blot (Figure 5A) and immunofluorescence (Figure 5B) analysis of phospho-STAT-1[Tyr701] levels and localization confirmed this evidence.

6-7. Effect of pSUPER.retro infection system on viability, cell cycle distribution, migration and soft-agar colony formation of HT29 cells. Moreover, in order to obtain more data on the effects of the constitutive COX-2 downregulation in HT29 pSUPER(+) cells, I performed four different assays to evaluate the proliferation profile and the invasiveness of these clones, compared to two different controls: HT29 wild type and HT29 pSUPER(-) cells. HT29 pSUPER(-) cells were selected with puromycin after infection with the retroviral vector, devoid of the anti-COX-2 shRNA expression cassette. Although the MTT proliferation assay (Figure 6A) and the cell cycle distribution analysis (Figure 6B) didn't show significant differences between controls and HT29 pSUPER(+), data from migration assay performed with Boyden chambers (Figure 7A and 7B) and soft-agar colony formation assay (Figure 7C) suggest that the stable knockdown of COX-2 gene by RNAi promotes a significant reduction of the migratory ability as well as a strong inhibition of colony formation in soft-agar in infected pSUPER(+) colon cancer cells. Interestingly, the loss of the malignant behaviour *in vitro* of pSUPER(+) HT29 cells did not seem to depend on an impairment of cell growth, since constitutive expression of anti-COX-2 shRNA in HT29 cells only slightly modified their proliferation profile and their cell cycle distribution, but it derived from a reduction of the ability to invade the extracellular matrix and to grow in anchorage-independent manner, which are indexes of an invasive and aggressive behaviour.

8. Efficiency of pSUPER.retro infection system. I tested the efficiency of the pSUPER.retro infection system on HUVEC and other cancer cell lines. The infection efficiency on HUVE cells was very low (less than 5%), even if repeated attempts were performed. In contrast, HT29 and HCA7 colon cancer cell lines, compared to HeLa cells (used as positive control), were easily infected showing higher efficiency levels (Figure 9). The infection efficiency for both HT29 and HCA7 was around 45%, whereas it was around 35% for HeLa cells.

9. COX-2 silencing mediated by enhanced pSUPER.retro system. In order to improve the efficiency and selectivity of COX-2 silencing in colon cancer cells, two different modifications were applied to the pSUPER.retro vector. In the basic form of the vector [pS(H1), described above], the transcription of the anti-COX-2 shRNA (shCOX-2) is driven by H1 human promoter for RNA pol III (Figure 9A.1) and the transcription STOP signal is represented by a repeat of five adenines. The H1 promoter allows the expression of shRNA, and then mature siRNA, in almost every human cell. In the light of a possible *in vivo* application, this could represent a limit for COX-2 silencing

only in tumoral tissues. For this reason, pS(H1) vector was modified and H1 promoter was substituted with the human *Cox-2* promoter [pS(COX2), Figure 9A.1] and with a promoter containing two sets (with the second set in the reverse orientation) of three copies of the TCF binding element (TBE) upstream of the Thymidine Kinase (TK) minimal promoter, derived from TOPFlash plasmid distributed by Upstate, USA [pS(TBE), Figure 9A.3]. In these two vectors, shCOX-2 is transcribed by the RNA pol II and then the transcription STOP signal was also substituted with the SV40 polyA signal. *Cox-2* promoter was chosen because it is highly activated in colon cancer cells that overexpress COX-2 protein. TBE promoter was chosen because it is bound by TCF-4/LEF-1 transcription factors and should promote the transcription of shCOX-2 only in cells in which the Wnt/β-catenin signalling pathway is strongly activated (*e.g.* colon cancer cells). As shown in Figure 9 (panels B and C), the efficiency of COX-2 silencing mediated by pS(COX-2) and pS(TBE) was evaluated in HT-29 and HCA-7 colon cancer cell lines and compared with the negative control [pS(-) empty vector, not expressing shCOX-2] and with the efficiency of pS(H1) vector. Levels of COX-2 protein, mRNA and siCOX-2 were analyzed in both cell lines. Data clearly show that enhanced pS(COX2) and, most of all, pS(TBE) vectors are more effective in silencing COX-2, both in HT-29 and HCA-7 cells, compared to pS(H1). This observation was confirmed by the increased levels of siCOX-2.

10. COX-2 silencing mediated by trans-kingdom RNA Interference (tkRNAi). Xiang and et al. (2006) demonstrated that it is possible to induce RNAi in mammalian cells after infection with engineered *E. Coli* strains expressing *Inv* and *HlyA* genes, which encode for two bacterial factors needed for successful transfer of shRNA in mammalian cells (Figure 10A). Four different *E. Coli* strains were produced after a co-transformation with pGB2Ω*inv-hly* plasmid and pS(-), pS(H1), pS(COX2) and pS(TBE) vectors, respectively. After the bacterial infection, the efficiency of the enhanced pSUPER.retro vectors was evaluated also in this system and the levels of COX-2 protein, mRNA and siCOX-2 were analyzed in HT-29 and HCA-7 cells (Figure 10, B and C). Data obtained were in great agreement with the data previously obtained after Lipofectamine transfection of HT-29 and HCA-7 cells with the same vectors. In HT-29 cells, the efficiency of pS(COX2) and pS(TBE) in silencing COX-2 protein was greatly enhanced compared to pS(H1) vector. The highest expression of siCOX-2, resulting in the highest COX-2 silencing, was obtained after infection with *E. Coli* transformed with pS(TBE) vector. In HCA-7 cells, while siCOX-2 expression didn't show great differences for the three vectors, only cells infected with *E. Coli* transformed with pS(TBE) resulted in a strong COX-2 inhibition.

FIGURE 1

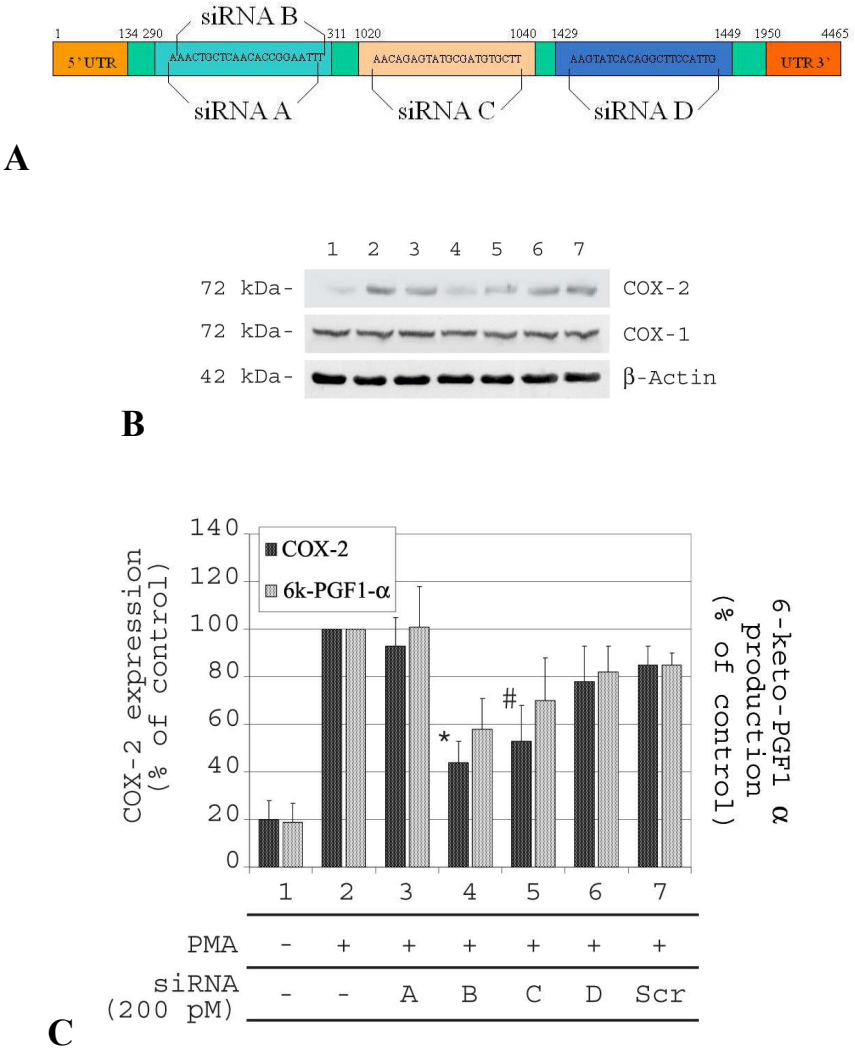


Figure 1. COX-2 specific knockdown by siRNAs in HUVE cells and evaluation of 6-keto-PGF1α production. HUVE cells were transiently transfected with siRNAs directed against COX-2 mRNA (sequences A, B, C, D; final concentration 200 pM; see panel **A**): COX-2 levels (dark bars) and 6-keto-PGF1α production (bright bars) were analysed by Western Blot and ELISA assay respectively (**B** and **C**). Panel **B** also shows COX-1 expression after siRNAs treatment. After the evaluation of the bands intensity by Image Master VDS software, both COX-2 and COX-1 levels were normalized against β-actin expression. All samples (lanes 2-7) except control in lane 1 were treated with PMA 40 nM. Lanes 3-6: samples treated with siRNA A, B, C and D, respectively. Lane 7: HUVE cells transfected with siRNA-Scr (scrambled), representing a negative control. Data are expressed as % of PMA-stimulated control value (lane 2) and represent the mean ± SEM of three independent experiments. * ($P<0.01$); # ($P<0.05$).

FIGURE 2

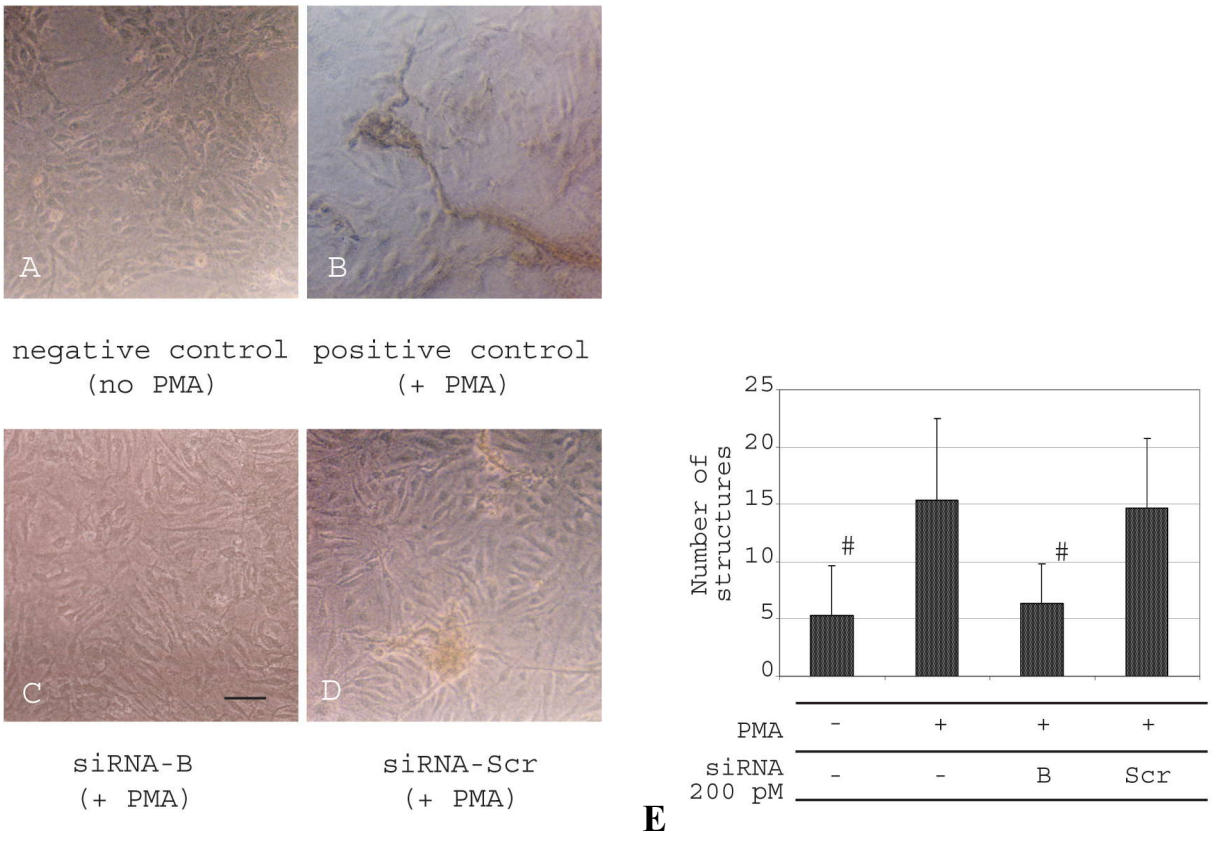


Figure 2. siRNA-B inhibition of PMA-induced angiogenesis on 3-D collagen gel. HUVE cells, seeded on 3-D collagen gels, were transfected with siRNA-B and siRNA-scrambled (**C** and **D** respectively; final concentration 200 pM) and treated for 48 h with PMA 40 nM in order to stimulate the early formation of capillary-like tubular structures. Results were compared with negative control (no PMA treatment, **A**) and PMA-stimulated positive control (**B**). The graph in panel **E** shows the number of capillary structures formed for each sample after treatments. All procedures are described under Material and Methods and results are expressed as mean ± SEM of three different experiments. # ($P<0.05$). Bar: 20 μm.

FIGURE 3

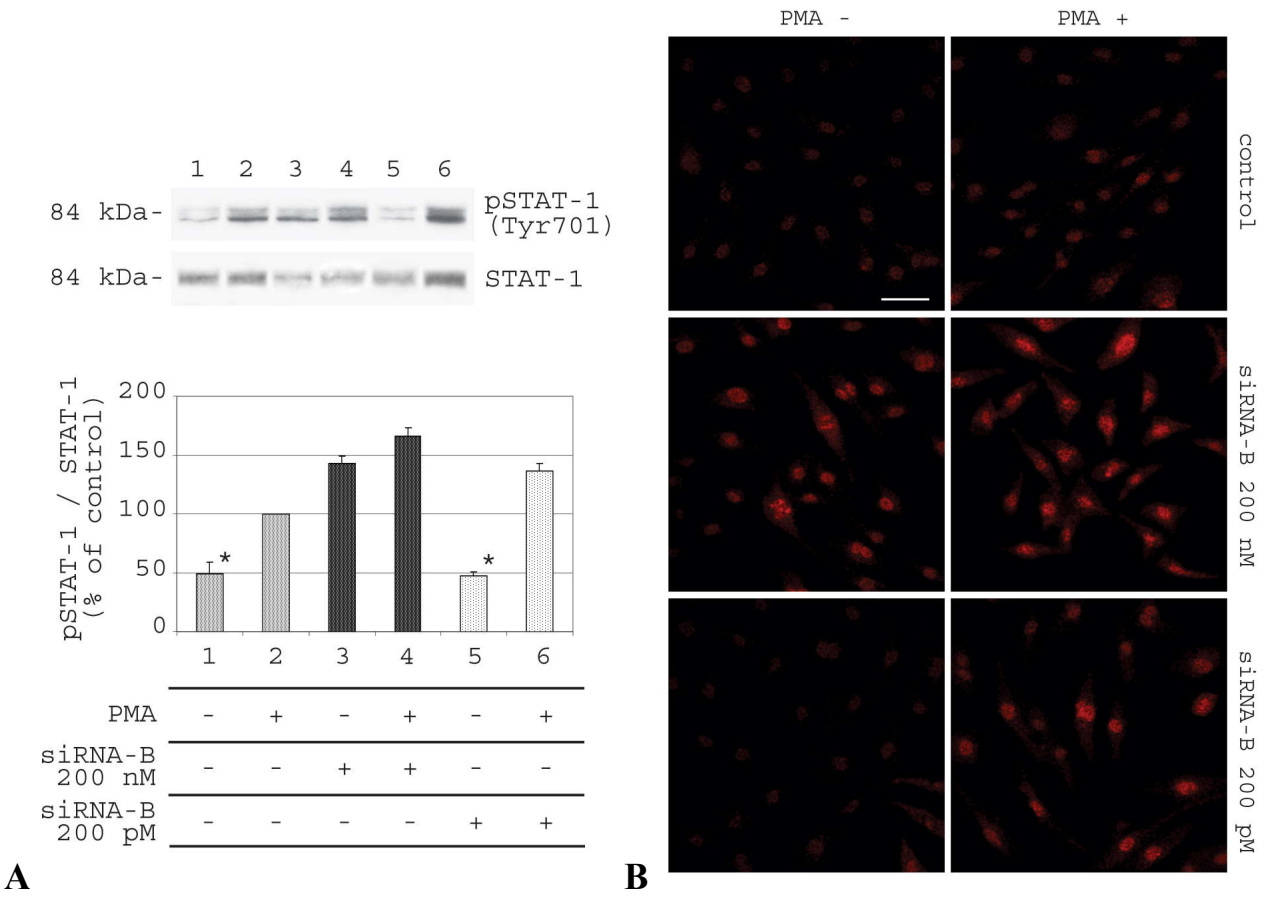


Figure 3. siRNA-B activates the interferon-signalling cascade in HUVEC only at high concentration. Cells were transiently transfected with siRNA-B directed against COX-2. Two different final concentrations were used (200 nM and 200 pM). Phospho-STAT-1 (Tyr701) and STAT-1 proteins expression was analysed by Western Blot and pSTAT-1 levels, normalized with respect to STAT-1 total levels, are reported in panel **A**. Samples in lanes 2, 4 and 6 were treated with PMA 40 nM and represent positive controls. Lanes 1: negative control (no PMA stimulation). Lanes 3 and 4: samples treated with siRNA-B 200 nM. Lanes 5 and 6: samples treated with siRNA-B 200 pM. Data are expressed as % of positive control value in lane 2 and represent the mean ± SEM of three independent experiments. The same treatments were used in an immunofluorescence assay to determine the phospho-STAT-1 protein levels and localization in siRNA-transfected HUVE cells (**B**). * ($P<0.01$). Bar: 20 μm.

FIGURE 4

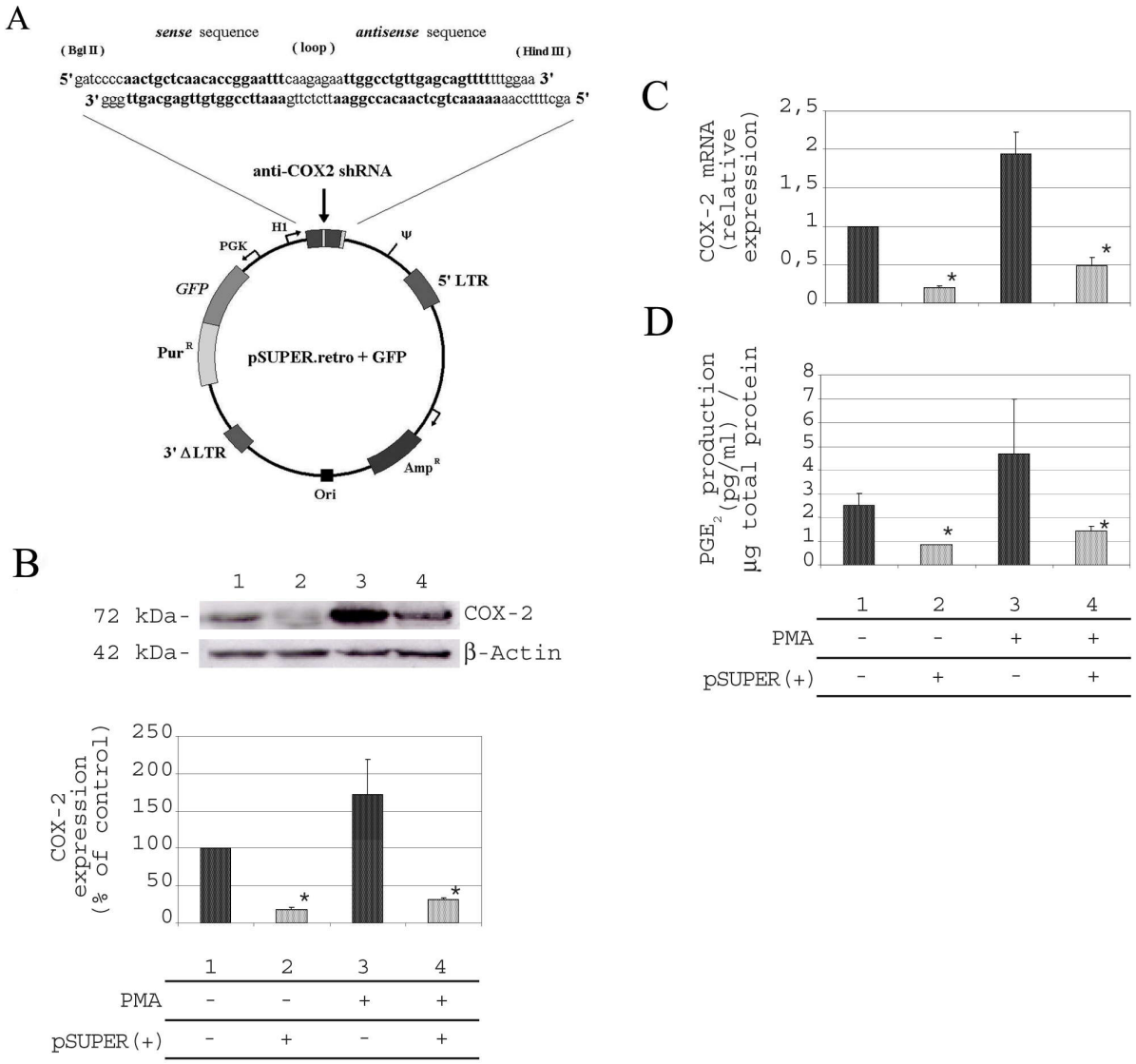


Figure 4. Stable knockdown of COX-2 gene by RNAi in HT-29 cells. (**A**) Scheme of pSUPER.retro vector (see details under Results Part 1). HT-29 cells were infected using pSUPER.retro system, selected and analysed for COX-2 protein and COX-2 mRNA levels by Western Blot and Real-Time PCR (**B** and **C**, respectively). PGE2 production (**D**) was evaluated by using an ELISA assay. COX-2 expression in infected cells (lanes 2 and 4) was compared with that of control cells (lanes 1 and 3), in the absence (lanes 1-2) or in the presence (lanes 3-4) of 40 nM PMA-stimulation. Data from Western Blot are expressed as % of PMA-stimulated control in lane 1. All results are expressed as mean ± SEM of three different experiments. * ($P<0.01$).

FIGURE 5

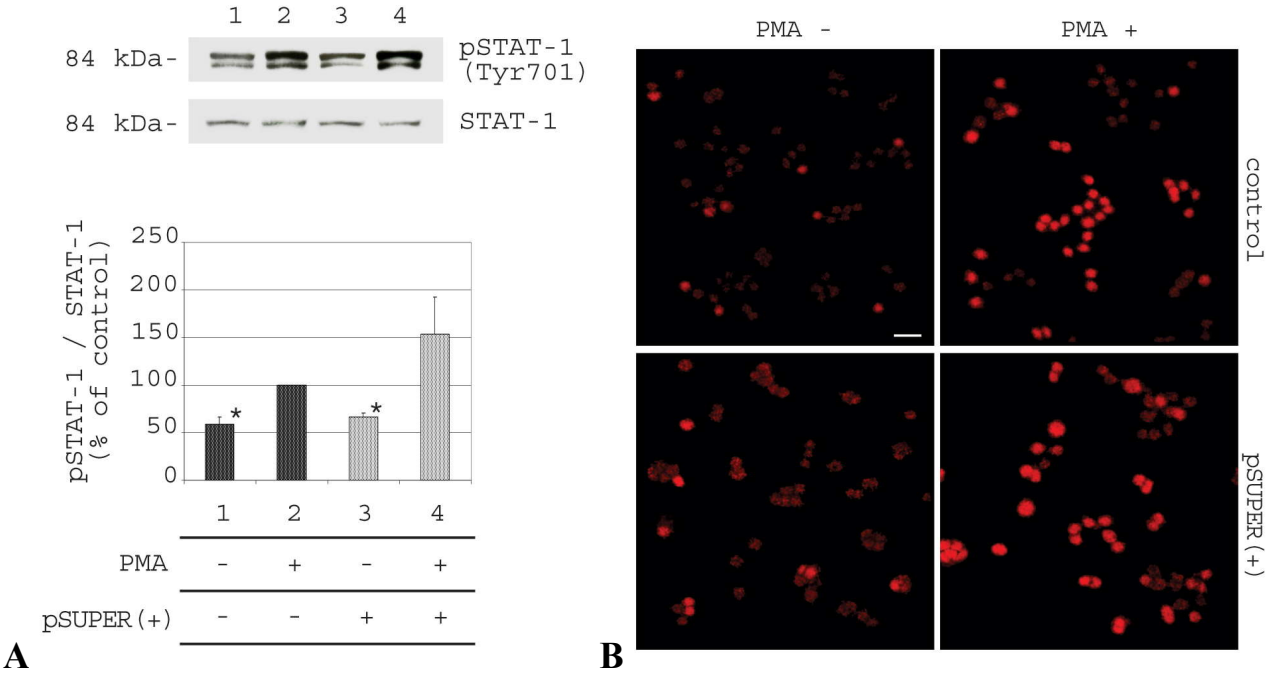
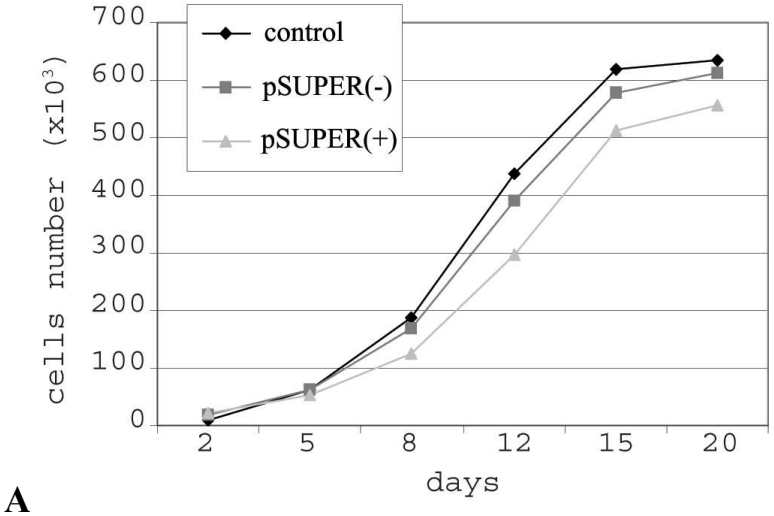
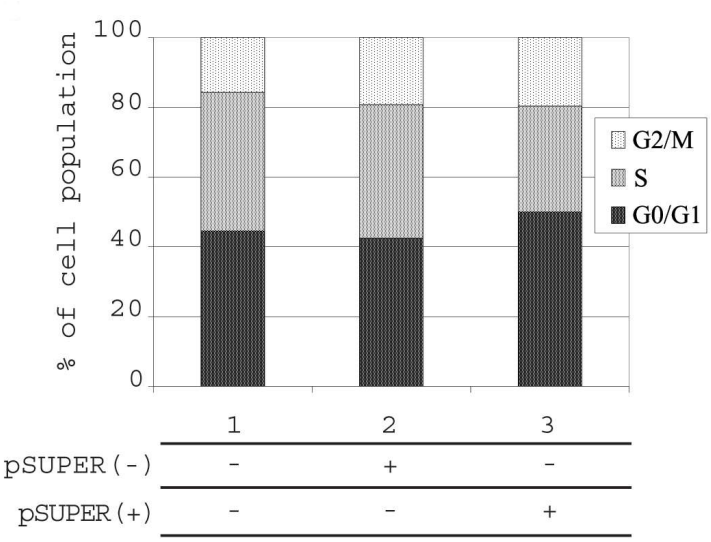


Figure 5. Effect of anti-COX-2 shRNA expression on the interferon-signalling cascade in HT29 pSUPER(+) cells. An analysis of STAT-1 phosphorylation in HT29 wild type cells and HT29 cells infected using pSUPER.retro system was performed. Phospho-STAT-1 (Tyr701) and STAT-1 proteins expression was analysed by Western Blot and pSTAT-1 levels, normalized with respect to STAT-1 total levels, are reported in panel **A**. Lanes 1 and 2: HT29 wild type. Lanes 3 and 4: HT29 pSUPER(+). Samples in lanes 2 and 4 were treated with PMA 40 nM. Data are expressed as % of positive control value in lane 2 and represent the mean ± SEM of three independent experiments. The same samples were analysed in an immunofluorescence assay to determine the phospho-STAT-1 protein level and localization (results are shown in panel **B**). * ($P<0.01$). Bar: 20 μm.

FIGURE 6



A



B

Figure 6. **Effect of pSUPER.retro infection system on the viability and the cell cycle distribution of HT29 cells.** Proliferation curves were determined by the MTT assay (**A**) and the cell cycle distribution analysis was carried out on $1x10^6$ cells/samples after 60 min of incubation with BrdU (**B**). Control: HT29 wild type cells (● and lane 1); pSUPER(-): HT29 cells infected with vector non expressing anti-COX2 shRNA (■ and lane 2); pSUPER(+): infected HT29 cells expressing shRNA against COX-2 mRNA (▲ and lane 3). All data represent the mean of three independent experiments.

FIGURE 7

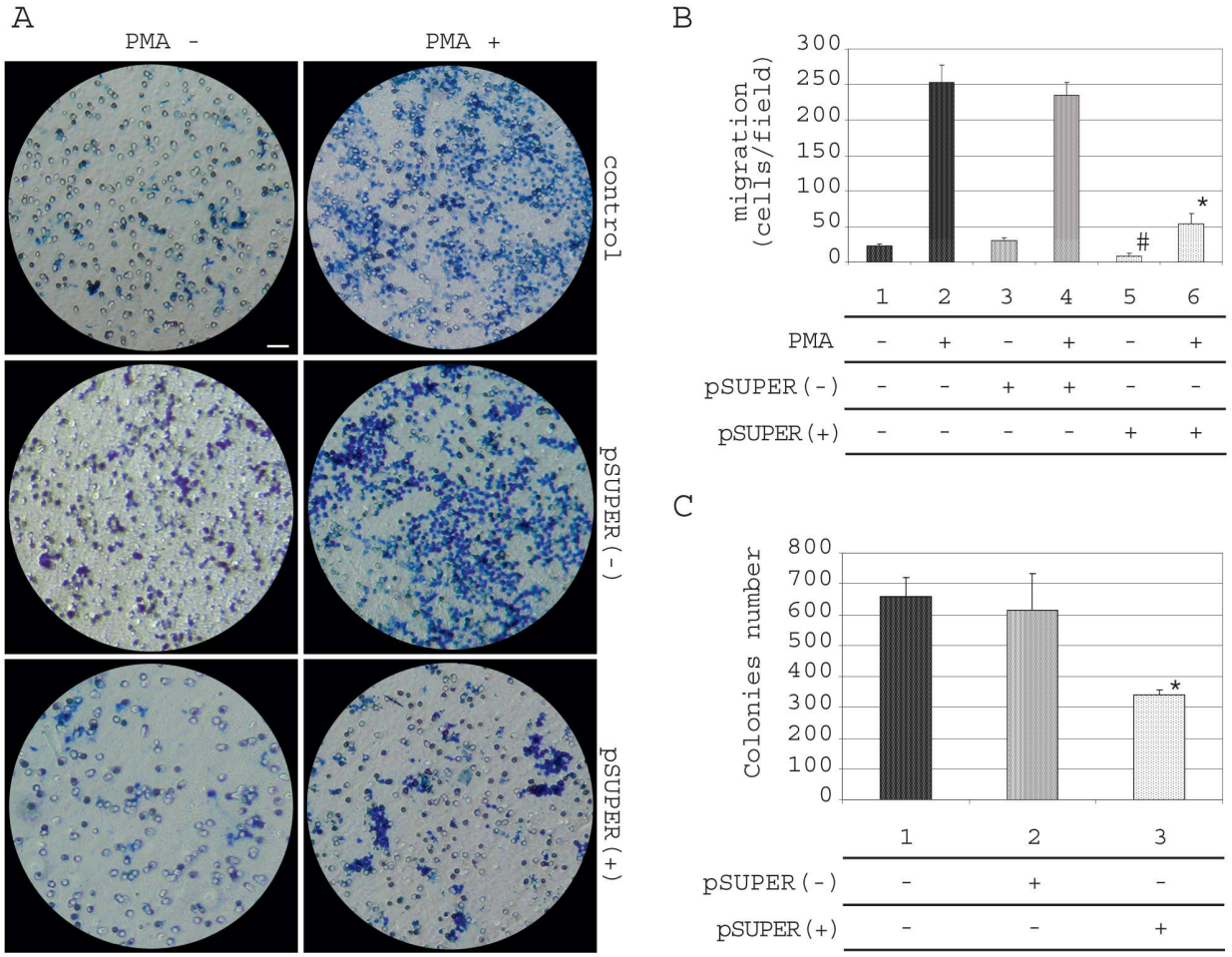


Figure 7. **Effect of pSUPER.retro infection system on migration and soft-agar colony formation in HT29 cells**. The migration assay was performed by using Boyden chambers and 8-μm polycarbonate membranes coated with Matrigel (40-fold dilution). After 24 h of incubation, cells that migrated through the Matrigel coated membranes were fixed, stained, photographed (**A**) and counted under light microscopy (**B**). Regarding the soft-agar colony formation assay, the number of colonies was evaluated 7 days after the seeding in soft-agar (**C**). Control: HT29 wild type cells; pSUPER(-): HT29 cells infected with vector non expressing anti-COX2 shRNA; pSUPER(+): infected HT29 cells expressing shRNA against COX-2 mRNA. In the migration assay samples were tested in the absence and presence of PMA 40 nM. Data in panels **B** and **C** represent the mean ± SEM of three independent experiments. * ($P<0.01$); # ($P<0.05$). Bar: 20 μm.

FIGURE 8

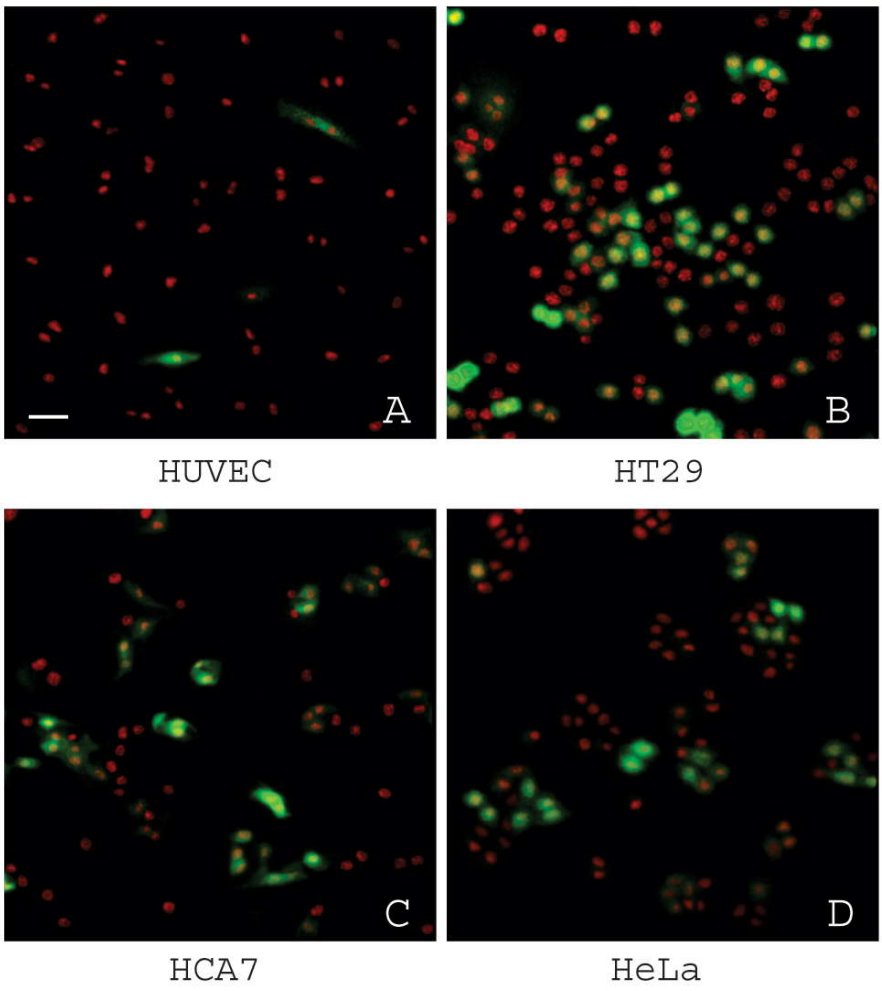


Figure 8. **Efficiency of pSUPER.retro infection system**. The images represent four different cell types that underwent the infection by pSUPER.retro system (**A**: HUVEC; **B**: HT29; **C**: HCA7; **D**: HeLa). Cells were fixed 24 h after infection and observed by using confocal microscopy. Nuclei were stained with propidium iodide and the infected cells appear GFP-positive since the pSUPER.retro vector contains an expressing cassette for GFP gene. Bar 20 μm.

FIGURE 9

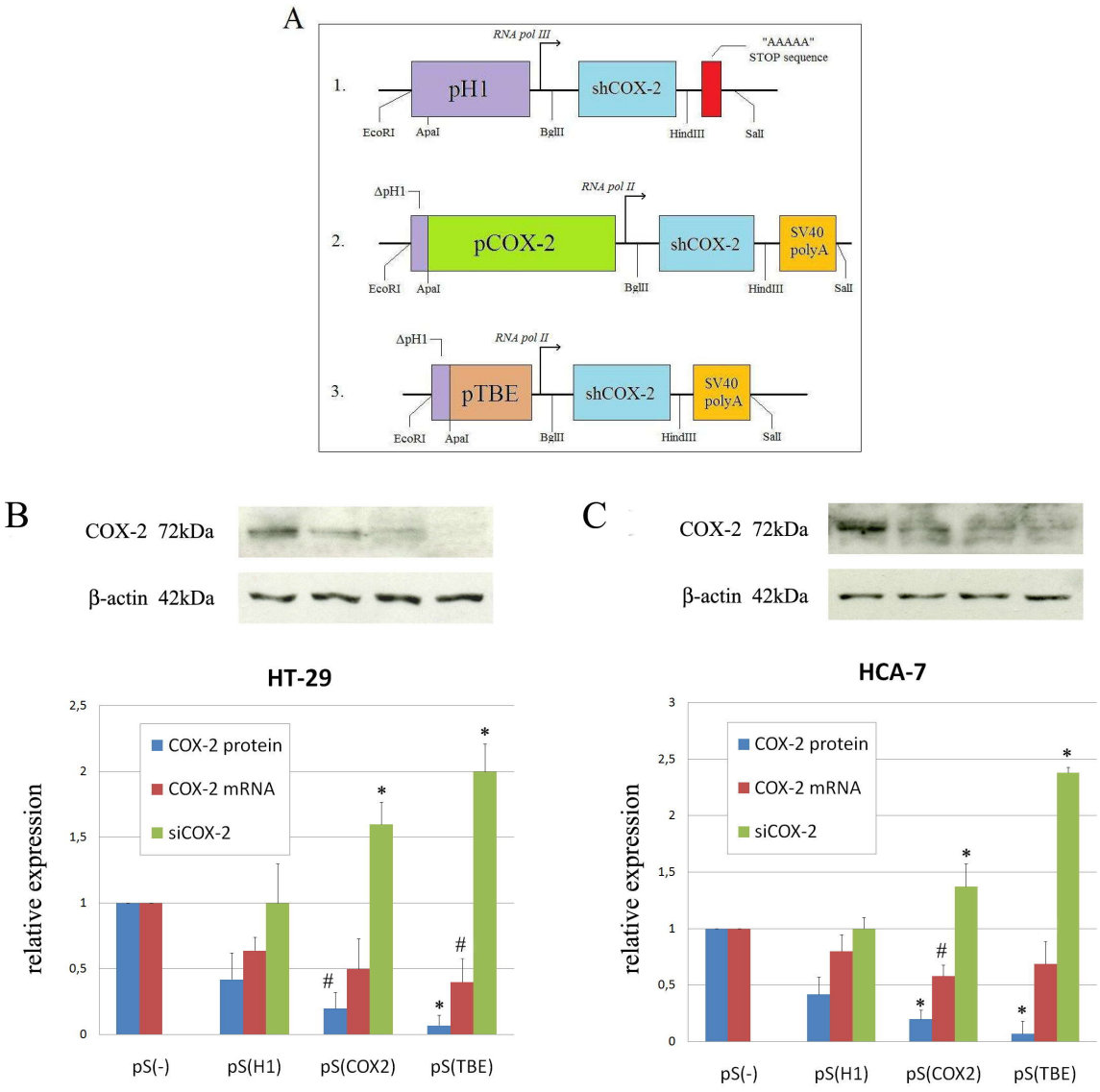


Figure 9. COX-2 silencing mediated by enhanced pSUPER.retro system. pSUPER.retro vector was modified in order to improve efficiency and specificity of COX-2 silencing. Modifications are shown in panel **A**. 1) pS(H1): original form of pSUPER.retro vector in which anti-COX-2 shRNA (shCOX-2) transcription is driven by H1 promoter for RNA pol III; 2) pS(COX2): in this vector H1 promoter was substituted with the whole promoter of *Cox-2* gene; 3) pS(TBE): in this vector H1 promoter was substituted with a promoter containing two sets (with the second set in the reverse orientation) of three copies of the TCF binding element (TBE) upstream of the Thymidine Kinase (TK) minimal promoter, derived from TOPFlash plasmid (Upstate, USA). In both pS(COX2) and pS(TBE), shCOX-2 transcription is driven by RNA pol II and the transcription STOP signal for RNA pol III (repeat of five adenines) was substituted with the SV40 polyA sequence. HT-29 (**B**) and HCA-7 (**C**) cells were transfected using Lipofectamine with pS(-) (pSUPER.retro vector not containing the expression cassette for shCOX-2, lane 1), p(H1) (lane 2), p(COX2) (lane 3) and p(TBE) (lane 4) vectors. Levels of COX-2 protein (blue bars), mRNA (red bars) and siCOX-2 (anti-COX-2 siRNA derived from shCOX-2, green bars) were evaluated with Western Blot and Real-Time PCR analysis. Data are shown as relative expression of negative control value in lane 1 and represent the mean ± SEM of three independent experiments. * ($P<0.01$); # ($P<0.05$).

FIGURE 10

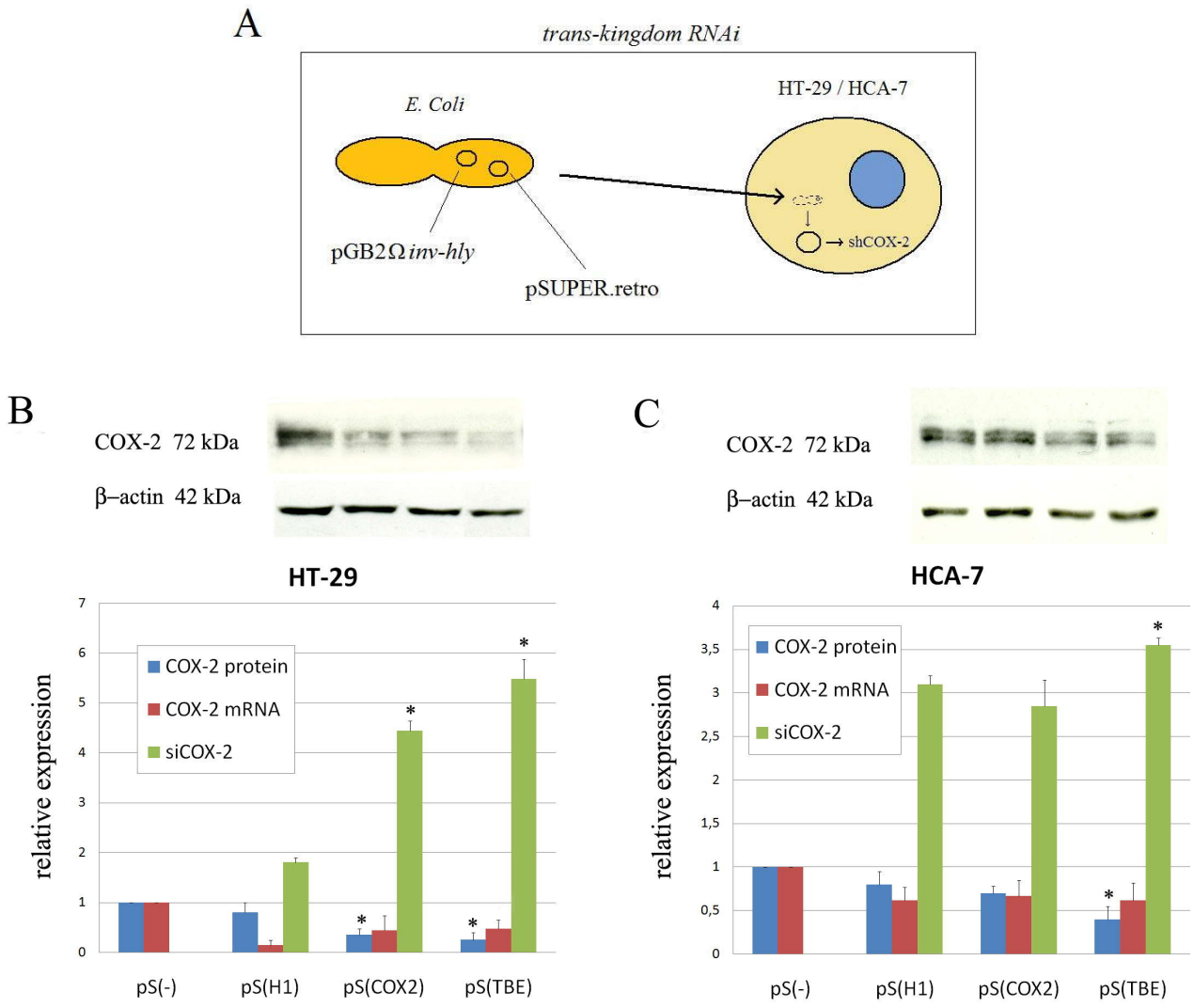


Figure 10. COX-2 silencing mediated by trans-kingdom RNA Interference (tkRNAi). (**A**) Schematic representation of tkRNAi. *E. Coli* strains were co-transformed with both pGB2Ω*inv-hly* and pSUPER.retro plasmids. Four different *E. Coli* strains were created, each containing one of the four different forms of pSUPER.retro vector previously described. HT-29 (**B**) and HCA-7 (**C**) were infected with the four recombinant *E. Coli* strains and levels of COX-2 protein (blue bars), mRNA (red bars) and siCOX-2 (anti-COX-2 siRNA derived from shCOX-2, green bars) were evaluated with Western Blot and Real-Time PCR analysis. Lanes 1-4: cells infected with *E. Coli* co-transformed with pS(-) (lane 1), pS(H1) (lane 2), pS(COX2) (lane 3) and pS(TBE) (lane 4). Data are shown as relative expression of negative control value in lane 1 and represent the mean ± SEM of three independent experiments. * ($P<0.01$).

Discussion part 1: COX-2 silencing RNAi-mediated

RNA Interference (RNAi) represents a brand new approach in the field of reverse genetics, since it is a potent tool capable of silencing genes in a high, long lasting and selective manner. The understanding of RNAi mechanism of action has soon disclosed a wide spectrum of possible applications, either *in vitro* or *in vivo*. In the past decade, many studies have shown that small dsRNA molecules are capable of downregulating genes with sequence homology and that the knockdown mediated by RNAi machinery is an ubiquitous phenomenon in eukaryotic cells. Much of the knowledge regarding RNAi comes from studies on *C. elegans*. In this organism, Mello and co-workers in 1998 demonstrated that microRNAs (miRNAs) physiologically regulate the expression of genes involved in worm development (Fire *et al,* 1998; Elbashir *et al,* 2001). Today, siRNAs and shRNAs are widely used by researchers to silence the expression of many target genes, because of their high specificity and their apparent non-toxicity. Moreover, systems based on DNA plasmids or retroviral vectors have provided new solutions to achieve a stable knockdown mediated by shRNAs.

In this work, I used a RNAi-based approach to obtain an efficient knockdown of COX-2 mRNA. The overexpression of COX-2 seems to play a critical role in many pathological processes. In particular, the concept that high levels of PGI2 and PGE2, the main products of arachidonate metabolism in vascular tissues and cancer cells respectively, stimulate both tumor-induced angiogenesis and tumor progression is widely accepted (Leahy *et al,* 2002; Iñiguez *et al,* 2003), whereas the overexpression of the inducible form of COX enzyme in colorectal cancer cells seems to promote tumor invasion, tumor growth and tumor metastasis (Gupta and Dubois, 2001; Yamauchi *et al,* 2002; Tuynman *et al,* 2004). Moreover, the possibility that the overexpression of genes implicated in human cancers may be due to a down-regulation or a general imbalance of specific miRNAs has been recently supported (Micheal *et al,* 2003; Calin *et al,* 2004; He *et al*, 2005; Lu *et al,* 2005) and it could be investigated in the case of colorectal cancer.

Data collected in this thesis show the effect of the transfection of four different sequences of anti-COX-2 siRNAs on HUVE cells, stimulated with PMA in order to overexpress COX-2 mRNA. It has been previously shown that PMA stimulates COX-2 expression in HUVEC, strongly increasing both mRNA and protein levels (Hirai et al, 1999). Under these conditions, the release of prostacyclin (PGI2) was also found to be highly augmented. It is well known that PGI2 stimulates angiogenesis, probably by acting on a nuclear receptor belonging to peroxisome proliferator-

activated receptors (PPARs) family. Two of the synthetic siRNAs tested (sequences B and C) resulted to be effective in downregulating COX-2 levels and enzymatic activity in HUVE cells in a specific manner, having no effect on COX-1 expression. Moreover, COX-2 silencing by siRNAs was observed even at very low concentrations (200 pM). Has to be mentioned that one of these active siRNA sequences (sequence B) overlaps the same sequence used by Denkert and co-workers (2003) to efficiently downregulate COX-2 expression in ovarian carcinoma cells. As a consequence of COX-2 downregulation and PGI2 reduction, endothelial cells transfected with siRNA-B failed to organize capillary-like tubular structures in 3-D collagen gel when stimulated with PMA, whereas control cells (non-transfected or transfected with a scrambled siRNA) rapidly formed several lengthened structures sprouting inside the gel.

It has been reported that synthetic siRNAs (Sledz *et al,* 2003; Judge *et al,* 2005), as well as short single-stranded RNAs (ssRNAs) (Kim *et al,* 2004) and some DNA vectors expressing shRNAs (Bridge *et al,* 2003), are able to trigger an interferon response *in vitro*. On the contrary, other findings demonstrate that is even possible to administer naked, synthetic siRNA to mice without inducing any interferon response (Heidel *et al,* 2004). In my experimental model I analyzed the phosphorylation status (on Tyr701) of STAT-1 protein as a marker of the interferon system activation (Brierley *et al*, 2005). As STAT-1 activation can be triggered by PMA treatment (Cohen *et al*, 2005), I compared the levels of STAT-1 phosphorylated isoform in HUVEC transfected with siRNA-B at two different doses (200 nM and 200 pM) with respect to PMA-stimulated control cells. While the treatment with a 200 nM dose of siRNA-B significantly increased STAT-1 phosphorylation levels, both in the absence or in the presence of PMA, the treatment with the same siRNA molecule at a low dose (200 pM) did not show any significant effect on STAT-1 phosphorylation when compared with controls. Similar results were obtained by analyzing phospho-STAT-1 expression and its nuclear translocation by immunofluorescence assay, indicating that low but effective doses of anti-COX-2 siRNA are devoid of effects on the interferon system.

Following the detection of a siRNA sequence able to efficiently interfere with COX-2 expression in HUVE cells, I focused my attention on a new RNAi strategy based on the use of a shRNA-expressing vector. The aim was to obtain a stable and efficient knockdown of COX-2 gene in HT-29 cells, a cell line derived from a human colorectal cancer and known to overexpress this enzyme. The approach I chose was based on pSUPER.retro technology (Brummelkamp *et al,* 2002). Many advantages came from this approach, first of all the possibility to achieve, by using inexpensive tools, a potent silencing of target genes that is also highly specific and long-lasting. Therefore, interesting results were achieved, since data from Western Blot, Real-Time PCR analysis and

ELISA assay clearly showed a strong and selective reduction of COX-2 protein and mRNA levels, with consequent inhibition of PGE2 production, in HT-29 cells infected with a pSUPER.retro vector expressing anti-COX-2 shRNA (shCOX-2) and then anti-COX-2 siRNA-B (siCOX-2) [HT-29 pSUPER(+)], even in the presence of PMA-induced COX-2 overexpression. These data support the evidence of a strong and long-lasting COX-2 mRNA degradation driven by siRNA molecules processed from shRNA precursors.

As previously tested in HUVEC treated with anti-COX-2 siRNA-B, I analyzed the effect of the permanent expression of shCOX-2 molecules on STAT-1 activation in HT-29 pSUPER(+) cells, detecting no significant increase neither in STAT-1 phosphorylation levels nor in phospho-STAT-1 nuclear accumulation. These data suggest that a vector-based stable expression of shRNA molecules induces a weaker interferon system response with respect to the transfection of synthetic siRNAs.

Performing experiments aimed to analyze the phenotype of COX-2-deficient HT-29 pSUPER(+) cells, no significant effects of COX-2 down-regulation on cell proliferation and cell cycle distribution were detected, observing only a slight decrease of the proliferation rate and a slight accumulation of cells in the G0/G1 phase. These results are in line with other data recently collected in a model of ovarian carcinoma cells (Denkert *et al*., 2003) and in a model of human hepatocellular carcinoma cells (Park *et al*, 2005) based on siRNA-mediated COX-2 down-regulation, suggesting that COX-2 itself is not decisively involved in the proliferation of human cancer cells as well.

However, interesting results were obtained by testing the *in vitro* invasive behavior of HT-29 pSUPER(+) cells and their anchorage-independent growth ability. PMA-stimulated HT-29 cells, expressing high levels of COX-2, resulted to be able to degrade and migrate through ECM components of Matrigel-coated membranes, which indicates their malignant behavior *in vitro,* associated with a well described invasive and metastatic ability *in vivo* (Kakiuchi *et al*, 2002). Moreover, HT-29 wild type cells easily formed colonies in soft-agar, which is an accepted criterion for transformation and an experimental condition that better represents tumor cells growth and invasiveness *in vivo.* The knockdown of COX-2 enzyme in HT-29 cells abrogated their ability to invade Matrigel-coated membranes in a Boyden chamber assay, either in the absence or in the presence of PMA-stimulation, and strongly impaired their anchorage-independent growth in soft agar basal conditions. These results support the involvement of COX-2 in the malignant behavior of human colon carcinoma cells and underline the relevance of a stable virus-based COX-2 knockdown mediated by RNA interference in order to impair the invasive and metastatic ability of CRC.

My study confirms the predominant role that RNA Interference is assuming in the field of gene silencing due to its high efficacy, specificity and non-toxicity. One of the novel targets in cancer therapies is to obtain a selective down-regulation of those genes overexpressed in tumor tissues. COX-2 is certainly one of them. Results reported here indicate an easy-to-use, powerful and high selective virus-based method to knockdown COX-2 gene in a stable and long-lasting manner, in colon cancer cells. Furthermore, they open up the possibility of an *in vivo* application of this anti-COX-2 retroviral vector, as therapeutic agent for human cancers overexpressing COX-2. In fact, a significant resistance of HUVE cells to the pSUPER.retro viral infection was observed in comparison with different types of human cancer cells, either CRC cells (HT-29 and HCA-7) and cervix carcinoma cells (HeLa). This observation indicates that endothelial cells are refractory to retroviral infection and suggests that this kind of virus-based approach might not affect the physiological prostaglandins production in vascular tissues, avoiding some of the well known side effects coming from therapies based on selective COX-2 inhibitors (coxibs) (Mamdani *et al,* 2004; Mitchell and Warner, 2005).

In order to improve the tumor selectivity, pSUPER.retro vector was modified for the shCOX-2 expression cassette. The aim was to gain a strong, specific transcription of shCOX-2 followed by COX-2 silencing only in cancer cells. It is well known that intracellular activation of different molecular pathways are responsible of COX-2 overexpression in colon cancer. This highlights that COX-2 promoter is overstimulated in tumor cells, compared with other normal tissues in which COX-2 gene is finely regulated. Moreover, there are some pathways that are typically activated in colon cancer cells: one of those is Wnt/β-catenin signalling pathway. β-catenin protein is frequently mutated in colon adenomas and adenocarcinomas and this alteration causes COX-2 overexpression mediated by transcriptional factors like TCF-4/LEF-1 that bind and activate COX-2 promoter. For this reason, my purpose was to put the shCOX-2 expression cassette under the control of TCF/LEF consensus sequences (TBEs, TCF Binding Elements) which can modulate the expression of the shRNA in those colon cancer cells presenting an high nuclear β-catenin. In pSUPER.retro vector previously used, the expression cassette of shCOX-2 is under control of RNA pol-III human promoter H1 [pS(H1) vector] that brings, after gene trasduction, a constitutive non-regulated transcription of siCOX-2 processed from the shCOX-2 precursor. This means that the COX-2 silencing system allows siCOX-2 production in every mammalian cell type where viral trasduction is effective. Thus, H1 promoter was substituted with the human *Cox-2* promoter [pS(COX2)] and with a promoter containing repeated copies of the TCF binding element (TBE) [pS(TBE)]. The transcription stop signal was also substituted with SV40 polyA signal. Following these modification, shCOX-2 transcription became strong and specific only in cells in which COX-2 and

Wnt/β-catenin signalling pathway are activated. These evidences were largely supported by data collected using HT-29 and HCA-7 colon cancer cells, in which the COX-2 silencing mediated by enhanced pS(COX2) and pS(TBE) vectors was evaluated. In particular, a stronger inhibition of *Cox-2* gene (at both protein and mRNA level) and a higher siCOX-2 production were achieved by using pS(TBE) vector, that represents not only the most effective, but also the most specific system to downregulate COX-2 in colon cancer cells.

Because of the many limits that a retroviral therapy could have in a possible *in vivo* treatment of CRC, the next goal was to render the enhanced RNAi-mediate COX-2 silencing more suitable for this kind of application. Xiang and et al. (2006) demonstrated that it is possible to induce RNAi in mammalian cells after infection with engineered *E. Coli* strains expressing *Inv* and *HlyA* genes, which encode for two bacterial factors needed for successful transfer of shRNA in mammalian cells. This system, called "trans-kingdom" RNAi (tkRNAi) could represent an optimal approach for the treatment of colorectal cancer, since *E. Coli* in normally resident in human intestinal flora and could easily vehicled to the tumor tissue. For this reason, I tested the improved COX-2 silencing mediated by pS(COX2) and pS(TBE) by using tkRNAi system. Results obtained in HT-29 and HCA-7 cell lines were in high agreement with data collected after the Lipofectamine transfection of pS(COX2) and pS(TBE) vectors in the same cell lines, previously discussed. These findings suggest that tkRNAi system for COX-2 silencing, in particular mediated by pS(TBE) vector, could represents a promising tool for the treatment of colorectal cancer.

Chapter V

Results Part 2: *miR-101* regulates COX-2 in cancer cells

1. COX-2 and selected miRNAs expression in six colon cancer cell lines. Figure 1A shows that six colon cancer cell lines (LoVo, HCA-7, LS-174T, HT-29, SW620 and Caco-2) express different levels of COX-2 protein and mRNA and it is important to note that there is not a direct correspondence between COX-2 protein and mRNA levels. The hypothesis is that in the six different cell lines, the efficiency of COX-2 mRNA translation is not the same, and this phenomenon could be due to a post-transcriptional regulation. Since microRNA-based RNAi is a form of post-transcriptional gene silencing, my purpose was to demonstrate a possible regulation of COX-2 protein expression by one or more microRNAs. The expression profile of six selected miRNAs (*miR-199a, miR-101-1, miR-101, let-7a, miR-100* and *miR-222*) was analyzed in the six colon cancer cell lines (Figure 1B). Selection of miRNAs was carried out using four different bioinformatical analyses and basing on data collected from literature (Table 1, detailed informations are shown in Supplementary Materials, section 1). Among the six miRNAs, only *miR-101(1)* seemed to correlate with COX-2 expression. In particular, its levels showed an inverse correlation with the COX-2 protein / mRNA ratio of the six cell lines. This ratio was taken as an index of COX-2 mRNA silencing. As shown in Figure 1C, colon lines with an high COX-2 protein / mRNA ratio have low levels of *miR-101(1)* (*e.g.* LS-174T) and lines with an low COX-2 protein / mRNA ratio have high levels of *miR-101(1)* (*e.g.* HCA-7).

2-3. Correlation between COX-2 and miR-101(1) expression in CRC tissues and metastases. Since *miR-101(1)* seemed to correlate with COX-2 expression, its levels were analyzed in tissue and metastases samples, derived for patients affected by CRC. Both Figures 2 and 3 show an evident correlation between *miR-101(1)* and COX-2 expression. COX-2 protein (Figure 2A), mRNA (Figure 2B) levels and their ratio (Figure 2C) were evaluated in tissues from six patients and a comparison between normal tissue and tumor tissue from the same patient was carried out. Panel D represents the expression of *miR-101(1)* in the same samples, inverse correlated with the COX-2 protein / mRNA ratio showed in panel C. COX-2 protein and mRNA levels were also analyzed in metastasis samples from eight CRC patients (Figure 3A). The ratio between protein and mRNA was compared to *miR-101(1)* levels, showing again an inverse correlation (Figure 3B).

4. miR-101(1) regulates COX-2 expression in colon cancer cell lines. Supported by the data obtained in colon cancer cell lines and tissues, a series of *in vitro* experiments was carried out to demonstrate a direct regulation of COX-2 expression mediated by *miR-101(1)*. Three colon cancer cell lines (LS-174T, HT-29 and HCA-7) were transfected with synthetic *miR-101(1)* using Lipofectamine and the silencing of COX-2 protein was evaluated. The effect of *miR-101(1)* transfection was compared to two different negative controls (cells treated only with Lipofectamine and cells transfected with *cel-miR-67*, a *C. Elegans* miRNA,) and a positive control (siCOX-2, previously tested in my experiments). The effect of the inhibitors of both *miR-101(1)* and *cel-miR-67* was also evaluated. All the RNA molecules were transfected at 200 nM final concentration. As expected, transfection of *miR-101(1)* led to a COX-2 silencing in all the three cell lines (Figure 4, A-B and C), compared to negative controls. Moreover, the transfection of *miR-101(1)* inhibitor (that blocks the action of *miR-101(1)* by pairing its molecule), induced an overexpression of COX-2 protein, slight in LS-174T cell and stronger in HT-29 and HCA-7 cells. Finally, Figure 4E shows the putative binding site of *miR-101(1)* in COX-2 3'-UTR region as detected by MiRanda software.

5. miR-101(1) downregulation is involved in COX-2 overexpression induced by hypoxia. In order to investigate a possible molecular pathway by which *miR-101(1)* regulates COX-2 expression, I started to analyze the involvement of *miR-101(1)* in COX-2 overexpression induced by hypoxia. The hypoxic context was chosen because it strongly enhance COX-2 expression in colon cancer cells. An hypoxic environment can be induced *in vitro* treating the cells with the iron chelator desferrioxamine (DFX). Figure 5 shows that COX-2 is overexpressed after 24 and 48 h of DFX 100 μM treatment in LS-174T (5A), HT-29 (5B) and HCA-7 (5C). The strongest induction of COX-2 was observed in HT-29 after 24 h and HCA-7 after 48 h while it was only slight in LS-174T. Surprisingly, in all the three cell lines COX-2 overexpression wasn't a consequence of increased mRNA levels. On the contrary, DFX treatment produced a significant decrease of COX-2 mRNA either after 24 h or 48 h. Moreover, a *miR-101(1)* transfection was performed on DFX treated cells. The transfected *miR-101(1)* strongly inhibited the COX-2 overexpression in LS-174T after 24 and 48 h, and in HT-29 and HCA-7 after 48 h. Under these conditions, the COX-2 protein / mRNA ratio was compared to *miR-101(1)* levels in all samples. For the three cell lines, the increased ratio was always coupled to a *miR-101(1)* decreased expression. After 48 h treatment with DFX and *miR-101(1)* transfection instead, the ratio values were comparable to the negative control samples, not treated with DFX while in LS-174T the ratio returned to normal levels already after 24 h (Figure 5, D-E and F).

FIGURE 1

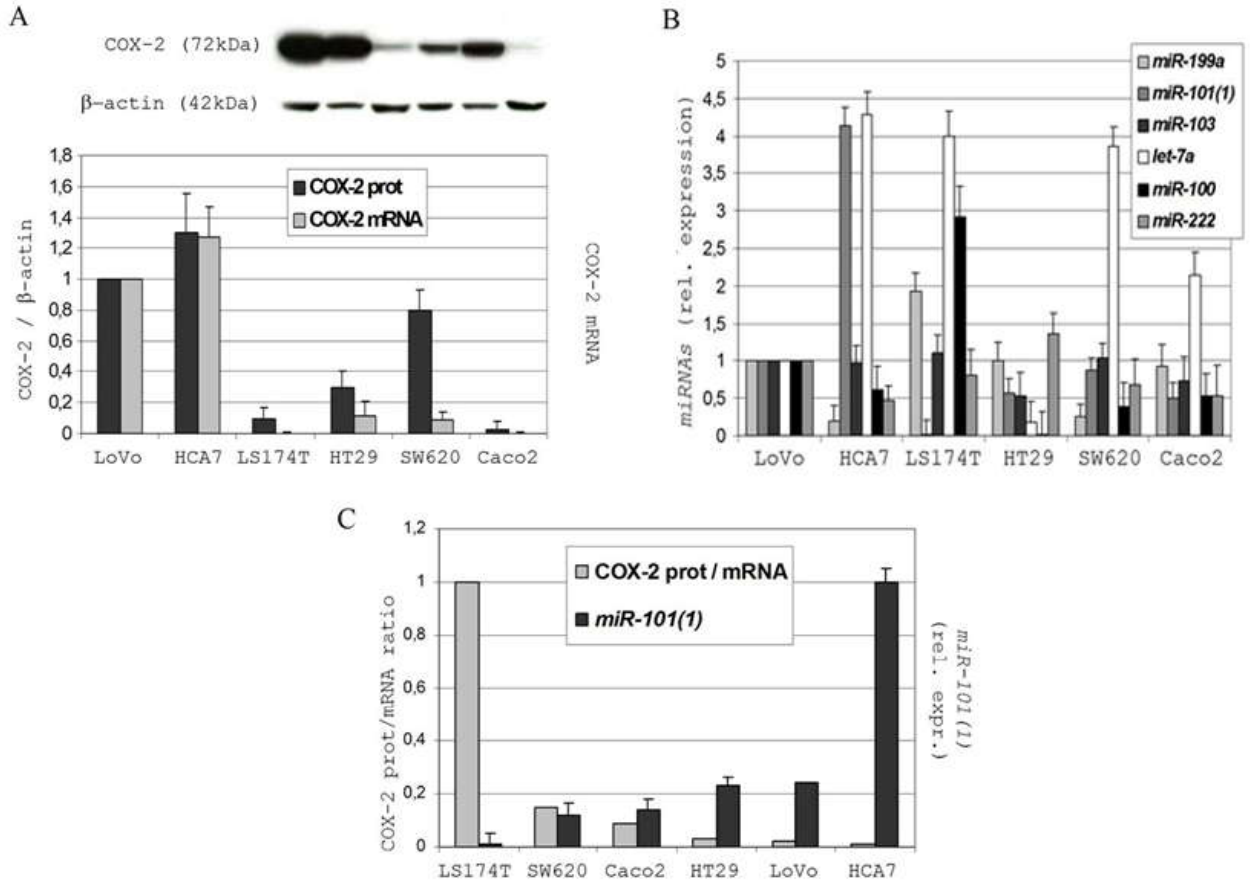


Figure 1. COX-2 and selected miRNAs expression in six colon cancer cell lines. (**A**) COX-2 protein (dark bars) and mRNA (light bars) levels were analyzed in six colon cancer cell by Western Blot and Real-Time PCR. COX-2 protein expression was normalized against β-actin protein and COX-2 mRNA expression was normalized against GUSB mRNA. (**B**) selected miRNAs expression was evaluated in the same cell lines samples by using Real-Time PCR and normalized against U6 RNA levels. (**C**) COX-2 protein / mRNA ratio (light bars) was taken as an index of COX-2 mRNA silencing and it was compared to *miR-101(1)* expression (dark bars) for the six colon cell lines. In all panels, data show the relative expression of each sample compared to the reference sample (LoVo cell line) and represent the mean ± SEM of three independent experiments.

TABLE 1

miRNA[1]	PicTar Score[2] (kcal/mol)	MiRanda Score[3] (kcal/mol)	Target Scan Score[4]	miRNA Target (hits)[5]	HCT-116 / Caco-2 (miR expr.)[6]	HCT-116 / HT-29 (ΔCt)[7]
hsa-miR-199a	7.6 (-21.9)	114 (-17.3)	-	0	0 / 0	-16.6 / -16.8
hsa-miR-101	2.2 (-23.8)	111 (-14.4)	0.14	1	229 / 24	-22.2 / -21.7
hsa-miR-103	0	123 (-18)	-	0	285 / 238	-13.1 / -12.9
hsa-let-7a	0	121 (-17.1)	-	0	92 / 3	-12.4 / -22.4
hsa-miR-100	0	103 (-16.1)	-	0	20 / 0	-11.7 / -21
hsa-miR-222	0	116 (-24.2)	-	0	242 / 22	-13.3 / -17.5

Table 1. Selection of six anti-COX-2 microRNAs candidates.

Six human microRNAs were selected as possible silencers of COX-2 mRNA translation. Selection was carried out using four different bioinformatical analyses and basing on data collected from literature. Detailed informations are shown in Supplementary Materials, section 1.

FIGURE 2

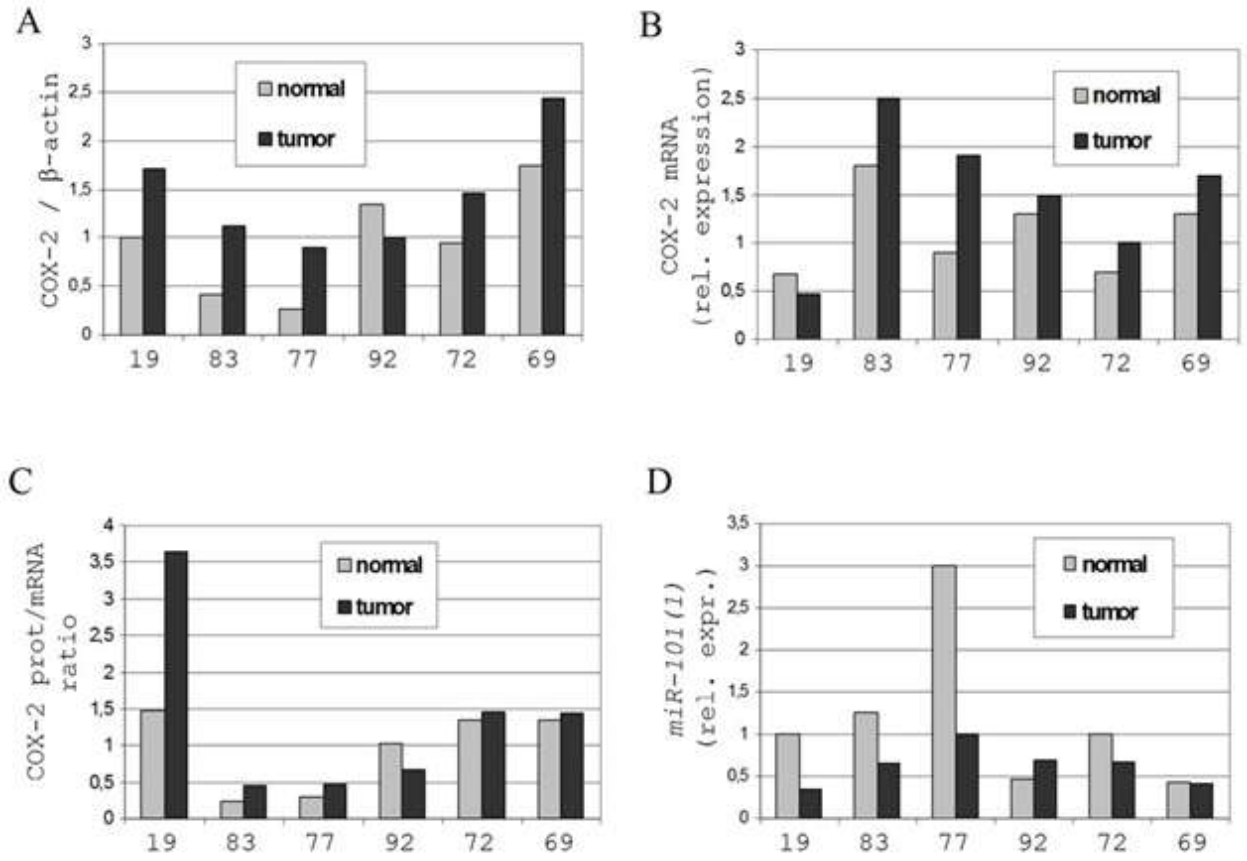


Figure 2. Correlation between COX-2 and *miR-101(1)* expression in CRC tissues. COX-2 protein and mRNA levels were analyzed in selected colon tissue samples from CRC patients by Western Blot and Real-Time PCR respectively. Densitometric analysis of COX-2 bands is available in Supplementary Materials, section 2. Data were normalized against β-actin protein and mRNA expression. For each patient, COX-2 protein (**A**) and mRNA (**B**) were evaluated in both normal (light bars) and tumor (dark bars) colon tissue. COX-2 protein / mRNA ratio (**C**) was analyzed and compared to *miR-101(1)* expression (**D**).

FIGURE 3

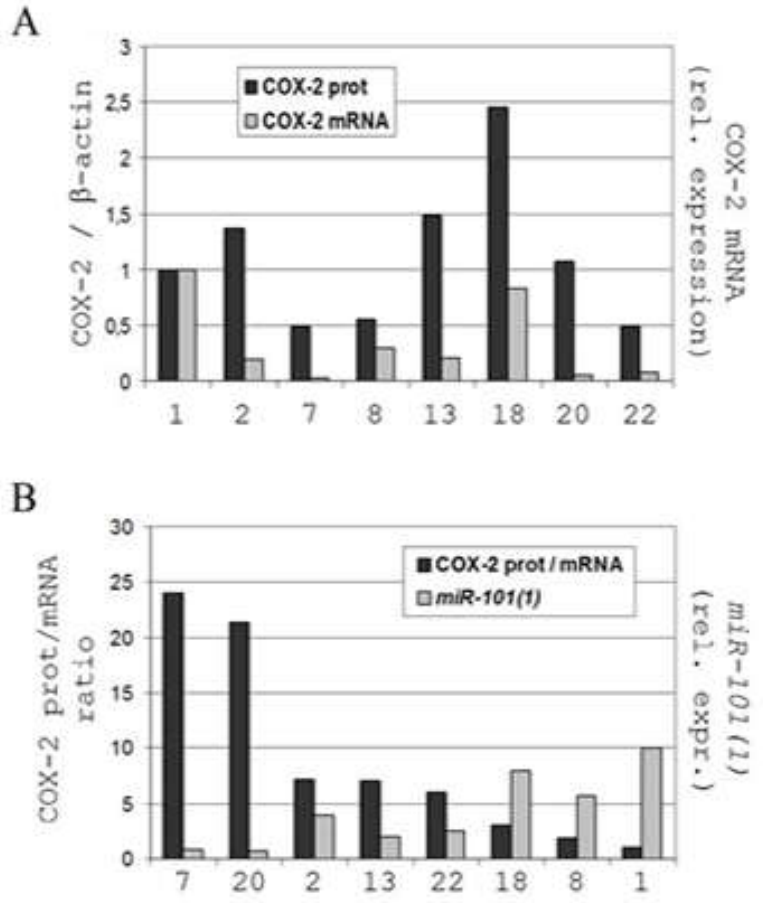


Figure 3. Correlation between COX-2 and *miR-101(1)* expression in CRC metastases. COX-2 protein and mRNA levels were analyzed in selected metastasis samples from CRC patients by Western Blot and Real-Time PCR respectively (**A**). Densitometric analysis of COX-2 bands is available in Supplementary Materials, section 2. Data were normalized against β-actin protein and mRNA expression. For each patient, COX-2 protein / mRNA ratio was analyzed and compared to *miR-101(1)* expression (**B**).

FIGURE 4

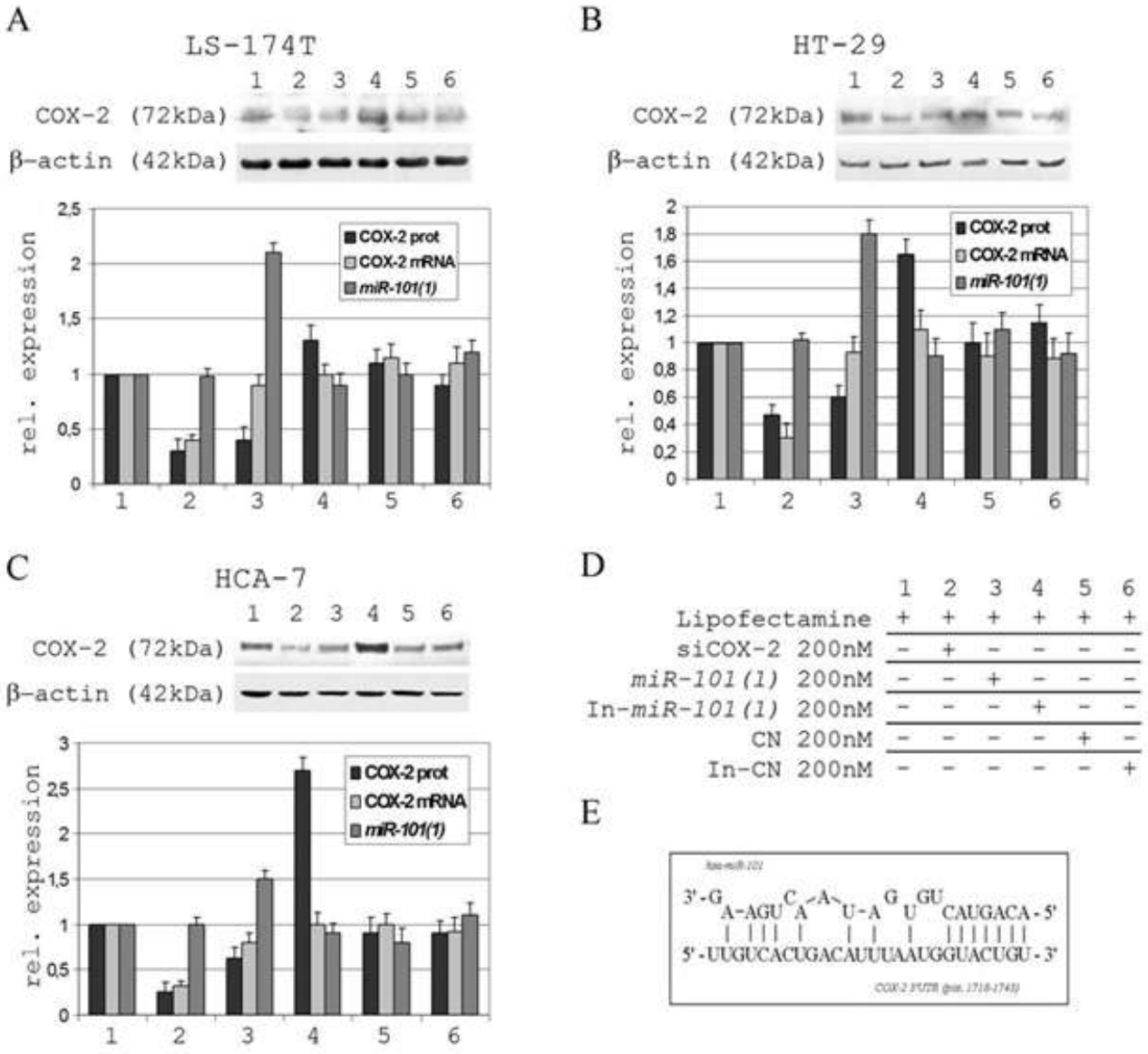


Figure 4. *miR-101(1)* regulates COX-2 expression in colon cancer cell lines. COX-2 regulation mediated by *miR-101(1)* was evaluated in three different colon cancer cell lines: LS-174T (**A**), HT-29 (**B**) and HCA-7 (**C**). Cell were transfected with: siRNA anti-COX2 (siCOX-2 positive control, lane 2); *miR-101(1)* (lane 3); *miR-101(1)* inhibitor (lane 4); miR-negative control (lane 5); miR-negative control inhibitor (lane 6). All treatments are described in **D**. COX-2 protein (dark bars) and mRNA (light bars) levels and *miR-101(1)* (medium bars) expression were analyzed by Western Blot and Real-Time PCR 48h after transfection and referred to the negative control sample in lane 1. Data represent the mean ± SEM of three independent experiments. **E**: putative binding site of *miR-101(1)* in COX-2 3'-UTR region as detected by MiRanda software.

FIGURE 5

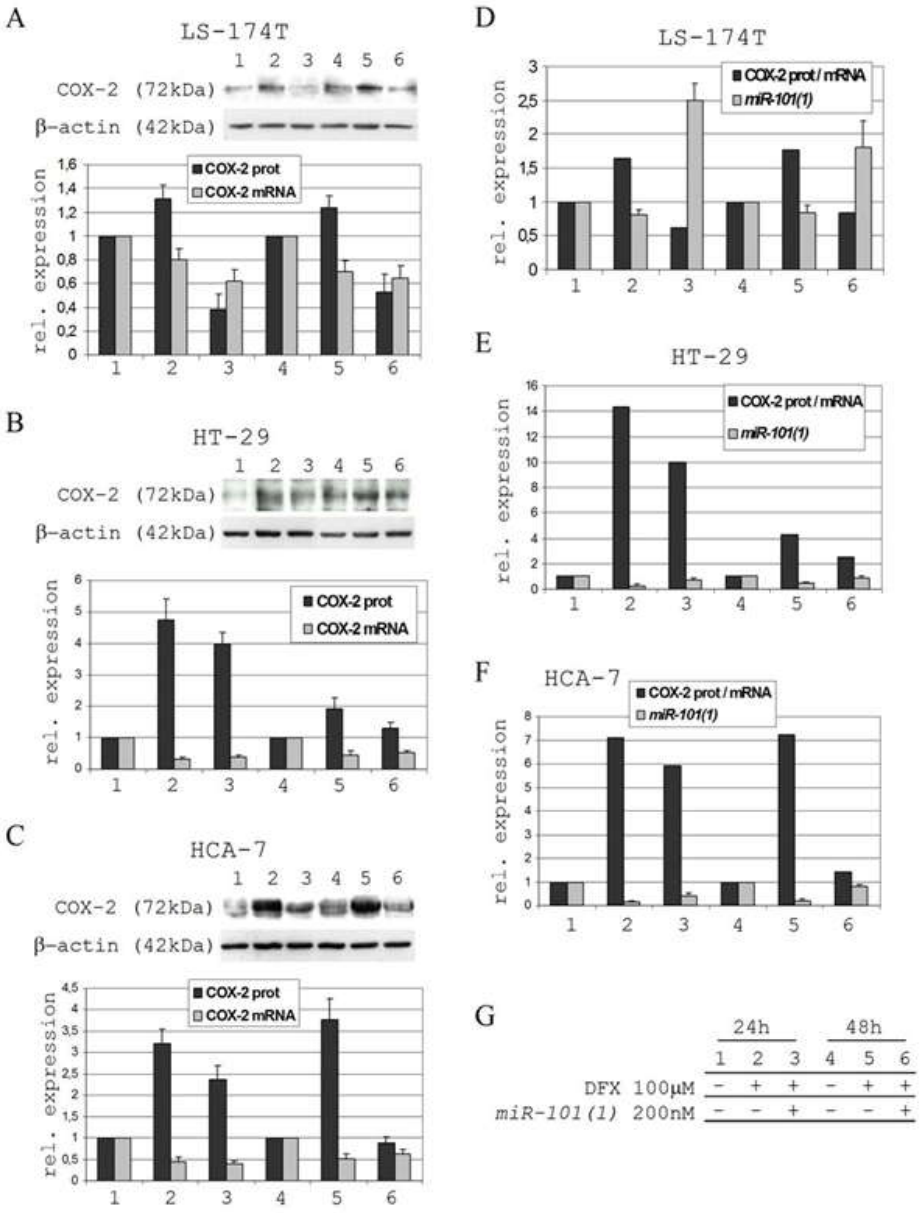


Figure 5. *miR-101(1)* downregulation is involved in COX-2 overexpression induced by hypoxia. *miR-101(1)* expression regulated by hypoxia was investigated in three different colon cancer cell lines: LS-174T (**A, D**), HT-29 (**B, E**) and HCA-7 (**C, F**). Cell were treated with desferrioxamine 100 μM (DFX , lanes 2, 3, 5 and 6) and transfected with *miR-101(1)* (lanes 3 and 6). **A**, **B** and **C**: COX-2 protein (dark bars) and mRNA (light bars) levels were analyzed by Western Blot and Real-Time PCR respectively 24 h and 48 h after transfection and referred to samples in lane 1 and 4. **D**, **E** and **F**: COX-2 protein / mRNA ratio (dark bars) was compared to *miR-101(1)* levels (light bars) analyzed with Real-Time PCR. Expression data refer to samples in lane 1 and 4. All treatments are described in **G**. Data represent the mean ± SEM of three independent experiments.

Discussion part 2: *miR-101* regulates COX-2 in cancer cells

As each microRNA (miRNA) is expected to regulate the translation of up to 100 mRNAs (Lim et al., 2005) it is clear that disturbances of the miRNA expression level (Calin et al., 2004), processing of the miRNA precursors (Sugito et al. ,2006), or mutations in the sequence of the miRNA, its precursor, or its target mRNA (Iwai and Naraba, 2005), may have detrimental effects on cell physiology. There is now ample evidence that the expression of miRNAs is altered in cancer, and that certain changes may be directly implicated in the carcinogenic process. A number of miRNAs have been shown to promote cell proliferation and survival, while others diminish cell proliferation and survival. These two classes of miRNAs may play a central role in cancer development as novel oncogenes and tumor suppressors, respectively. In general, the majority of miRNAs are downregulated in cancer specimens (Lu et al., 2005). In normal tissues, some of these miRNAs have been documented to inhibit the translation of proto-oncogenes by targeting the 3'ends of their mRNAs. Such miRNAs are therefore considered as "tumor suppressor miRNAs" (TS-miRs) since their normal function is to control the expression of an oncogene (Calin et al., 2002). Conversely, certain miRNAs seem to be upregulated in cancer and may act as "oncomiRs" since they can enable the downregulation of a tumor suppressor (O'Donnell et al., 2005; Chan et al., 2005).

Components of the miRNA/RNAi pathway may be altered during the progressive development of colorectal cancer (CRC) as well. Michael et al., (2003) firstly demonstrated a reduced accumulation of *miR-143* and *miR-145* in colorectal neoplasia. They supposed that altered accumulation of these mature miRNAs may reflects early changes in the cellular composition of tumors, compared with normal mucosae. Moreover, *hsa-miR-342* showed an anti-apoptotic effect in the colorectal cancer cell line HT-29, suggesting that this microRNA could function as a proapoptotic tumor suppressor (Grady et al., 2008). On the contrary, many other studies were carried out to find out an upregulation in miRNA expression patterns in CRC. In particular, *hsa-miR-200c, hsa-let-7g* (Nakajima et al., 2006), various members of the oncogenic miR-17-92 family (Dews et al., 2006; Lanza et al., 2007), *miR-34a* (Tazawa et al., 2007), *miR-21* (Tanzer and Stadler, 2004; Chan et al., 2005; Iorio et al., 2005; Si et al., 2007) and other miRNAs as *miR-20a miR-106a*, *miR-181b*, *miR-203* (Schetter et al., 2008) were find to be significantly overexpressed in the colorectal tumor samples compared to the corresponding normal, acting as oncomiRs.

Basing on the data from literature, the first aim of this second part of my research was to elucidate a possible relation between cyclooxygenase-2 (COX-2) overexpression in CRC and altered miRNA

expression patterns. Since *Cox-2* gene expression, as for other human genes, could be modulated by the machinery of RNAi, the hypothesis was that overexpressed COX-2 protein in colon cancer cells could be the result of decreased levels of one or more tumor suppressor miRNAs. In this thesis, I clearly show an inverse correlation between COX-2 expression and the human *miR-101(1)* levels in colon cancer cell lines, tissues and metastases. Moreover, I demonstrate that the *in vitro* modulating of *miR-101(1)* expression in colon cancer cell lines leads to significant variations in COX-2 expression. In these experiments, an important parameter was considered as an index of COX-2 silencing mediated by miRNA: the COX-2 protein / mRNA ratio was compared to the expression profile of *miR-101(1)* in each sample analyzed. Since the most common mechanism of gene silencing mediated by miRNA is based on the translational repression of cellular mRNAs (Pillai et al., 2005; Nottrott et al., 2006), the assumption was that high levels of miRNA, *miR-101(1)* in my case, should correspond to low values of COX-2 protein / mRNA ratio and lower levels of *miR-101(1)* should correspond to higher levels of the same ratio. miRNAs can also induce a rapid degradation of the mRNAs target, as in the small interfering RNA (siRNAs) pathway, if a perfect base complementarity exists between miRNA and mRNA (Bagga et al., 2005). Nevertheless, it can be excluded that *miR-101(1)* silences COX-2 messenger by this latter mechanism. In fact, analyzing the putative binding site of *miR-101(1)* in COX-2 3'-UTR region as detected by predictive software, there is not a complete pairing of *miR-101(1)* on COX-2 mRNA (Figure 4E).

The starting point of my research was the selection of possible miRNA candidates for the COX-2 silencing. After a computational analysis of all the known human miRNAs and the COX-2 mRNA 3'UTR region, the expression profile of six selected miRNAs (*miR-199a, miR-101-1, miR-101, let-7a, miR-100* and *miR-222*) was analyzed in six colon cancer cell lines (LoVo, HCA-7, LS-174T, HT-29, SW620 and Caco-2). Surprisingly, *miR-101(1)* levels showed a perfect inverse correlation with the COX-2 protein / mRNA ratio of these cells. In order to demonstrate a direct regulation of COX-2 protein expression mediated by *miR-101(1)*, further *in vitro* tests were carried out in LS-174T, HT-29 and HCA-7 cells. In all the three cases, the transfection of synthetic exogenous *miR-101(1)* or its inhibitor, induced a significant silencing or upregulation of COX-2, respectively. It must be notice that the highest level of COX-2 silencing (induced by transfected *miR-101-1*) and the lowest level of COX-2 upregulation (induced by transfected *miR-101-1* inhibitor) were recorded in LS-174T cells that normally express the lowest level of endogenous *miR-101(1)*, compared to the other cell lines. On the contrary, the lowest level of COX-2 silencing and the highest level of COX-2 upregulation were recorded in HCA-7 cells that normally express the highest level of *miR-101(1)*. All these findings demonstrate that *miR-101(1)* regulates COX-2 expression in human cells. However, it is not to be excluded that *miR-101(1)* could regulate COX-2 expression not directly, but

through a silencing mechanism acting on other cellular factors involved in COX-2 regulation. To discard this last hypothesis, I performed a Luciferase assay in which *miR-101(1)* "seed" region of COX-2 3'UTR mRNA was cloned downstream the luciferase gene in pGL3.basic reporter plasmid. Transfection of both plasmid and *miR-101(1)* in LS-174T determined a decreased amount of luciferase protein production (compared to positive and negative controls), showing the evidence of a direct interaction between *miR-101(1)* and COX-2 mRNA (preliminary data, not shown). These data are supported by the work of Chakrabarty et al. (2007) that recently demonstrated that two miRNAs, *mmu-miR-101a* and *mmu-miR-199a**, are spatiotemporally expressed in the mouse uterus during implantation coincident with expression of COX-2, critical for implantation. The *in vitro* gain- and loss-of-function experiments showed that COX-2 expression is posttranscriptionally regulated by these two miRNAs.

It is well known that COX-2 is overexpressed in colorectal cancer (Sinicrope et al., 1999) and this contributes to an increased apoptosis resistance (Moss et al., 1996) and invasiveness (Tsujii et al., 1997) of the tumor cells and an increased tumor angiogenesis (Tsujii et al., 1998) in colon tissues. Since *miR-101(1)* was found to regulate COX-2, I started to investigate the role of *miR-101(1)* in colon tumorigenesis. The analysis of COX-2 protein-mRNA and *miR-101(1)* levels in different colon tissues (normal vs. tumor) and metastases from patients affected by CRC, confirmed the inverse correlation between COX-2 and *miR-101(1),* previously described in the *in vitro* experiments. Combining together data collected *in vitro* and ex vivo, I can show evidences that, not only *miR-101(1)* regulates COX-2 in colon cancer cell lines but, more interestingly, alterations of its expression profile could be involved in colorectal cancer growth and development.

The main question now is: if COX-2 overexpression depends on a *miR-101(1)* expression impairment, who controls *miR-101(1)* repression in the CRC pathology? I will try to answer to this question carrying on my personal research on RNAi, COX-2 and colon cancer and some data were already collected. In particular, I started to investigate *miR-101(1)* regulation in the hypoxic environment.

Adaptation to hypoxia is critical for tumor cell growth and survival and is achieved largely by transcriptional activation of genes that facilitate short- and long-term adaptive responses (Semenza, 2002). Hypoxia induces COX-2 in colorectal tumor cells and this upregulation is mediated directly by hypoxia-inducible factor 1 (HIF-1) (Kaidi et al. (2006). An hypoxic environment can be induced *in vitro* treating the cells with the iron chelator desferrioxamine (DFX) (Woo et al. 2006). For this reason I decided to analyze the effect of hypoxia on *miR-101(1)* expression and a great enthusiasm derived from this sequence of experiments. In fact, treating LS-174T, HT-29 and HCA-7 colon

cancer cell lines with DFX, I observed a strong decrease in *miR-101(1)* levels, and this phenomenon was always coupled to a COX-2 overexpression. Moreover, COX-2 overexpression DFX-induced was completely abrogated when DFX treated cells were also transfected with synthetic *miR-101(1)*. Even if further investigations are needed, I can conclude that hypoxia-induced COX-2 overexpression, fundamental for cancer cells survival *in vivo*, could be also connected with the RNAi mechanism mediated by *miR-101(1)*.

In conclusion, in this second part of my thesis I demonstrated that COX-2 regulation can be directed by the mechanism of RNA Interference, through the action of human *miR-101(1)*. *miR-101(1)* silences COX-2 *in vitro*, and this evidence was found in LS-174T, HT-29 and HCA-7 colon cancer cell lines. Moreover, a reduced accumulation of *miR-101(1)* was found both in tissues and metastases samples derived from patients affected by colorectal cancer. As a consequence, *miR-101(1)* deregulation in cancer tissues could represent one of the main causes underlying COX-2 enzyme overexpression in CRC. Finally, preliminary studies pointed out that *miR-101(1)* impairment could be a consequence of the adaptation of cancer cells to the hypoxic environment that strongly characterize CRC tissues.

Chapter VI

Materials and Methods

Cell Lines. HUVE cells were isolated from freshly collected umbilical cords. Cells were grown in M199 medium supplemented with 20% foetal calf serum, 100 mg/ml ECGS, 100 mg/ml heparin, 2 mM L-glutamine and antibiotics (penicillin 100 U/ml and streptomycin 100 mg/ml. Cells were maintained at 37° C in 5% CO_2 and used for experiments between 3rd and 5th passage. HT-29, SW-620 and LoVo (S, doxorubicin-sensitive) human colon cancer cells were obtained from American Type Culture Collection (Manassas, VA, USA). HCA-7, Caco-2 and LS-174T cells were obtained from European Collection of Cell Cultures (ECACC; Salisbury, Wiltshire, UK). All the cell lines were cultured at 37°C in 5% CO2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated Foetal Calf Serum (FCS), 2 mM L-glutamine, penicillin 100 U/ml and streptomycin 100 mg/ml. FCS and DMEM were purchased from Cambrex Biowittaker, USA. All the other reagents were purchased from Sigma (Sigma Chemical, St. Louis, USA).

Synthetic siRNAs Design. Four different siRNAs against COX-2 mRNA were designed as suggested by Elbashir and collaborators (2002) and chemically synthesized (PROLIGO Primers and Probes, USA). The identified target sequences on COX-2 mRNA (NM000963) were respectively: bases 290-310 (siRNA-A, 5'aaactgctcaacaccggaatt3'), bases 291-311 (siRNA-B, 5'aactgctcaacaccggaattt3'), bases 1020-1040 (siRNA-C, 5'aacagagtatgcgatgtgctt3') and bases 1429-1449 (siRNA-D, 3'aagtatcacaggcttccattg5'). We also used a scrambled siRNA (Ambion, USA) as a negative control, with no significant homology to any known gene sequences in human, mouse and rat genome. All siRNA sequences were controlled for their specificity by using BLAST database and did not show any homology to other human gene.

siRNAs Transfection. HUVE cells were seeded in 25 cm^2 flasks (150.000 cells/flask) and grown up to 50% confluence. After 24 h, cells were transfected with siRNAs by using Oligofectamine reagent (Invitrogen, USA) according to the manufacturer's instructions. Briefly, each siRNA (400 fmoles) was diluted in 350 μl of serum-free medium. For each flask, 10 μl of Oligofectamine

reagent was incubated with 40 μl of serum-free medium for 10 minutes at room temperature. 50 μl of Oligofectamine solution were added to diluted siRNAs. After incubation for 20 minutes at room temperature, siRNAs-Oligofectamine complexes were added to flasks containing 1.6 ml of serum-free medium, resulting in 2 ml of total transfection volume and in a siRNA final concentration of 200 pM. After 4 h of incubation at 37°C, 3 ml of complete medium containing FCS and phorbol 12-myristate 13-acetate (PMA) was added (final concentration: 10% serum and 40 nM PMA).

Western Blot. Cells were scraped and lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 100 mM NaCl, 1% Triton X-100 and protease inhibitors mixture), additionated with 5 mM NaF, 1 mM Na_3VO_4, 10 mM β-glycerolphosphate, for the phospho-STAT-1 immunoblotting experiments. Cell lysates were incubated 1 h on ice and centrifuged at 12000g to collect supernatants. Protein concentration in supernatants was evaluated by using the Lowry method. After addition of SDS-PAGE sample buffer and boiling, 40 μg of denatured proteins were separated in 12% SDS-PAGE and then transferred to nitrocellulose papers. After the blotting, nitrocellulose papers were incubated with specific antibodies. The primary antibodies used were: polyclonal anti-COX-2 (Cayman Chemicals, USA), anti-phospho-STAT-1[Tyr701] (Cell Signaling, USA) and anti-β-actin (Sigma, USA); monoclonal anti-COX-1 (Cayman Chemicals, USA) and anti-STAT-1 p84/p91 (Santa Cruz Biotechnology, USA). Secondary antibodies (HRP-conjugated) were purchased from Santa Cruz Biotechnology, USA. Immunolabelling was visualised by using the ECL procedure (Amersham Biosciences, USA). Bands were quantified by using a densitometric image analysis software (Image Master VDS, Pharmacia Biotech, Uppsala, Sweden). Normalization was made against β-actin expression.

Determination of COX-2 Activity. Conditioned media from HUVE cells were collected 48 h after transfection with siRNAs and stored at -80°C. COX-2 enzymatic activity was evaluated by measuring the 6-keto-PGF1α release in conditioned media by using an ELISA assay (Assay Designs, USA). 6-keto-PGF1α production was then related to the protein concentration in cell lysates. PGE2 levels in HT29 culture media were evaluated by using an ELISA assay (Cayman Chemicals, USA) and related to the protein concentration in cell lysates.

In Vitro Angiogenesis Test. *In vitro* angiogenesis was evaluated by seeding HUVE cells on a 3-D collagen gel in a 24-well plate and transfecting them with siRNAs. Briefly, collagen gel was prepared by adding 8 volumes of collagen solution (3 mg/ml, Roche Applied Science, USA) to 2 volumes of a mixture containing M199 5X, HEPES 0.02 M and $NaHCO_3$ 7.5 mg/ml. After pH adjusting to 7.2-7.4, the mixture was quickly dispensed to the wells and gelification was achieved at 37°C. After gelification, wells were washed twice with M199. HUVE cells ($5x10^4$/well) were seeded onto the gels and then transfected with siRNAs (final concentration 200 pM) by using Oligofectamine reagent (Invitrogen, USA) according to the manufacturer's instructions. *In vitro* capillary-like formation was stimulated with 40 nM PMA, examined 48 h after transfection by using a phase contrast microscope and the number of tubular capillary-like structures per well was counted.

Cloning anti-COX-2 short hairpin RNA (shCOX-2) into pSUPER.retro Vector. Constructs, coding for anti-COX-2 shRNA, were prepared as described by Brummelkamp *et al* (2002a). pSUPER.retro vector (Oligoengine, Seattle, WA, USA), based on the murine stem cell virus (MSCV) genome, was a kind gift of Dr. P. Chieco (C.R.B.A. lab, Bologna, Italy). Forward and reverse sequences for sh(COX-2) construct were 5'-gatccccaactgctcaacaccggaatttcaagagaattccggtgttgagcagtttttttggaa-3' and 5'-agcttttccaaaaaaactgctcaacaccggaattctcttgaaattccggtgttgagcagttggg-3', respectively, as shown in Figure 1. 64 nt-containing oligos were synthesized and purchased from PROLIGO (USA). Sequence design started from the most effective anti-COX-2 synthetic siRNA (siCOX-2, sequence B). Steps for cloning oligonucleotides into pSUPER.retro vector were made accordingly to pSUPER RNAi system protocol (www.oligoengine.com). Forward and reverse oligonucleotides were annealed to form a duplex. The annealed oligos were then ligated into the BglII–HindIII cleavage site within the pSUPER.retro vector pre-linearized with the same restriction enzymes. Recombinant vector containing inserts was transformed into competent *E. coli* cells. After selection in ampicillin-containing medium, colonies were recovered and checked for the presence of recombinant pSUPER.retro vector.

Virus Production and Cell Infections. Anti-COX-2 pSUPER.retro vector was transfected into Phi-NX ("Phoenix") packaging cell line kindly provided by Dr. P. Chieco (C.R.B.A lab, Bologna, Italy) to produce ecotropic retroviral supernatants. Phoenix cells were cultured in Dulbecco's modified

Eagle's medium (DMEM) supplemented with 10% foetal calf serum and pre-treated with Chloroquine at final concentration of 25 μM. The day before transfection, Phoenix cells were seeded in 10 cm dishes ($3x10^6$ cells/dish) in order to reach 60% confluence at the time of transfection. Cells were transfected with 10 μg of viral vector DNA by using calcium-phosphate precipitation method. 48 h after transfection, culture medium was filtered through a 0.45 μm filter and the viral supernatant was used for HT29 cells infection after addition of 8 μg/ml of polybrene (Sigma, USA). After infection, HT29 cells were incubated at 32°C in 5% CO_2 for 6 h. Then medium was changed with fresh medium and HT29 cells were allowed to recover for 48 h at 37°C in 5% CO_2. Infection efficiency was examined under a fluorescence microscope to check the GFP expression. Infected cells were selected by adding puromycin (1 μg/ml) for 48 h to the culture medium. COX-2 expression in HT29 wild type cells and in HT29 pSUPER(+) clones (producing anti-COX-2 shRNA) was analyzed by Western Blot and by Real-Time Polymerase Chain Reaction, as described in this section. The same procedure was used to infect HUVE, HCA7 and HeLa cells and to obtain HT29 pSUPER(-) clones infected with empty vector, not expressing anti-COX-2 shRNA. Infection efficiencies were evaluated by using confocal microscopy analysis.

RNA Extraction and Real-Time PCR. Total RNA was extracted using Eurozol reagent (CELBIO, Milan, Italy) according to the manufacturer's instructions. RNA was quantified by spectrophotometry and analysed by electrophoresis on 1% agarose/formaldehyde denaturing gel to exclude the presence of RNA degradation. Extracted RNA samples were then treated with DNase I, to remove any genomic DNA contamination, by using DNA-*free* kit (Ambion, USA) and reverse-transcripted by using RevertAid™ First Strand cDNA Synthesis Kits (Fermentas, Canada). miRNAs were reverse-transcribed with stem-loop RT-PCR technique (Chen et al. 2005). COX-2 mRNA and miRNAs levels were analyzed by Real-Time PCR by using SYBR supermix kit and a Bio-Rad iCycler system (Bio-Rad, USA) according to the manufacturer's instructions. The melting curve data were collected to check PCR specificity. Each cDNA sample was analysed as triplicate and corresponding samples with no cDNAs were included as negative controls. COX-2 mRNA levels were normalized against GUSB mRNA while miRNAs expression was normalized against RNA U6 levels. All relative expressions were calculated by using Ct values and all primers and parameters for RT-PCR and Real-Time PCR are summarized in Supplementary Materials, section 3.

MTT Assay. HT29 cells [wild type, pSUPER(-) and pSUPER(+)] were seeded in 24-well plates ($1x10^4$ cells/well) and the MTT assay was performed in triplicate at days 2, 5, 8, 12, 15 and 20. Briefly, medium was replaced with fresh complete medium (450 µl). 50 µl of PBS containing 5 mg/ml MTT (Sigma, USA) was added to each well. In the absence of light, samples were incubated for 2 hours and precipitates were resuspended by adding 100 µl of 10% SDS solution to each well. Absorbance was measured spectrophotometrically on a plate reader (Bio-Rad, USA) at 570 nm.

BrdU Labelling Index. The cell cycle distribution of HT29 cells [wild type, pSUPER(-) and pSUPER(+)] was evaluated by using a cytofluorimeter. Briefly, $1x10^6$ cells were seeded in complete medium. After 24 h from seeding, cell cultures were incubated with 10 µM BrdU (Sigma, USA) for 1 h in a CO_2 atmosphere at 37°C. Harvested cells were fixed in 70% ethanol for 30 min. After DNA denaturation with 2N HCl for 30 min at room temperature, cells were washed with 0.1M $Na_2B_4O_7$ (pH 8.5). Cells were then processed for indirect immunofluorescence staining, using α-BrdU (Becton Dickinson, Milan, Italy) diluted 1:4 as a primary MAb, and stained with 20 µg/ml Propidium Iodide before flow cytometry analysis (FACSCalibur, Becton Dickinson, Milan, Italy).

Cell Migration Assay. Migration assay was performed by using Boyden chambers (New Technologies Group, Italy) with 8-µm pore polycarbonate membranes (New Technologies Group, Italy). Membranes were coated with Matrigel (Sigma, USA) at 40-fold dilution. Assay was performed using fresh DMEM supplemented with 10% heat-inactivated FCS as chemoattractant agent. HT29 cells [wild type, pSUPER(-) and pSUPER(+)] were added into the upper chamber at high density ($500x10^3$ cells) either in the absence or presence of PMA 40 nM stimulation and then incubated for 24 hours at 37°C. Following incubation, membranes were disassembled and nonmigratory cells on the upper surface of the membrane were wiped with a cotton swab. Cell invasion was determined by counting under light microscopy the number per optical fields (200X magnification) of the cells that migrated to the lower side of each membrane, after fixing and staining membranes with 2% Toluidine Blue.

Soft-Agar Colony Formation Assay. Anchorage-independent growth was determined in 0.33% agarose (SeaPlaque, FMC BioProducts Rockland, ME, USA). HT29 cell suspensions [wild type, pSUPER(-) and pSUPER(+); 1000 cells/sample] were plated in a semisolid medium (DMEM

supplemented with 10% FCS and 0.5% agar). Dishes were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and colonies were counted (under light microscopy) after 7 days.

Confocal Microscopy and Immunofluorescence Analysis. To visualize the localization of phospho-STAT-1 (Tyr701) protein, HUVE and HT29 cells were grown on cover slides, washed in TBS buffer, fixed with 4% paraformaldehyde, permeabilized with PBS-Triton X-100 0.1% and quenched with cold 0.1% sodium borohydride in TBS. Cells were treated with blocking buffer (PBS containing 10% horse serum and 1% BSA) and then incubated with phospho-STAT-1 (Tyr701) primary antibody diluted 1:100 in TBS-BSA 1%. After washings, cells were incubated with secondary anti-rabbit TRITC conjugated antibody (Dako, Denmark) diluted 1:50 in TBS-BSA 1%. Finally, slides were washed and mounted in glycerol-PBS medium containing 50 mg/ml DABCO. To evaluate pSUPER.retro infection system efficiency, cells (HUVE, HT29, HCA7 and HeLa) were seeded on cover slides 24 h after infection, fixed with 4% paraformaldehyde and permeabilized with PBS-Triton X-100 0.1%. Nuclei were stained with propidium iodide 0.05 μg/ml and slides were mounted, after washings, in glycerol-PBS medium containing 50 mg/ml DABCO. The imaging was performed on a confocal microscope (Leica, Germany) equipped with an argon/krypton laser. Optical sections were obtained at increments of 0.5 μm in the Z-axis and were digitized with a scanning mode format of 512 x 512 pixels. The image processing and the volume rendering were performed using the Leica TCS software.

Production of pS(COX2) and pS(TBE) Vectors. Basic pSUPER.retro vector, not expressing shCOX-2 [pS(-)] was modified as follow: after digestion of pS(-) with HindIII and SalI restriction enzymes, SV40 polyA signal sequence derived from pSG5-HER plasmid was introduced. To obtain pS(COX2) vector, entire human Cox-2 promoter sequence (derived from HCA-7 genomic DNA) was firstly subcloned with shCOX-2 sequence in pcDNA-3.1(-) plasmid (Invitrogen, USA) between ApaI and BamHI restriction sites and then the whole sequence was cloned in pS(-)+SV40polyA after digestion with ApaI and BglII restriction enzymes. To obtain pS(TBE) vector, a promoter containing two sets (with the second set in the reverse orientation) of three copies of the TCF binding element (TBE, derived from TOPFlash plasmid, Upstate, USA) was subcloned with shCOX-2 sequence in pcDNA-3.1(-) plasmid between ApaI and BamHI restriction sites and then the whole sequence was cloned in pS(-)+SV40polyA after digestion with ApaI and BglII restriction

enzymes. All primers used for SV40 polyA signal, Cox-2 promoter and TBE promoter amplification and cloning are shown in Supplementary Materials, section 4.

pSUPER.retro Vectors Transfection. HT-29 and HCA-7 cells were seeded in a 6-wells plate ($8x10^5$ and $4x10^5$ cells/well) at 60% confluence. After 24 h, cells were transfected with by using Lipofectamine 2000 transfection reagent (Invitrogen, USA) according to the manufacturer's instructions. Briefly, 2 μg of each pSUPER.reto vector [pS(-), pS(H1), pS(COX2) and pS(TBE)] was diluted in 400 μl of serum-free medium. For each well, 9 μl of Lipofectamine was incubated with 400 μl of serum-free medium for 5 minutes at room temperature. Lipofectamine solution was added to diluted vectors. After 20 minutes of incubation at room temperature, DNA-Lipofectamine complexes were added to each well. After 6 h of incubation at 37°C, transfection medium was replaced with 1 ml of complete medium containing 10% FCS and 2 μg/ml puromycin. After 48 h transfected cells were lysated for Western Blot and Real-Time PCR analyses.

tkRNAi Infections. *E. Coli* cells were co-transformed with both pGB2Ω*inv-hly* plasmid (kindly gift of prof. Courvalin, Pasteur Institute, Paris) and pSUPER.retro vectors. After selection with ampicillin 100 μg/ml and streptomycin 25 μg/ml, four different *E. Coli* strains were created, each containing pGB2Ω*inv-hly* plasmid and one of the four different forms of pSUPER.retro vector previously described. HT-29 and HCA-7 cells were seeded in a 6-wells plate ($8x10^5$ and $4x10^5$ cells/well) at 50% confluence. After 24 h, cells were infected with engineered *E. Coli*, previously grown o.n. in LB medium at 37°C, diluted in cells medium 1:1250. After 3 h incubation at 37°C, 5% CO_2 cells were washed and fresh complete medium containing antibiotics was replaced. After 18 h of incubation at 37°C, puromycin was added to the medium, 2 μg/ml final concentration. After 24 h infected cells were lysated for Western Blot and Real-Time PCR analyses.

Patients and CRC Tissues. Formalin-fixed, paraffin-embedded samples from 14 CRC patients were used in this investigation. Tissue samples frozen in liquid nitrogen, immediately after surgical procedure, were used for Western Blot and Real-Time PCR analyses.

miRNAs Transfections. *miR-101(1)* (5'-uacaguacugugauaacugaa-3'), miR-negative control (cel-miR-67: 5'-ucacaaccuccuagaaagaguaga-3') and their inhibitors used for transfection were purchased from Dharmacon (Lafayette, CO, USA). Anti-COX-2 siRNA (siCOX-2, 5'-aactgctcaacaccggaattt-3') was purchased from PROLIGO Primers and Probes, USA. LS-174T, HT-29 and HCA-7 cells were seeded in a 12-wells plate ($4x10^5$ and $2x10^5$ cells/well) at 60% confluence. After 24 h, cells were transfected with by using Lipofectamine 2000 transfection reagent (Invitrogen, USA) according to the manufacturer's instructions. Briefly, miRNAs and siCOX-2 (80 pmoles) were diluted in 200 µl of serum-free medium. For each well, 4.5 µl of Lipofectamine was incubated with 200 µl of serum-free medium for 5 minutes at room temperature. Lipofectamine solution was added to diluted RNAs. After 20 minutes of incubation at room temperature, RNAs-Lipofectamine complexes were added to each well, with a RNA final concentration of 200 nM. After 6 h of incubation at 37°C, transfection medium was replaced with 1 ml of complete medium containing 10% FCS. After 48 h transfected cells were lysated for Western Blot and Real-Time PCR analyses.

Statistical analysis. Data were expressed as mean ± SEM. Differences were analysed by Student's *t* test and considered statistically significant at $P<0.05$ and $P<0.01$.

Supplementary Materials

Section 1.

(1) hsa-miRNAs sequences (http://microrna.sanger.ac.uk/sequences)

hsa-mir-199a: CCCAGUGUUCAGACUACCUGUUC

hsa-mir-101(1): UACAGUACUGUGAUAACUGAAG

hsa-mir-103: AGCAGCAUUGUACAGGGCUAUGA

hsa-let-7a: UGAGGUAGUAGGUUGUAUAGUU

hsa-mir-100: AACCCGUAGAUCCGAACUUGUG

hsa-mir-222: AGCUACAUCUGGCUACUGGGUCUC

(2) PicTar (http://pictar.bio.nyu.edu)

"Seed" sequence on: Homo sapiens prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) (PTGS2), **3'UTR** (pos. 1950-4465 from NM_000963**)**

- miR-199a: 1722

- miR-101(1): 1892

(3) MiRanda (http://www.microrna.org/miranda_new.html)

Alignments on: Homo sapiens prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) (PTGS2), **3'UTR** (pos. 1950-4465 from NM_000963**)**

- miR-199a: 1201-1232

- miR-101(1): 1718-1743

- miR-103: 1222-1247

- let-7a: 1215-1240

- miR-100: 2061-2087

- miR-222: 997-1021

General setting: score threshold = 80; energy threshold = -14 kcal/mol; scaling factor = 2

(4) Estimated false discovery rate (http://genes.mit.edu/tscan/ucsc.html)

(5) http://cbio.mskcc.org/cgi-bin/mirnaviewer/mirnaviewer.pl

(6) Expression level of the miRNAs detected by miRNA serial analysis of gene expression (miRAGE). Cummins et al. 2005 Table 2

(7) Data are presented as ΔCT [CT miRNA - CT U6 RNA]. Jiang et al. 2005.Supplemental Table 5.

Section 2.

Densitometric analysis of COX-2 and β-actin bands from Western Blot performed on CRC tissue and metastasis samples.

Tissue samples (N, normal; T, tumor):

Sample	19N	19T	83N	83T	77N	77T	92N	92T	72N	72T	69N	69T
COX-2	1,00	0,89	0,74	2,29	1,14	5,59	1,06	2,25	4,71	4,70	2,54	3,34
β-actin	1,00	0,52	1,76	2,03	4,23	6,21	0,79	2,25	5,01	3,22	1,45	1,37

Metastasis Samples:

Sample	1	2	7	8	13	18	20	22
COX-2	1,92	4,44	2,32	2,15	3,51	4,64	2,11	0,89
β-actin	6,68	11,16	16,56	13,71	8,07	6,58	6,89	6,48

Section 3.

Primers and parameters for RT-PCR

mRNA, miRNA	sequence
COX-2	5’-ctgatgcgtgaagtgctg-3’
GUSB	5’-gtatctctctcgcaaaaggaac-3’
U6	5’-aaaatatggaacgcttcacg-3’
hsa-miR-199a	5’-gtcgtatccagtgcagggtccgaggtattcgcactggatacgacgaacagg-3’
hsa-miR-101(1)	5’-gtcgtatccagtgcagggtccgaggtattcgcactggatacgaccttcagt-3’
hsa-miR-103	5’-gtcgtatccagtgcagggtccgaggtattcgcactggatacgactcatagc-3’
hsa-let-7a	5’-gtcgtatccagtgcagggtccgaggtattcgcactggatacgacaactata-3’
hsa-miR-100	5’-gtcgtatccagtgcagggtccgaggtattcgcactggatacgaccacaagt-3’
hsa-miR-222	5’-gtcgtatccagtgcagggtccgaggtattcgcactggatacgacgagaccc-3’

Step 1	20 min	15°C
Step 2	10 min	25°C
Step 3	40 min	42°C
Step 4	10 min	75°C

Primers and parameters for real-time PCR

cDNAs	Forward Primer	Reverse Primer
COX-2	5'-cctgtgcctgatgattgc-3'	5'-ctgatgcgtgaagtgctg-3'
GUSB	5'-tggtataagaagtatcagaagcc-3'	5'-gtatctctctcgcaaaaggaac-3'
U6	5'-cttcggcagcacatatact-3'	5'-aaaatatggaacgcttcacg-3'
hsa-miR-199a	5'-cccagtgttcagactacct-3'	5'-tgcagggtccgaggtat-3'
hsa-miR-101(1)	5'-gggtactgtgataactgaagg-3'	5'-tgcagggtccgaggtat-3'
hsa-miR-103	5'-cgagcagcattgtacagg-3'	5'-tgcagggtccgaggtat-3'
hsa-let-7a	5'-ggtgaggtagtaggttgtatag-3'	5'-tgcagggtccgaggtat-3'
hsa-miR-100	5'-ggaacccgtagatccgaa-3'	5'-tgcagggtccgaggtat-3'
hsa-miR-222	5'-agctacatctggctactgg-3'	5'-tgcagggtccgaggtat-3'

HOLD	95°C	2 min
Cycle 1 (x5)	95°C	30 sec
	46°C	20 sec
	72°C	30 sec
Cycle 2 (x35)	95°C	30 sec
	51°C	20 sec
	72°C 30 sec	30 sec
HOLD	48°C	60 sec
Cycle (x104)	+0,5°C/cycle	10 sec/cycle

U6 and miRNAs cDNAs were diluted 1000 fold before real-time PCR amplification.

Section 4.

Primers	Sequences
SV40 polyA FW	5’-CCCAAGCTTAAATAAAGCAATAGCATCAC-3’
SV40 polyA REV	5’-TAGAGTCGACCAGACATGATAAGAT-3’
COX-2 promoter FW1	5’-CCTGAGCACTACCCATGATA-3’
COX-2 promoter REV1	5’-CCGAGAGAACCTTCCTTTTTA-3’
COX-2 promoter FW2	5’-CGGGCCCTGAGCACTACCCATGATA-3’
COX-2 promoter REV2	5’-CGGGATCCACCGAGAGAACCTTCC-3’
TBE promoter FW	5’-CGGGCCCAAGCTATCAAAGGG-3’
TBE promoter REV	5’-CGGGATCCGGCGCCTCAGCTGGC-3’

Collaborations

1. Selective inhibition of prostacyclin synthase activity by rofecoxib (2007)

Griffoni C, Spisni E, Strillacci A, Toni M, Bachschmid MM, Tomasi V.

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The development of cyclooxygenase-2 (COX-2) selective inhibitors prompted studies aimed at treating chronic inflammatory diseases and cancer by using this new generation of drugs. Yet, several recent reports pointed out that long-term treatment of patients with COX-2 selective inhibitors (especially rofecoxib) caused severe cardiovascular complicances. The aim of this study was to ascertain whether, in addition to inhibiting COX-2, rofecoxib may also affect prostacyclin (PGI2) level by inhibiting PGI2 forming enzyme (prostacyclin synthase, PGIS). In order to evaluate if selective (celecoxib, rofecoxib) and non-selective (aspirin, naproxen) anti-inflammatory compounds could decrease PGI2 production in endothelial cells by inhibiting PGIS, we analyzed the effect of anti-inflammatory compounds on the enzyme activity by ELISA assay after addition of exogenous substrate, on PGIS protein levels by Western blotting and on its subcellular distribution by confocal microscopy. We also analyzed the effect of rofecoxib on PGIS activity in bovine aortic microsomal fractions enriched in PGIS. This study demonstrates an inhibitory effect of rofecoxib on PGIS activity in human umbilical vein endothelial (HUVE) cells and in PGIS-enriched bovine aortic microsomal fractions, which is not observed by using other anti-inflammatory compounds. The inhibitory effect of rofecoxib is associated neither to a decrease of PGIS protein levels nor to an impairment of the enzyme intracellular localization. The results of this study may explain the absence of a clear relationship between COX-2 selectivity and cardiovascular side effects. Moreover, in the light of these results we propose that novel selective COX-2 inhibitors should be tested on PGI2 synthase activity inhibition.

2. Cyclooxygenase-2/Carbonic anhydrase-IX up-regulation promotes invasive potential and hypoxia survival in colorectal cancer cells (2008)

Sansone P [1,2], Piazzi G [1,5#], Paterini P [1,4,5#], Strillacci A [4], Ceccarelli C [5], Minni F [6], Biasco G [5], Chieco P [1], and Bonafè M [1, 3].

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Inflammation promotes colorectal carcinogenesis. Tumor growth often generates an hypoxic environment in the inner tumor mass. We here show that, in colon cancer cells, the expression of the pro-inflammatory enzyme Cyclooxygenase-2 (COX-2) associates with that of the hypoxia survival gene Carbonic Anhydrase-IX (CA-IX). The modulation of COX-2 gene expression by the stable infection of a specific short hairpin RNA (shCOX-2) in colorectal cancer cells reveals that CA-IX gene expression relies upon the capacity of COX-2 gene and of COX-2 products PGE_2 to activate ERK1/2 pathway. In normoxic environment, shCOX-2 infected/CA-IX siRNA transfected colorectal cancer cells show a reduced level of active Metalloproteinase-2 (MMP-2) that associates with a decreased extracellular matrix invasion capacity. In presence of hypoxia that increases COX-2 gene expression and PGE_2 production, shCOX-2 infected/CA-IX siRNA transfected colorectal cancer cells disclose a blunted survival capability. At a high cell density, a culture condition associated with mild pericellular hypoxia, that promotes the expression of COX-2/CA-IX genes and enhances the invasive potential of colon cancer cells, shCOX-2/CA-IX siRNA transfected cells show an impaired invasive potential. Finally, in human colon cancer tissues, COX-2/CA-IX protein expression level, assessed by western blot and immunohistochemistry, correlate each other and both increase with tumor stage. In conclusion, these data indicate that COX-2/CA-IX interplay promotes the aggressive behaviour of colorectal cancer cells.

Publications

1. Strillacci A, Griffoni C, Spisni E, Manara MC, Tomasi V (2006). RNA interference as a key to knockdown overexpressed cyclooxygenase-2 gene in tumour cells. *Br J Cancer*. 94(9):1300-10.

2. Spisni E, Toni M, Strillacci A, Galleri G, Santi S, Griffoni C, Tomasi V (2006). Caveolae and caveolae constituents in mechanosensing: effect of modeled microgravity on cultured human endothelial cells. *Cell Biochem Biophys*. 46(2): 155-164

3. Tomasi V, Griffoni C, Santi S, Lenaz P, Iorio RA, Strillacci A, Spisni E (2006). Assays for membrane and intracellular signalling events. *Angiogenesis Assays. A critical appraisal of current techniques*. (CA Stanton, C Lewis and R Bicknell). John Wiley & Sons, Ltd. pp: 139-157.

4. Griffoni C, Spisni E, Strillacci A, Toni M, Bachschmid MM, Tomasi V (2007). Selective inhibition of prostacyclin synthase activity by rofecoxib. *J Cell Mol Med*. 11(2):327-38.

5. Sansone P, Piazzi G, Paterini P, Strillacci A, Ceccarelli C, Minni F, Biasco G, Chieco P, Bonafè M (2008). Cyclooxygenase-2/Carbonic anhydrase-IX interplay promotes malignant features in colorectal cancer cells. *J Pathol* (submitted).

6. Strillacci A, Griffoni C, Sansone P, Spisni E, Tomasi V (2008). miR-101 is involved in cyclooxygenase-2 regulation in human colon cancer cells. (in preparation).

7. Strillacci A, Griffoni C, Sansone P, Spisni E, Tomasi V (2008). COX-2 silencing mediate by "trans-kingdom" RNA Interference in colon cancer cells. (in preparation).

References

Akao Y, Nakagawa Y, Naoe T (2006a). let-7 microRNA functions as a potential growth suppressor in human colon cancer cells. *Biol Pharm Bull* **29**: 903– 6.

Akao Y, Nakagawa Y, Naoe T (2006b). MicroRNAs 143 and 145 are possible common onco-microRNAs in human cancers. *Oncol Rep*; **16**: 845– 50.

Akarsu ES, Palaoglu O, and Ayhan IH (1989) Iloprost-induced writhing in mice and its suppression by morphine. *Methods Find Exp Clin Pharmacol* **11:** 273-275.

Akira S, Kishimoto (1997). TNF. IL6 and NF-kappa B in cytokine gene regulation. *Adv Immunol.*; **65**: 1–46.

Allen A and Garner A (1980) Mucus and bicarbonate secretion in the stomach and their possible role in mucosal protection. *Gut* **21:** 249-262.

Allport V and Bennett P (2001) Cyclooxygenase enzymes and human labour, in *Therapeutic Roles of Selective COX-2 Inhibitors* (Vane JR and Botting RM eds) pp 252-273, William Harvey Press, London.

Almer G, Guegan C, Teismann P, Naini A, Rosoklija G, Hays AP, Chen C, and Przedborski S (2001) Increased expression of the pro-inflammatory enzyme cyclooxygenase-2 in amyotrophic lateral sclerosis. *Ann Neurol* **49:** 176-185.

Andersen K, Launer LJ, Ott A, Hoes AW, Breteler MMB, and Hofman A (1995) Do nonsteroidal anti-inflammatory drugs decrease the risk for Alzheimer's disease? The Rotterdam Study. *Neurology* **45:** 1441-1445.

Aronoff DM, Boutaud O, Marnett LJ, and Oates JA (2003) Inhibition of prostaglandin H2 synthases by salicylate is dependent on the oxidative state of the enzymes. *J Pharmacol Exp Ther* **304:** 589-595.

Bachelor MA, Cooper SJ, Sikorski ET and Bowden GT (2005) Inhibition of p38 mitogen-activated protein kinase and phosphatidylinositol 3-kinase decreases UVB-induced activator protein-1 and cyclooxygenase-2 in a SKH-1 hairless mouse model, *Mol. Cancer Res.* **3:**90–99.

Bagga S, Bracht J, Hunter S, Massirer K, Holtz J, Eachus R, *et al*. (2005). Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. *Cell*; **122**: 553– 63.

Bahari HM, Ross IN, and Turnberg LA (1982) Demonstration of a pH gradient across the mucus layer on the surface of human gastric mucosa *in vitro*. *Gut* **23:** 513-516.

Ballou LR, Botting RM, Goorha S, Zhang J, and Vane JR (2000) Nociception in cyclooxygenase isozyme-deficient mice. *Proc Natl Acad Sci USA* **97:** 10272-10276.

Bandeira-Melo C, Phoofolo M, and Weller PF (2001) Extranuclear lipid bodies, elicited by CCR3-mediated signaling pathways, are the sites of chemokineenhance leukotriene C4 production in eosinophils and basophils. *J Biol Chem* **276:** 22779-22787.

Baron JA (2003) Epidemiology of non-steroidal anti-inflammatory drugs and cancer. *Prog Exp Tumor Res* **37:** 1-24.

Barreau C, Paillard L and Osborne HB (2005). AU-rich elements and associated factors: are there unifying principles?. *Nucleic Acids Res.* **33:**7138–7150.

Bayly CI, Black WC, Leger S, Ouimet N, Ouellet M, and Percival MD (1999) Structure-based design of COX-2 selectivity into flurbiprofen. *Bioorg Med Chem Lett* **9:** 307-312.

Behrens J, von Kries J P, Kuhl M, Bruhn L, Wedlich D, Grosschedl R, and Birchmeier W (1996). Functional interaction of β-catenin with the transcription factor LEF-1. Nature (Lond.), **382***:* 638–642.

Ben-Av P, Crofford LJ, Wilder RL, and Hla T (1995) Induction of vascular endothelial growth factor expression in synovial fibroblasts by prostaglandin E and interleukin-1: a potential mechanism for inflammatory angiogenesis. *FEBS Lett* **372:** 83-87.

Bernstein E, Caudy AA, Hammond SM, Hannon GJ. 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 409:363–66

Betz M and Fox BS (1991) Prostaglandin E_2 inhibits the production of Th1 lymphokines but not Th2 lymphokines. *J Immunol* **146:** 108-113.

Bhattacharyya SN, Habermacher R, Martine U, Closs EI, Filipowicz W (2006). Relief of microRNA-mediated translational repression in human cells subjected to stress. *Cell*; **125**: 1111–24.

Bienz, M, and Clevers (2000). H. Linking colorectal cancer to Wnt signaling. *Cell*, **103**:311–320.

Bombardier C, Laine L, Reicin A, Shapiro D, Burgos–Vargas R, Davis B, Day R, Ferraz MB, Hawkey CJ, Hochberg MC, Kvien TK, Schnitzer TJ (2000). Comparison of upper gastrointestinal toxicity of rofecoxib and naproxen in patients with rheumatoid arthritis. VIGOR Study Group. *N Engl J Med* **343**(21): 1520–1528.

Bombardieri S, Cattani P, Ciabattoni G, Di Munno O, Pasero G, Patrono C, Pinca E, and Pugliese F (1981) The synovial prostaglandin system in chronic inflammatory arthritis: differential effects of steroidal and nonsteroidal anti-inflammatory drugs. *Br J Pharmacol* **73:** 893-901.

Bonazzi A, Mastyugin V, Mieyal PA, Dunn MW, Laniado-Schwartzman M (2000). Regulation of cyclooxygenase-2 by hypoxia and peroxisome proliferators in the corneal epithelium. *J Biol Chem.* **275**:2837–44.

Boutaud O, Aronoff DM, Richardson JH, Marnett LJ, and Oates JA (2002) Determinants of the cellular specificity of acetaminophen as an inhibitor of prostaglandin H(2) synthases. *Proc Natl Acad Sci USA* **99:** 7130-7135.

Bozza PT, Payne JL, Morham SG, Langenbach R, Smithies O, and Weller PF (1996) Leukocyte lipid body formation and eicosanoid generation: cyclooxygenaseindependent inhibition by aspirin. *Proc Natl Acad Sci USA* **93:** 11091-11096.

Bozza PT and Weller PF (2001) Arachidonyl triflouromethyl ketone induces lipid body formation in leukocytes. *Prostaglandins Leukot Essent Fatty Acids* **64:** 227-230.

Breder CD, Dewitt D, and Kraig RP (1995) Characterization of inducible cyclooxygenase in rat brain. *J Comp Neurol* **355:** 296-315.

Breitner JC, Welsh KA, Helms MJ, Gaskell PC, Gau B, Roses AD, Pericak-Vance MA, and Saunders AM (1995) Delayed onset of Alzheimer's disease with nonsteroidal anti-inflammatory and histamine H2 blocking drugs. *Neurobiol Aging* **16:** 523-530.

Brengues M, Teixeira D, Parker R (2005). Movement of eukaryotic mRNAs between polysomes and cytoplasmic processing bodies. *Science.* **310**: 486– 9.

Brennecke J, Stark A, Russell RB, Cohen SM (2005). Principles of microRNA-target recognition. *PLoS Biol*; **3**: e85.

Bridge AJ, Pebernard S, Ducraux A, Nicoulaz AL, Iggo R (2003). Induction of an interferon response by RNAi vectors in mammalian cells. *Nat. Genet.* **34**:263–64.

Brierley MM, Fish EN (2005) Stats: multifaceted regulators of transcription. *J Interferon Cytokine Res* **25**: 733 – 744.

Broe GA, Grayson DA, Creasey HM, Waite LM, Casey BJ, Bennett HP, Brooks WS, and Halliday GM (2000) Anti-inflammatory drugs protect against Alzheimer disease at low doses. *Arch Neurol* **57:** 1586-1591.

Buck M, Poli V, van der Geer P, Chojkier M, Hunter T (1999). Phosphorylation of rat serine 105 or mouse threonine 217 in C/EBP beta is required for hepatocyte proliferation induced by TGF alpha. *Mol Cell.* **4**: 1087–1092.

Brummelkamp TR, Bernards R, Agami R (2002). Stable suppression of tumorigenicity by virus-mediated RNA interference. *Cancer Cell* **2:**243–47.

Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, *et al*. (2002). Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A.* **99**: 15524– 9.

Calin GA, Ferracin M, Cimmino A, Di Leva , Shimizu M, Wojcik SE, *et al*. (2005). A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. *N Engl J Med*. **353**: 1793– 801.

Calin GA, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S, *et al*. (2004). Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci U S A*. **101**: 2999– 3004.

Catella-Lawson F, McAdam B, Morrison B, Kapoor S, Kujubu D, Antes L, Lasseter KC, Quan H, Gertz BJ, and FitzGerald GA (1999). Effects of specific inhibition of cyclooxygenase-2 on sodium balance, hemodynamics, and vasoactive eicosanoids. *J Pharmacol Exp Ther* **289:** 735-741.

Chakrabarty A, Tranguch S, Daikoku T, Jensen K, Furneaux H, Dey SK (2007). MicroRNA regulation of cyclooxygenase-2 during embryo implantation. *Proc Natl Acad Sci U S A*. **104**(38):15144-9.

Chan JA, Krichevsky AM, Kosik KS (2005). Micro RNA-21 is an antiapoptotic factor in human glioblastoma cells. *Cancer Res*. **65**: 6029– 33.

Chandrasekharan NV, Dai H, Roos KL, Evanson NK, Tomsik J, Elton TS, and Simmons DL (2002). COX-3, a cyclooxygenase-1 variant inhibitied by acetaminophen and other analgesic/antipyretic drugs: cloning, structure, and expression. *Proc Natl Acad Sci USA* **99:** 13926-13931.

Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, Barbisin M, Xu NL, Mahuvakar VR, Andersen MR, Lao KQ, Livak KJ, Guegler KJ (2005). Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res*. **33**(20):e179.

Chen JJ, Huang WC and Chen CC (2005). Transcriptional regulation of cyclooxygenase-2 in response to proteasome inhibitors involves reactive oxygen species-mediated signalling pathway and recruitment of CCAAT/enhancer-binding protein {delta} and CREB-binding protein, *Mol. Biol. Cell.* **16**: 5579–5591.

Chen YN, Bienkowski MJ, and Marnett LJ (1987) Controlled tryptic digestion of prostaglandin H synthase. Characterization of protein fragments and enhanced rate of proteolysis of oxidatively inactivated enzyme. *J Biol Chem* **262:** 16892-16899.

Cohen S, Dovrat S, Sarid R, Huberman E, Salzberg S (2005) JAK-STAT signaling involved in phorbol 12-myristate 13-acetate- and dimethylsulfoxide-induced 2'-5' oligoadenylate synthetase expression in human HL-60 leukemia cells. *Leuk Res* **29:** 923 – 931

Chun KS and Surh YJ (2004). Signal transduction pathways regulating cyclooxygenase-2 expression: Potential molecular targets for chemoprevention, *Biochem. Pharmacol.* **68:** 1089–1100.

Cipollone F and Patrono C (2002) Cyclooxygenase-2 polymorphism: putting a brake on the inflammatory response to vascular injury? *Arterioscler Thromb Vasc Biol* **22:** 1516-1518.

Coffey RJ, Hawkey CJ, Damstrup L, Graves-Deal R, Daniel VC, Dempsey PJ, Chinery R, Kirkland SC, DuBois RN, Jetton TL, et al. (1997) Epidermal growth factor receptor activation induces nuclear targeting of cyclooxygenase-2, basolateral release of prostaglandins and mitogenesis in polarizing colon cancer cells. *Proc Natl Acad Sci USA* **94:** 657-662.

Cok SJ and Morrison AR (2001). The 3′-untranslated region of murine cyclooxygenase-2 contains multiple regulatory elements that alter message stability and translational efficiency, *J. Biol. Chem.* **276:** 23179–23185.

Crofford LJ (2000) Clinical experience with specific COX-2 inhibitors in arthritis. *Curr Pharm Des* **6:** 1725-1736.

Crofford LJ, Wilder RL, Ristimaki AP, Sano H, Remmers EF, Epps HR, and Hla T (1994) Cyclooxygenase-1 and –2 expression in rheumatoid synovial tissues. Effects of interleukin-1 beta, phorbol ester, and corticosteroids. *J Clin Investig* **93:** 1095-1101.

Csiki I, Yanagisawa K, Haruki N, et al. (2006). Thioredoxin-1 modulates transcription of cyclooxygenase-2 via hypoxia-inducible factor-1α in non-small cell lung cancer. *Cancer Res*. **66**:143–50.

Cummins JM, He Y, Leary RJ, Pagliarini R, Diaz LA Jr, Sjoblom T, Barad O, Bentwich Z, Szafranska AE, Labourier E, Raymond CK, Roberts BS, Juhl H, Kinzler KW, Vogelstein B, Velculescu VE (2006). The colorectal microRNAome. *Proc Natl Acad Sci U S A*. **103**(10):3687-92.

Dean JL, Brook M, Clark AR and Saklatvala J (1999). p38 mitogen-activated protein kinase regulates cyclooxygenase-2 mRNA stability and transcription in lipopolysaccharide-treated human monocytes, *J. Biol. Chem.* **274:** 264–269.

Deng D, Deng G, Smith MF, Zhou J, Xin H, Powell SM, and Lu Y (2002) Simultaneous detection of CpG methylation and single nucleotide polymorphism by denaturing high performance liquid chromatography. *Nucleic Acids Res* **30:** E13.

Deng WG, Zhu Y, Wu KK (2004). Role of p300 and PCAF in regulating cyclooxygenase-2 promoter activation by inflammatory mediators. *Blood.* **103**: 2135–2142.

Deng WG, Zhu Y, Wu KK (2003). Up-regulation of p300 binding and p50 acetylation in tumor necrosis factor-alpha-induced cyclooxygenase-2 promoter activation. *J Biol Chem.* **278**: 4770–4777.

Denkert C, Furstenberg A, Daniel PT, Koch I, Kobel M, Weichert W, Siegert A, Hauptmann S (2003) Induction of G0/G1 cell cycle arrest in ovarian carcinoma cells by the anti-inflammatory drug NS-398, but not by COX-2-specific RNA interference. *Oncogene* **22:** 8653 – 8661

Descombes P, Schibler UA (1991). liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. *Cell.* **67**: 569–579.

Devillier P, Acker GM, Advenier C, Regoli D, and Frossard N (1991) Respiratory epithelium releases relaxant prostaglandin E_2 through activation of substance P (NK_1) receptors. *Am Rev Respir Dis* **139:** A351.

DeWitt DL, El-Harith EA, Kraemer SA, Andrews MJ, Yao EF, Armstrong RL, and Smith WL (1990) The aspirin and heme-binding sites of ovine and murine prostaglandin endoperoxide synthases. *J Biol Chem* **265:** 5192-5198.

DeWitt DL and Smith WL (1988) Primary structure of prostaglandin G/H synthase from sheep vesicular gland determined from the complementary DNA sequence. *Proc Natl Acad Sci USA* **85:** 1412-1416.

Dews M, Homayouni A, Yu D; et al. (2006). Augmentation of tumor angiogenesis by a Myc-activated microRNA cluster. *Nat Genet.* **38**(9):1060-1065.

Dey I, Keller K, Belley A, and Chadee K (2003) Identification and characterization of a cyclooxygenase-like enzyme from Entamoeba histolytica. *Proc Natl Acad Sci USA* **100:** 13561-13566.

Diederichs S, Haber DA (2006). Sequence variations of microRNAs in human cancer: alterations in predicted secondary structure do not affect processing. *Cancer Res.* **66**: 6097– 104.

Ding C and Jones G (2002) Lumiracoxib (novartis). *Drugs* **5:** 1168-1172.

Dixon DA (2003) Regulation of COX-2 expression in human cancers. *Prog Exp Tumor Res* **37:** 52-71.

Dixon DA, Kaplan CD, McIntyre TM, Zimmerman GA and Prescott SM (2000). Post-transcriptional control of cyclooxygenase-2 gene expression. The role of the 3′-untranslated region, *J. Biol. Chem.* **275:** 11750–11757.

Dixon DA, Tolley ND, King PH, Nabors LB, McIntyre TM, Zimmerman GA and Prescott SM (2001). Altered expression of the mRNA stability factor HuR promotes cyclooxygenase-2 expression in colon cancer cells, *J. Clin. Invest.* **108**: 1657–1665.

Doller A, Huwiler A, Muller R, Radeke HH, Pfeilschifter H and Eberhardt W (2007). Protein kinase C alpha-dependent phosphorylation of the mRNA-stabilizing factor HuR, *Mol. Biol. Cell* **18**: 2137–2148.

Dubois RN, Abramson SB, Crofford L, Gupta RA, Simon LS, Van De Putte LB, and Lipsky PE (1998) Cyclooxygenase in biology and disease. *FASEB J* **12:** 1063-1073.

Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, and DuBois RN (1994) Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology* **107:** 1183-1188.

Eberhardt W, Doller A, Akool S and Pfeilschifter J (2007). Modulation of mRNA stability as a novel therapeutic approach, *Pharmacol. Ther.* **114**: 56–73.

Eis PS, Tam W, Sun L, Chadburn A, Li Z, Gomez MF, *et al*. (2005). Accumulation of miR-155 and BIC RNA in human B cell lymphomas. *Proc Natl Acad Sci U S A.* **102**: 3627– 32.

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., Tuschl, T. (2001a) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**,494-498.

Elbashir, S. M., Lendeckel, W., Tuschl, T. (2001b) RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev.* **15**,188-200.

Eliopoulos A.G., Dumitru C.D., Wang C.C., Cho J. and Tsichlis P.N.(2002). Induction of COX-2 by LPS in macrophages is regulated by Tpl2-dependent CREB activation signals, *EMBO J.* **21**: 4831–4840.

Enright AJ, John B, Gaul U, Tuschl T, Sander C, Marks DS (2003). MicroRNA targets in Drosophila. *Genome Biol.* **5**: R1.

Evanson NK (2002) Post-transcriptional regulation of chicken and mammalian cyclooxygenase 2. Honors Thesis, Brigham Young University, Provo, UT.

Evett GE, Xie W, Chipman JG, Robertson DL, and Simmons DL (1993) Prostaglandin G/H synthase isoenzyme 2 epxression in fibroblasts: regulation by dexamethasone, mitogens, and oncogenes. *Arch Biochem Biophys* **306:** 169-177.

Fels OAS, Pawlowski NA, Abraham EL, and Cohn ZA (1986) Compartmentalized regulation of macrophage arachidonic acid metabolism. *J Exp Med* **163:** 752-757.

Ferreira SH (1972) Prostaglandins, aspirin-like drugs and analgesia. *Nature (Lond)* **240:** 200-203.

Ferreira SH, Nakamura M, and Abreu Castro MS (1978) The hyperalgesic effects of prostacyclin and PGE_2. *Prostaglandins* **16:** 31-37.

Ferreri NR, An SJ, and McGiff JC (1999) Cyclooxygenase-2 expression and function in the medullary thick ascending limb. *Am J Physiol* **277:** F360-F368.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., Mello, C. C. (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **391**,806-811

FitzGerald GA and Patrono C (2001) The coxibs, selective inhibitors of cyclooxygenase-2. *N Engl J Med* **345:** 433-442.

FitzGerald GA, Pedersen AK, and Patrono C (1983) Analysis of prostacyclin and thromboxane biosynthesis in cardiovascular disease. *Circulation* **67:** 1174-1177.

Fletcher BS, Kujubu DA, Perrin DM, Herschman HR (1992). Structure of the mitogen-inducible TIS10 gene and demonstration that the TIS10-encoded protein is a functional prostaglandin G/H synthase. *J Biol Chem.* **267**: 4338–4344.

Flower RJ and Vane JR (1972) Inhibition of prostaglandin synthetase in brain explains the anti-pyretic activity of paracetamol (4-acetamidophenol). *Nature (Lond)* **240:** 410-411.

Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM, and Evans RM (1995) 15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. *Cell* **83:** 803-812.

Fu JY, Masfarrer JL, Seibert K, Raz A, and Needleman P (1990) The induction and suppression of prostaglandin H_2 synthase (cyclooxygenase) in human monocytes. *J Biol Chem* **265:** 16737-16740.

Fuller RW, Dixon CMS, Dollery CT, and Barnes PJ (1986) Prostaglandin D_2 potentiates airway responses to histamine and methacholine. *Am Rev Respir Dis* **133:** 252-254.

Funk CD, Funk LB, Kennedy ME, Pong AE, and Fitzgerald GA (1991) Human platelet/erythroleukemia cell prostaglandin G/H synthase: cDNA cloning, expression and gene chromosomal assignment. *FASEB J* **5:** 2304-2312.

Garavito RM and DeWitt DL (1999) The cyclooxygenase isoforms: structural insights into the conversion of arachidonic acid to prostaglandins. *Biochim Biophys Acta* **1441:** 278-287.

Gardner B, Zhu LX, Sharma S, Tashkin DP, and Dubinett SM (2003) Methanandamide increases COX-2 expression and tumor growth in murine lung cancer. *FASEB J* **17:** 2157-2159.

Garzon R, Pichiorri F, Palumbo T, Visentini M, Aqeilan R, Cimmino A, *et al*. MicroRNA gene expression during retinoic acid-induced differentiation of human acute promyelocytic leukemia. *Oncogene* 2007; **26**: 4148– 57.

Gibb W and Sun M (1996) Localization of prostaglandin H synthase type 2 protein and mRNA in term human fetal membranes and decidua. *J Endocrinol* **150:** 497-503.

Gierse JK, Koboldt CM, Walker MC, Seibert K, and Isakson PC (1999) Kinetic basis for selective inhibition of cyclo-oxygenases. *Biochem J* **339:** 607-614.

Gilroy DW, Colville-Nash PR, Willis D, Chivers J, Paul-Clarke MJ, and Willoughby DA (1999) Inducible cyclooxygenase may have anti-inflammatory properties. *Nat Med* **5:** 698-701.

Giovannucci E, Egan KM, Hunter DJ, Stampfer MJ, Colditz GA, Willett WC, Speizer FE (1995). Aspirin and the risk of colorectal cancer in women. *N Engl J Med.* **333**(10): 609–614.

Golding MC, Long CR, Carmell MA, Hannon GJ, Westhusin ME (2006). Suppression of prion protein in livestock by RNA interference. *Proc. Natl. Acad. Sci. USA* **103**:5285–90

Grady WM, Parkin RK, Mitchell PS, Lee JH, Kim YH, Tsuchiya KD, Washington MK, Paraskeva C, Willson JK, Kaz AM, Kroh EM, Allen A, Fritz BR, Markowitz SD, Tewari M (2008). Epigenetic silencing of the intronic microRNA hsa-miR-342 and its host gene EVL in colorectal cancer. *Oncogene*. In press.

Greco A, Ajmone-Cat MA, Nicolini A, Sciulli MG, and Minghetti L (2003) Paracetamol effectively reduces prostaglandin E2 synthesis in brain macrophages by inhibiting enzymatic activity of cyclooxygenase but not phospholipase and prostaglandin E synthase. *J Neurosci Res* **71:** 844-852.

Greig GM, Francis DA, Falgueyret JP, Ouellet M, Percival MD, Roy P, Bayly C, Mancini JA, and O'Neill GP (1997) The interaction of arginine 106 of human prostaglandin G/H synthase-2 with inhibitors is not a universal component of inhibition mediated by nonsteroidal anti-inflammatory drugs. *Mol Pharmacol* **52:** 829-838.

Gretzer B, Maricic N, Respondek M, Schuligoi R, and Peskar BM (2001) Effects of specific inhibition of cyclo-oxygenase-1 and cyclo-oxygenase-2 in the rat stomach with normal mucosa and after acid challenge. *Br J Pharmacol* **132:** 1565-1573.

Grimm D, Streetz KL, Jopling CL, Storm TA, Pandey K, et al. (2006). Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature* 441:537–41

Grishok, A., Tabara, H., Mello, C. C. (2000) Genetic requirements for inheritance of RNAi in C. elegans. *Science* **287**,2494-2497

Guo, S., Kemphues, K. J. (1995) par-1, a gene required for establishing polarity in C. elegans embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. *Cell* **81**,611-620

Gupta RA and Dubois RN (2001). Colorectal cancer prevention and treatment by inhibition of Cox-2. *Nature Rev Cancer* **1:** 11 – 21.

Habenicht AJ, Goerig M, Grulich J, Rothe D, Gronwald R, Loth U, Schettler G, Kommerell B, and Ross R (1985) Human platelet-derived growth factor stimulates prostaglandin synthesis by activation and by rapid de novo synthesis of cyclooxygenase. *J Clin Investig* **75:** 1381-1387.

Halliday G, Robinson SR, Shepherd C, and Kril J (2000) Alzheimer's disease and inflammation: a review of cellular and therapeutic mechanisms.*Clin Exp Pharmacol Physiol* **27:** 1-4.

Hamberg M and Samuelsson B (1973) Detection and isolation of an endoperoxide intermediate in prostaglandin biosynthesis. *Proc Natl Acad Sci USA* **70:** 899-903.

Hamberg M, Sanz A, and Castresana C (1999) α-Oxidation of fatty acids in higher plants. *J Biol Chem* **274:** 24503-24513.

Hamberg M, Svensson J, and Samuelsson B (1974) Prostaglandin endoperoxides. A new concept concerning the mode of action of prostaglandins. *Proc Natl Acad Sci USA* **71:** 3824-3828.

Hamilton, A. J., Baulcombe, D. C. (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* **286**: 950-952.

Hammond, S. M., Bernstein, E., Beach, D., Hannon, G. J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature* **404**: 293-296

Han JW, Sadowski H, Young DA, and Macara IG (1990) Persistent induction of cyclooxygenase in p60v-src transformed 3T3 fibroblasts. *Proc Natl Acad Sci USA* **87:** 3373-3377.

Harris RC (1996) The macula densa: recent developments. *J Hypertens* **14:** 815-822.

Harris RC, McKanna JA, Akai Y, Jacobson HR, Dubois RN, and Breyer MD (1994) Cyclooxygenase-2 is associated with the macula densa of rat kidney and increases with salt restriction. *J Clin Investig* **94:** 2504-2510.

Hasuwa H, Kaseda K, Einarsdottir T, Okabe M. 2002. Small interfering RNA and gene silencing in transgenic mice and rats. *FEBS Lett.* **532**:227–30

Hatanaka K, Harada Y, Kawamura M, Ogino M, Saito M, and Katori M (1996) Cell types expressing COX-2 in rat carrageenin-induced pleurisy. *Jpn J Pharmacol* **71 (Suppl I):** 304P.

Hayaishi O (1991) Molecular mechanisms of sleep-wake regulation: roles of prostaglandins D_2 and E_2. *FASEB J* **5:** 2575-2581.

He H, Jazdzewski K, Li W, Liyanarachchi S, Nagy R, Volinia S, *et al*. The role of microRNA genes in papillary thyroid carcinoma. *Proc Natl Acad Sci U S A* 2005; **102**: 19075– 80.

Heidel JD, Hu S, Liu XF, Triche TJ and Davis ME (2004). Lack of interferon response in animals to naked siRNAs. *Nature Biotechnol* **22:** 1579 – 1582.

Hemler M and Lands WE (1976). Purification of the cyclooxygenase that forms prostaglandins: demonstration of two forms of iron in the holoenzyme. *J Biol Chem* **251:** 5575-5579.

Henderson AS, Jorm AF, Christensen H, Jacomb PA, and Korten AE (1997) Aspirin, anti-inflammatory drugs and risk of dementia. *Int J Geriatr Psychiatry* **12:** 926-930.

Higgs EA, Moncada S, and Vane JR (1978) Inflammatory effects of prostacyclin (PGI_2) and 6-oxo-PGF_{1a} in the rat paw. *Prostaglandins* **16:** 153-162.

Hla T (1996) Molecular characterization of the 5.2 KB isoform of the human cyclooxygenase-1 transcript. *Prostaglandins* **51:** 81-85.

Hla T and Neilson K (1992) Human cyclooxygenase-2 cDNA. *Proc Natl Acad Sci USA* **89:** 7384-7388.

Hla T, Ristimaki A, Appleby S, and Barriocanal JG (1993) Cyclooxygenase gene expression in inflammation and angiogenesis. *Ann N Y Acad Sci* **696:** 197-204.

Ho L, Purohit D, Haroutunian V, Luterman JD, Willis F, Naslund J, Buxbaum JD, Mohs RC, Aisen PS, and Pasinetti GM (2001) Neuronal cyclooxygenase 2 expression in the hippocampal formation as a function of the clinical progression of Alzheimer Diseases. *Arch Neurol* **58:** 487-492.

Holgate ST (1986) The pathophysiology of bronchial asthma and targets for its drug treatment. *Agents Actions* **18:** 281-287.

Holtzman MJ, Turk J, and Shornick LP (1992) Identification of a pharmacologically distinct prostaglandin H synthase in cultured epithelial cells. *J Biol Chem* **267:** 21438-21445.

Howe LR, Subbaramaian K, Chung WJ, Dannenberg AJ and Brown MCA (1999). Transcriptional activation of cyclooxygenase-2 in Wnt-1-transformed mouse mammary epithelial cells. *Cancer Res*. **59***:* 1572–1577.

Hörnsten L, Su C, Osbourn AE, Garosi P, Hellman U, Wernstedt C, and Oliw EH (1999) Cloning of linoleate diol synthase reveals homology with prostaglandin H synthases. *J Biol Chem* **274:** 28219-28224.

Hunt RH, Harper S, Watson DJ, Yu C, Quan H, Lee M, Evans JK, and Oxenius B (2003) The gastrointestinal safety of the COX-2 selective inhibitor etoricoxib assessed by both endoscopy and analysis of upper gastrointestinal events. *Am J Gastroenterol* **98:** 1725-1733.

Iadecola C, Niwa K, Nogawa S, Zhao X, Nagayama M, Araki E, Morham S, and Ross ME (2001) Reduced susceptibility to ischemic brain injury and N-methyl-D-aspartate-mediated neurotoxicity in cyclooxygenase-2-deficient mice. *Proc Natl Acad Sci USA* **98:** 1294-1299.

Inoue H, Kosaka T, Miyata A, Hara S, Yokoyama C, Nanayama T and Tanabe T (1995). Structure and expression of the human prostaglandin endoperoxide synthase 2 gene, *Adv. Prostaglandin Thromboxane Leukotriene Res.* **23**: 109–111.

Iñiguez MA, Rodriguez A, Volpert OV, Fresno M, Redondo JM (2003) Cyclooxygenase-2: a therapeutic target in angiogenesis. *TRENDS Mol Med* **9:** 73 – 78.

Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, Sabbioni S, *et al*. MicroRNA gene expression deregulation in human breast cancer. *Cancer Res* 2005; **65**: 7065– 70.

Hirai K, Ezumi Y, Nishida E, Uchiyama T, Takayama H (1999) Comparative study of vanadate-and phorbol ester-induced cyclo-oxygenase-3 expression in human endothelial cells. *Thromb Haemost* **82:** 1545 – 1552.

Iwai N, Naraba H (2005). Polymorphisms in human pre-miRNAs. *Biochem Biophys Res Commun*. **331**: 1439– 44.

Jackson AL, Bartz SR, Schelter J, Kobayashi SV, Burchard J, et al. (2003). Expression profiling reveals off-target gene regulation by RNAi. *Nat. Biotechnol.* **21**:635–37.

Jackson AL, Burchard J, Leake D, Reynolds A, Schelter J, et al. (2006). Position-specific chemical modification of siRNAs reduces "off-target" transcript silencing. *RNA* **12**:1197–205

Jaffe EA and Weksler BB (1979) Recovery of endothelial cell prostacyclin production after inhibition by low doses of aspirin. *J Clin Investig* **63:** 532-535.

Jarving R, Jarving I, Kurg R, Brash AR, and Samel N (2004) On the evolutionary origin of cyclooxygenase (COX) isozymes: characterization of marine invertebrate COX genes points to

independent duplication events in vertebrate and invertebrate lineages. *J Biol Chem* **279:** 13624-13633.

Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, *et al*. (2005) RAS is regulated by the let-7 microRNA family. *Cell*; **120**: 635– 47.

Judge AD, Bola G, Lee AC, MacLachlan I.(2006). Design of noninflammatory synthetic siRNA mediating potent gene silencing in vivo. Mol. Ther. **13**:494–505.

Judge AD, Sood V, Shaw JR, Fang D, McClintock K, MacLachlan I. 2005. Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. Nat. Biotechnol. **23**:457–62.

Kaidi A, Qualtrough D, Williams AC and Paraskeva C (2006) Direct Transcriptional Up-regulation of Cyclooxygenase-2 by Hypoxia-Inducible Factor (HIF)-1 Promotes Colorectal Tumor Cell Survival and Enhances HIF-1 Transcriptional Activity during Hypoxia. *Cancer Res.* **66**: 6683-6691.

Kakiuchi Y, Tsuji S, Tsujii M, Murata H, Kawai N, Yasumaru M, Kimura A, Komori M, Irie T, Miyoshi E, Sasaki Y, Hayashi N, Kawano S, Hori M (2002) Cyclooxygenase-2 activity altered the cell-surface carbohydrate antigens on colon cancer cells and enhanced liver metastasis. *Cancer Res* **62:** 1567 – 1572.

Kariko K, Bhuyan P, Capodici J, Weissman D. 2004. Small interfering RNAs mediate sequence-independent gene suppression and induce immune activation by signaling through toll-like receptor 3. *J. Immunol.* **172**:6545–49

Kawahito Y, Kondo M, Tsubouchi Y, Hashiramoto A, Bishop-Bailey D, Inoue K, Kohno M, Yamada R, Hla T, and Sano H (2000) 15-deoxy-delta(12,14)-PGJ(2) induces synoviocyte apoptosis and suppresses adjuvant-induced arthritis in rats. *J Clin Investig* **106:** 189-197.

Kelkenberg U, Wagner AH, Sarhaddar J, Hecker M, von der Leyen, H.E. (2002). CCAAT. /enhancer-binding protein decoy oligodeoxynucleotide inhibition of macrophage-rich vascular lesion formation in hypercholesterolemic rabbits. *Arterioscler Thromb Vasc Biol.*; **22**: 949–954.

Kennedy CR, Zhang Y, Brandon S, Guan Y, Coffee K, Funk CD, Magnuson MA, Oates JA, Breyer MD, and Breyer RM (1999) Salt-sensitive hypertension and reduced fertility in mice lacking the prostaglandin EP_2 receptor. *Nat Med* **5:** 217-220.

Kinzler KW, and Vogelstein B (1996). Lessons from hereditary colorectal cancer. *Cell*, **87***:* 159–170.

Kitzler J, Hill E, Hardman R, Reddy N, Philpot R, and Eling TE (1995) Analysis and quantitation of splicing variants of the TPA-inducible PGHS-1 mRNA in rat tracheal epithelial cells. *Arch Biochem Biophys* **316:** 856-863.

Kluiver J, Haralambieva E, de Jong D, Blokzijl T, Jacobs S, Kroesen BJ, *et al*. (2006). Lack of BIC and microRNA miR-155 expression in primary cases of Burkitt lymphoma. *Genes Chromosomes Cancer*; **45**: 147– 53.

Koeduka T, Matsui K, Akakabe Y, and Kajiwara T (2000) Molecular characterization of fatty acid α-hydroperoxide-forming enzyme (α-oxygenase) in rice plants. *Biochem Soc Trans* **28:** 765-768.

Konheim YL and Wolford JK (2003) Association of a promoter variant in the inducible cyclooxygenase-2 gene (PTGS2) with type 2 diabetes mellitus in Pima Indians. *Hum Genet* **113:** 377-381.

Kozak KR, Crews BC, Morrow JD, Wang LH, Ma YH, Weinander R, Jakobsson PJ, and Marnett LJ (2002) Metabolism of the endocannabinoids, 2-arachidonylglycerol and anandamide, into prostaglandin, thromboxane, and prostacyclin glycerol esters and ethanolamides. *J Biol Chem* **277:** 44877-44885.

Kujubu DA, Fletcher BS, Varnum BC, Lim RW, and Herschman HR (1991) TIS 10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. *J Biol Chem* **266:** 12866-12872.

Kurumbail RG, Stevens AM, Gierse JK, McDonald JJ, Stegeman RA, Pak JY, Gildehaus D, Miyashiro JM, Penning TD, Seibert K, et al. (1996) Structural basis for selective inhibition of cyclooxygenase-2 by anti-inflammatory agents. *Nature (Lond)* **384:** 644-648.

Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. Identification of tissue-specific microRNAs from mouse. *Curr Biol* 2002; **12**: 735– 9.

Langenbach R, Loftin CD, Lee C, and Tiano H (1999) Cyclooxygenase-deficient mice. A summary of their characteristics and susceptibilities to inflammation and carcinogenesis. *Ann N Y Acad Sci* **889:** 52-61.

Langenbach R, Morham SG, Tiano HF, Loftin CD, Ghanayem BI, Chulada PC, Mahler JF, Lee CA, Goulding EH, Kluckman KD, et al. (1995) Prostaglandin synthase 1 gene disruption in mice reduces arachidonic acid-induced inflammation and indomethacin-induced gastric ulceration. *Cell* **83:** 483-492.

Lanza G, Ferracin M, Gafà R, Veronese A, Spizzo R, Pichiorri F, Liu CG, Calin GA, Croce CM, Negrini M (2007). mRNA/microRNA gene expression profile in microsatellite unstable colorectal cancer. *Mol Cancer.* **6**:54.

Leahy KM, Ornberg RL, Wang Y, Zweifel BS, Koki AT, Masferrer JL (2002) Cyclooxygenase-2 inhibition by celecoxib reduces proliferation and induces apoptosis in angiogenic endothelial cells in vivo. *Cancer Res* **62:** 625 – 631.

Lee H.K. and Jeong S. (2006). Beta-Catenin stabilizes cyclooxygenase-2 mRNA by interacting with AU-rich elements of 3′-UTR, *Nucleic Acids Res.* **34**: 5705–5714.

Lee S.H., Soyoola E., Chanmugam P., Hart S., Sun W., Zhong H., Liou S., Simmons D. and Hwang D. (1992). Selective expression of mitogen-inducible cyclooxygenase in macrophages stimulated with lipopolysaccharide, *J. Biol. Chem.* **267**: 25934–25938.

Lewis BP, Burge CB, Bartel DP. 2005. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* **120**:15–20.

Lewis DL, Hagstrom JE, Loomis AG, Wolff JA, Herweijer H. 2002. Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. *Nat. Genet.* **32**:107–8

Li L, Lin X, Staver M, Shoemaker A, Semizarov D, et al. 2005. Evaluating hypoxia-inducible factor-1alpha as a cancer therapeutic target via inducible RNA interference in vivo. *Cancer Res.* **65**:7249–58.

Li S, Wang Y, Matsumura K, Ballou LR, Moreham SG, and Blatteis CM (1999) The febrile response to lipopolysaccharide is blocked in cyclooxygenase-2-/-mice. *Brain Res* **825:** 86-94.

Liao J, Piwien-Pilipuk G, Ross SE, Hodge CL, Sealy L, MacDougald OA, Schwartz J. (1999) CCAAT/enhancer-binding protein beta (C/EBPbeta) and C/EBPdelta contribute to growth hormone-regulated transcription of c-fos. *J Biol Chem.* **274**: 31597–31604.

Lim GP, Yang F, Chu T, Chen P, Beech W, Teter B, Tran T, Ubeda O, Ashe KH, Frautschy SA, et al. (2000) Ibuprofen suppresses plaque pathology and inflammation in a mouse model for Alzheimer's disease. *J Neurosci* **20:** 5709-5714.

Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castle J, *et al*. (2005). Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature*; **433**: 769– 73.

Lin X, Ruan X, Anderson MG, McDowell JA, Kroeger PE, et al. (2005). siRNA-mediated off-target gene silencing triggered by a 7 nt complementation. *Nucleic Acids Res.* **33**:4527–35

Liou JY, Shyue SK, Tsai MJ, Chung CL, Chu KY, and Wu KK (2000) Colocalization of prostacyclin synthase with prostaglandin H synthase-1 (PGHS-1) but not phorbol ester-induced PGHS-2 in cultured endothelial cells. *J Biol Chem* **275:** 15314-15320.

Liu CH, Chang SH, Narko K, Trifan OC, Wu MT, Smith E, Haudenschild C, Lane TF, and Hla T (2001) Overexpression of cyclooxygenase-2 is sufficient to induce tumorigenesis in transgenic mice. *J Biol Chem* **276:** 18563-18569.

Liu Y., Borchert G.L. and Phang J.M. (2004). Polyoma enhancer activator 3, an ets transcription factor, mediates the induction of cyclooxygenase-2 by nitric oxide in colorectal cancer cells, *J. Biol. Chem*. **279**: 18694–18700.

Liu J, Carmell MA, Rivas FV, Marsden CG, Thomson JM, et al. (2004a). Argonaute2 is the catalytic engine of mammalian RNAi. *Science* **305**:1437–41.

Loftin CD, Trivedi DB, Tiano HF, Clark JA, Lee CA, Epstein JA, Morham SG, Breyer MD, Nguyen M, Hawkins BM, et al. (2001) Failure of ductus arteriosus closure and remodeling in neonatal mice deficient in cyclooxygenase-1 and cyclooxygenase-2. *Proc Natl Acad Sci USA* **98:** 1059-1064.

Loll PJ, Picot D, and Garavito RM (1995) The structural basis of aspirin activity inferred from the crystal structure of inactivated prostaglandin H2 synthase. *Nat Struct Biol* **2:** 637-643.

Lu J, Getz G, Miska EA, varez-Saavedra E, Lamb J, Peck D, *et al*. (2005). MicroRNA expression profiles classify human cancers. *Nature*; **435**: 834– 8.

Lujambio A, Ropero S, Ballestar E, Fraga MF, Cerrato C, Setien F, *et al*. (2007). Genetic unmasking of an epigenetically silenced microRNA in human cancer cells. *Cancer Res*; **67**: 1424–9.

Luong C, Miller A, Barnett J, Chow J, Ramesha C, and Browner MF (1996) Flexibility of the NSAID binding site in the structure of human cyclooxygenase-2. *Nat Struct Biol* **3:** 927-933.

Lusis AJ (2000) Atherosclerosis. *Nature (Lond)* **407:** 233-241.

Lysz TW and Needleman P (1982) Evidence for two distinct forms of fatty acid cyclooxygenase in brain. *J Neurochem* **38:** 1111-1117.

Lysz TW, Zweig A, and Keeting PE (1988) Examination of mouse and rat tissues for evidence of dual forms of the fatty acid cyclooxygenase. *Biochem Pharmacol* **37:** 921-927.

Ma Y, Creanga A, Lum L, Beachy PA. 2006. Prevalence of off-target effects in *Drosophila* RNA interference screens. *Nature* **443**:359–63.

Mahmoud NN, Boolbol SK, Dannenberg AJ, Mestre JR, Bilinski RT, Martucci C, Newmark HL, Chadburn A, Bertagnolli MM (1998). The sulfide metabolite of sulindac prevents tumors and restores enterocyte apoptosis in a murine model of familial adenomatous polyposis. *Carcinogenesis* **19**(1): 87–91.

Majima M, Isono M, Ikeda Y, Hayashi I, Hatanaka K, Harada Y, Katsumata O, Yamashina S, Katori M, and Yamamoto S (1997) Significant roles of inducible cyclooxygenase (COX)-2 in angiogenesis in rat sponge implants. *Jpn J Pharmacol* **75:** 105-114.

Malkowski MG, Ginell SL, Smith WL, and Garavito RM (2000) The productive conformation of arachidonic acid bound to prostaglandin synthase. *Science (Wash DC)* **289:** 1933-1937.

Mamdani M, Juurlink DN, Lee DS, Rochon PA, Koop A, Naglie G, Austin PC, Laupacis A and Stukel TA (2004) Cyclo-oxygenase-2 inhibitors versus non-selective non-steroidal anti-inflammatory drugs and congestive heart failure outcomes in elderly patients: a population-based cohort study. *Lancet* **363:** 1751 – 1756.

Marks F, Furstenberger G, Neufang G, and Muller-Decker K (2003) Mouse skin as a model for cancer chemoprevention by nonsteroidal anti-inflammatory drugs. *Cancer Res* **163:** 46-47.

Marnett LJ (2000) Cyclooxygenase mechanisms. *Curr Opin Chem Biol* **4:** 545-552.

Marnett LJ and Kalgutkar AS (1998) Design of selective inhibitors of cyclooxygenase-2 as nonulcerogenic anti-inflammatory agents. *Curr Opin Chem Biol* **2:** 482-490.

Marques JT, Devosse T, Wang D, Zamanian-Daryoush M, Serbinowski P, et al. 2006. A structural basis for discriminating between self and nonself double-stranded RNAs in mammalian cells. *Nat. Biotechnol* **24**:559–65.

Marshall PJ and Kulmacz RJ (1988) Prostaglandin H synthase: distinct binding sites for cyclooxygenase and peroxidase substrates. *Arch Biochem Biophys* **266:** 162-170.

Martin SE, Caplen NJ. 2006. Mismatched siRNAs downregulate mRNAs as a function of target site location. *FEBS Lett.* **580**:3694–98

Martin SE, Jones TL, Thomas CL, Lorenzi PL, Nguyen DA, et al. 2007. Multiplexing siRNAs to compress RNAi-based screen size in human cells. *Nucleic Acids Res*. doi:10.1093/nar/gkm141.

Marx J (2001) Cancer research. Anti-inflammatories inhibit cancer growth – but how? *Science (Wash DC)* **291:** 581-582.[

Matranga C, Tomari Y, Shin C, Bartel DP, Zamore PD. 2005. Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. *Cell* **123**:607–20

Matsusaka T, Fujikawa K, Nishio Y, Mukaida N, Matsushima K, Kishimoto T, Akira S. (1993). Transcription factors NF-IL6 and NF-kappa B synergistically activate transcription of the inflammatory cytokines, interleukin 6 and interleukin 8. *Proc Natl Acad Sci U S A.* **90**: 10193–10197.

May, R.C. and Plasterk, R.H. (2005). RNA interference spreading in *C. elegans*. *Methods Enzymol.* **392**, 308–315.

Mazière P and Enright AJ (2007). Prediction of microRNA targets. *Drug Discov Today*.**12(11-12)**:452-8.

McAdam BF, Catella-Lawson F, Mardini IA, Kapoor S, Lawson JA, and FitzGerald GA (1999) Systemic biosynthesis of prostacyclin by cyclooxygenase (COX)-2: the human pharmacology of a selective inhibitor of COX-2. *Proc Natl Acad Sci USA* **96:** 272-277.

McCaffrey AP, Meuse L, Pham TT, Conklin DS, Hannon GJ, Kay MA. 2002. RNA interference in adult mice. *Nature* **418**:38–39.

McEvoy A.N., Bresnihan B., FitzGerald O. and Murphy E.P. (2004). Cyclooxygenase 2-derived prostaglandin E2 production by corticotropin-releasing hormone contributes to the activated cAMP response element binding protein content in rheumatoid arthritis synovial tissue, *Arthritis Rheum.* **50**: 1132–1145.

Meade EA, Smith WL, and DeWitt DL (1993) Expression of the murine prostaglandin (PGH) synthase-1 and PGH synthase-2 isozymes in Cos-1 cells. *J Lipid Mediat* **6:** 119-129.

Merlie JP, Fagan D, Mudd J, and Needleman P (1988) Isolation and characterization of the complementary DNA for sheep seminal vesicle prostaglandin endoperoxide synthase (cyclooxygenase). *J Biol Chem* **263:** 3550-3553.

Michael MZ, O' Connor SM, van Holst Pellekaan NG, Young GP, James RJ (2003). Reduced accumulation of specific microRNAs in colorectal neoplasia. Mol Cancer Res. **1(12)**:882-91.

Mifflin R.C., Saada J.I., Di Mari J.F., Valentich J.D., Adegboyega P.A. and Powell D.W. (2004). Aspirin-mediated COX-2 transcript stabilization via sustained p38 activation in human intestinal myofibroblasts, *Mol. Pharmacol.* **65**: 470–478.

Miller TA (1983) Protective effects of prostaglandins against gastric mucosal damage: current knowledge and proposed mechanisms. *Am J Physiol* **245:** G601-G623.

Miller VM, Xia H, Marrs GL, Gouvion CM, Lee G, et al. 2003. Allele-specific silencing of dominant disease genes. *Proc. Natl. Acad. Sci. USA* **100**:7195–200.

Mitchell JA, Belvisi MG, Akarasereenont P, Robbins RA, Kwon O-J, Croxtall J, Barnes PJ, and Vane JR (1994) Induction of cyclo-oxygenase-2 by cytokines in human pulmonary epithelial cells: regulation by dexamethasone. *Br J Pharmacol* **113:** 1008-1014.

Mitchell JA, Larkin S, and Williams TJ (1995) Cyclooxygenase-2: regulation and relevance in inflammation. *Biochem Pharmacol* **50:** 1535-1542.

Mitchell JA, Saunders M, Barnes PJ, Newton R, and Belvisi MG (1997) Sodium salicylate inhibits cyclo-oxygenase-2 activity independently of transcription factor (nuclear factor kappaB) activation: role of arachidonic acid. *Mol Pharmacol* **51:** 907-912.

Mitchell JA and Warner TD (2005) Discontinuation of Vioox. *Lancet* **365:** 27 – 28.

Miyamoto T, Ogino N, Yamamoto S, and Hayaishi O (1976) Purification of prostaglandin endoperoxide synthetase from bovine vesicular gland microsomes. *J Biol Chem* **251:** 2629-2636.

Moncada S, Needleman P, Bunting S, and Vane JR (1976) Prostaglandin endoperoxide and thromboxane generating systems and their selective inhibition. *Prostaglandins* **12:** 323-329.

Moore SD, Brodt-Eppley J, Cornelison LM, Burk SE, Slater DM, and Myatt L (1999) Expression of prostaglandin H synthase isoforms in human myometrium at parturition. *Am J Obstet Gynecol* **180:** 103-109.

Morteau O, Morham SG, Sellon R, Dieleman LA, Langenbach R, Smithies O, and Sartor RB (2000) Impaired mucosal defense to acute colonic injury in mice lacking cyclooxygenase-1 or cyclooxygenase-2. *J Clin Investig* **105:** 469-478.

Mousses S, Caplen NJ, Cornelison R, Weaver D, Basik M, et al. 2003. RNAi microarray analysis in cultured mammalian cells. *Genome Res.* **13**:2341–47.

Murata T, Ushikubi F, Matsuoka T, Hirata M, Yamasaki A, Sugimoto Y, Ichikawa A, Aze Y, Tanaka T, Yoshida N, et al. (1997) Altered pain perception and inflammatory response in mice lacking prostacyclin receptor. *Nature (Lond)* **388:** 678-682.

Murphy DJ and Vance J (1999) Mechanisms of lipid-body formation. *Trends Biochem Sci* **24:** 109-115.

Nakajima G, Hayashi K, Xi Y, Kudo K, Uchida K, Takasaki K, Yamamoto M, Ju J (2006). Non-coding MicroRNAs hsa-let-7g and hsa-miR-181b are Associated with Chemoresponse to S-1 in Colon Cancer. *Cancer Genomics Proteomics*.**3(5)**:317-324.

Napoli, C., Lemieux, C., Jorgensen, R. (1990) Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. *Plant Cell* **2**,279-289

Neeraja S, Sreenath AS, Reddy PR, and Reddanna P (2003) Expression of cyclooxygenase-2 in the testis. *Reprod Biomed Online* **6:** 302-309.

Nieminen R., Lahti A., Jalonen U., Kankaanranta H. and Moilanen E. (2006). JNK inhibitor SP600125 reduces COX-2 expression by attenuating mRNA in activated murine J774 macrophages, *Int. Immunopharmacol.* **6**: 987–996.

Nottrott S, Simard MJ, Richter JD (2006). Human let-7a miRNA blocks protein production on actively translating polyribosomes. *Nat Struct Mol Biol*; **13**: 1108– 14.

O'Banion MK (1999) COX-2 and Alzheimer's disease: potential roles in inflammation and neurodegeneration. *Expert Opin Investig Drugs* **8:** 1521-1536.

O'Banion MK, Sadowski HB, Winn V, and Young DA (1991) A serumand glucocorticoid-regulated 4-kilobase mRNA encodes a cyclooxygenase-related protein. *J Biol Chem* **266:** 23261-23267.

O'Banion MK, Winn V, and Young DA (1992) cDNA cloning and functional activity of a glucocorticoid-regulated inflammatory cyclooxygenase. *Proc Natl Acad Sci USA* **89:** 4888-4892.

O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, Mendell JT (2005). c-Myc-regulated microRNAs modulate E2F1 expression. *Nature*; **435**: 839– 43.

Ogino N, Ohki S, Yamamoto S, and Hayaishi O (1978) Prostaglandin endoperoxide synthetase from bovine vesicular gland microsomes: inactivation and activation by heme and other metalloporphyrins. *J Biol Chem* **253:** 5061-5068.

Ohki S, Ogino N, Yamamoto S, and Hayaishi O (1979) Prostaglandin hydroperoxidase, an integral part of prostaglandin endoperoxide synthetase from bovine vesicular gland microsomes. *J Biol Chem* **254:** 829-836.

Ohrt T, Merkle D, Birkenfeld K, Echeverri CJ, Schwille P. 2006. In situ fluorescence analysis demonstrates active siRNA exclusion from the nucleus by Exportin 5. *Nucleic Acids Res.* **34**:1369–80

Oida H, Namba T, Sugimoto Y, Ushikubi F, Ohishi H, Ichikawa A, and Narumiya S (1995) In situ hybridization studies of prostacyclin receptor mRNA expression in various mouse organs. *Br J Pharmacol* **116:** 2828-2837.

Oliw EH, Su C, Skogstrom T, and Benthin G (1998) Analysis of novel hydroperoxides and other metabolites of oleic, linoleic, and linolenic acids by liquid chromatography-mass spectormetry and ion trap MSn. *Lipids* **33:** 843-852.

Osborn JL, Kopp UC, Thames MD, and DiBona GF (1984) Interactions among renal nerves, prostaglandins, and renal arterial pressure in the regulation of renin release. *Am J Physiol* **247:** F706-F713.

Oshima M, Dinchuk JE, Kargman SL, Oshima H, Hancock B, Kwong E, Trzaskos JM, Evans JF, and Taketo MM (1996) Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). *Cell* **87:** 803-809.

Ota A, Tagawa H, Karnan S, Tsuzuki S, Karpas A, Kira S, *et al*. Identification and characterization of a novel gene, C13orf25, as a target for 13q31-q32 amplification in malignant lymphoma. *Cancer Res* 2004; **64**: 3087– 95. Ouellet M and Percival MD (2001) Mechanism of acetaminophen inhibition of cyclooxygenase isoforms. *Arch Biochem Biophys* **387:** 273-280.

Pablos JL, Santiago B, Carreira PE, Galindo M, and Gomez-Reino JJ (1999) Cyclooxygenase-1 and -2 are expressed by human T cells. *Clin Exp Immunol* **115:** 86-90.

Papafili A, Hill MR, Brull DJ, McAnulty RJ, Marshall RP, Humphries SE, and Laurent GJ (2002) Common promoter variant in cyclooxygenase-2 represses gene expression: evidence of role in acute-phase inflammatory response. *Arterioscler Thromb Vasc Biol* **22:** 1641-1646.

Park BH, Vogelstein B, and Kinzler KW (2001) Genetic disruption of PPARdelta decreases the tumorigenicity of human colon cancer cells. *Proc Natl Acad Sci USA* **98:** 2598-2603.

Park S.W., Sung M.W., Heo D.S., Inoue H., Shim S.H. and Kim K.H. (2005). Nitric oxide upregulates the cyclooxygenase-2 expression through the cAMP-response element in its promoter in several cancer cell lines, *Oncogene* **24**: 6689–6698.

Parker CW (1986) Leukotrienes and prostaglandins in the immune system. *Adv Prostaglandin Thromboxane Leukot Res* **16:** 113-134.

Parker JS, Barford D. 2006. Argonaute: a scaffold for the function of short regulatory RNAs. *Trends Biochem. Sci.* **31**:622–30.

Pasricha PJ, Bedi A, O'Connor K, Rashid A, Akhatar AJ, Zahurak ML, Piantadosi S, Hamilton SR, and Giardiello FM (1995) The effects of sulindac on colorectal proliferation and apoptosis in familial adenomatous polyposis. *Gastroenterology* **109:** 994-998.

Patrignani P, Panara MR, Greco A, Fusco O, Natoli C, Iacobelli S, Cipollone F, Ganci A, Creminon C, Maclouf J, et al. (1994) Biochemical and pharmacological characterization of the cyclooxygenase activity of human blood prostaglandin endoperoxide synthases. *J Pharmacol Exp Ther* **271:** 1705-1712.

Pavord ID and Tattersfield AE (1995) Bronchoprotective role for endogenous prostaglandin E2. *Lancet* **344:** 436-438.

Pekarsky Y, Santanam U, Cimmino A, Palamarchuk A, Efanov A, Maximov V, *et al*. (2006). Tcl1 expression in chronic lymphocytic leukemia is regulated by miR-29 and miR-181. *Cancer Res*; **66**: 11590– 3.

Pettus B.J., Bielawski J., Porcelli A.M., Reames D.L., Johnson K.R., Morrow J., Chalfant C.E., Obeid L.M. and Hannun Y.A. (2003). The sphingosine kinase 1/sphingosine-1-phosphate pathway mediates COX-2 induction and PGE2 production in response to TNF-alpha, *FASEB J.* **17**: 1411–1421.

Phillips K., Kedersha N., Shen L., Blackshear P.J. and Anderson P. (2004). Arthritis suppressor genes TIA-1 and TTP dampen the expression of tumor necrosis factor alpha, cyclooxygenase 2, and inflammatory arthritis, *Proc. Natl. Acad. Sci. U. S. A.* **101**: 2011–2016.

Phillips RK, Wallace MH, Lynch PM, Hawk E, Gordon GB, Saunders BP, Wakabayashi N, Shen Y, Zimmerman S, Godio L, et al.; FAP Study Group (2002) A randomized, double blind, placebo controlled study of celecoxib, a selective cyclooxygenase 2 inhibitor, on duodenal polyposis in familial adenomatous polyposis. *Gut* **50:** 857-860.

Picot D, Loll P, and Garavito M (1994) The X-ray crystal structure of the membrane protein prostaglanind H2 synthase-1. *Nature (Lond)* **367:** 243-249.

Pierce KL, Gil DW, Woodward DF, and Regan JW (1995) Cloning of human prostanoid receptors. *Trends Pharmacol Sci* **16:** 253-256.

Pillai RS, Bhattacharyya SN, Artus CG, Zoller T, Cougot N, Basyuk E, *et al*. (2005). Inhibition of translational initiation by Let-7 MicroRNA in human cells. *Science*; **309**: 1573– 6.

Plant MH and Laneuville O (1999) Characterization of a novel transcript of prostaglandin endoperoxide H synthase 1 with a tissue-specific profile of expression. *Biochem J* **344:** 677-685.

Plescia OJ, Smith A, and Grinwich K (1975) Subversion of the immune system by tumor cells and the role of prostaglandins. *Proc Natl Acad Sci USA* **72:** 1848-1852.

Pollard M and Luckert PH (1981) Treatment of chemically induced intestinal cancers with indomethacin. *Proc Soc Exp Biol Med* **167:** 161-164.

Portanova JP, Zhang Y, Anderson GD, Hauser SD, Masferrer JL, Seibert K, Gregory SA, and Isakson PC (1996) Selective neutralization of prostaglandin E2 blocks inflammation, hyperalgesia, and interleukin 6 production in vivo. *J Exp Med* **184:** 883-891.

Ramer R, Weinzierl U, Schwind B, Brune K, and Hinz B (2003) Ceramide is involved in r(+)-methanandamide-induced cyclooxygenase-2 expression in human neuroglioma cells. *Mol Pharmacol* **64:** 1189-1198.

Reddy BS, Maruyama H, and Kelloff G (1987) Dose-related inhibition of colon carcinogenesis by dietary piroxicam, a nonsteroidal anti-inflammatory drug, during different stages of rat colon tumor development. *Cancer Res* **47:** 5340-5346.

Reynolds A, Anderson EM, Vermeulen A, Fedorov Y, Robinson K, et al. 2006. Induction of the interferon response by siRNA is cell type- and duplex length-dependent. *RNA* **12**:988–93

Reynolds A, Leake D, Boese Q, Scaringe S, Marshall WS, Khvorova A. 2004. Rational siRNA design for RNA interference. *Nat. Biotechnol.* **22**:326–30.

Rieke CJ, Mulichak AM, Garavito RM, and Smith WL (1999) The role of arginine 120 of human prostaglandin endoperoxide H synthase-2 in the interaction with fatty acid substrates and inhibitors. *J Biol Chem* **274:** 17109-17114.

Roberts LJ 2nd, Sweetman BJ, Lewis RA, Austen KF, and Oates JA (1980) Increased production of prostaglandin D2 in patients with systemic mastocytosis. *N Engl J Med* **303:** 1400-1404.

Rodriguez A, Griffiths-Jones S, Ashurst JL, Bradley A (2004). Identification of mammalian microRNA host genes and transcription units. *Genome Res*; **14**: 1902– 10.

Romano, N., Macino, G. (1992) Quelling: transient inactivation of gene expression in Neurospora crassa by transformation with homologous sequences. *Mol. Microbiol.* **6**,3343-3353

Rossi A, Kapahi P, Natoli G, Takahashi T, Chen Y, Karin M, and Santoro MG (2000) Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of IκB kinase. *Nature (Lond)* **403:** 103-108.

Roth GJ, Machuga ET, and Ozols J (1983) Isolation and covalent structure of the aspirin-modified, active site region of prostaglandin synthetase. *Biochemistry* **22:** 4672-4675.

Roth GJ, Siok CJ, and Ozols J (1980) Structural characteristics of prostaglandin synthetase from sheep vesicular glands. *J Biol Chem* **255:** 1301-1304.

Roth GJ, Stanford N, and Majerus PW (1975) Acetylation of prostaglandin synthase by aspirin. *Proc Natl Acad Sci USA* **72:** 3073-3076.

Rowlinson SW, Crews BC, Goodwin DC, Schneider C, Gierse JK, and Marnett LJ (2000) Spatial requirements for 15-(R)-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid synthesis within the cyclooxygenase active site of murine COX-2. Why acetylated COX-1 does not synthesize 15-(R)-hete. *J Biol Chem* **275:** 6586-6591.

Rubinson DA, Dillon CP, Kwiatkowski AV, Sievers C, Yang L, et al. 2003. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat. Genet.* **33**:401–6

Saito Y, Liang G, Egger G, Friedman JM, Chuang JC, Coetzee GA, *et al*. (2006). Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. *Cancer Cell*; **9**: 435– 43.

Samad TA, Moore KA, Sapirstein A, Billet S, Allchorne A, Poole S, Bonventre JV, and Woolf CJ (2001) Interleukin-1β-mediated induction of COX-2 in the CNS contributes to inflammatory pain hypersensitivity. *Nature (Lond)* **410:** 471-475.

Samuelsson B (1963) Isolation and identification of prostaglandins from human seminal plasma. 18. Prostaglandins and related factors. *J Biol Chem* **238:** 3229-3234.

Sandler RS, Halabi S, Baron JA, Budinger S, Paskett E, Keresztes R, Petrelli N, Pipas JM, Karp DD, Loprinzi CL, et al. (2003) A randomized trial of aspirin to prevent colorectal adenomas in patients with previous colorectal cancer. *N Engl J Med* **348:** 883-890.

Sang N, Stiehl DP, Bohensky J, Leshchinsky I, Srinivas V, Caro J. (2003). MAPK signaling up-regulates the activity of hypoxia-inducible factors by its effects on p300. *J Biol Chem.* **278**:14013–9.

Sano H, Hla T, Maier JA, Crofford LJ, Case JP, Maciag T, and Wilder RL (1992) In vivo cyclooxygenase expression in synovial tissues of patients with rheumatoid arthritis and osteoarthritis and rats with adjuvant and streptococcal cell wall arthritis. *J Clin Investig* **89:** 97-108.

Sanz A, Moreno JI, and Castresana C (1998) PIOX, a new pathogen-induced oxygenase with homology to animal cyclooxygenase. *Plant Cell* **10:** 1523-1537.

Saunders MA, Sansores-Garcia L, Gilroy DW, Wu KK (2001). Selective suppression of CCAAT/enhancer-binding protein beta binding and cyclooxygenase-2 promoter activity by sodium salicylate in quiescent human fibroblasts. *J Biol Chem.*; **276**: 18897–18904.

Scacheri PC, Rozenblatt-Rosen O, Caplen NJ, Wolfsberg TG, Umayam L, et al. 2004. Short interfering RNAs can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells. *Proc. Natl. Acad. Sci. USA* **101**:1892–97.

Scarfo LM, Weller PF, and Farber HW (2001) Induction of endothelial cell cytoplasmic lipid bodies during hypoxia. *Am J Physiol Heart Circ Physiol* **280:** H294-H301.

Scharf S, Mander A, Ugoni A, Vajda F, and Christophidis N (1999) A double blind, placebo-controlled trial of diclofenac/misoprostol in Alzheimer's disease. *Neurology* **53:** 197-201.

Schetter AJ, Leung SY, Sohn JJ, Zanetti KA, Bowman ED, Yanaihara N, Yuen ST, Chan TL, Kwong DL, Au GK, Liu CG, Calin GA, Croce CM, Harris CC (2008). MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. *JAMA*. **299(4)**:425-36.

Schmedtje JF, Ji YS, Liu WL, DuBois RN, Runge MS (1996). Hypoxia induces cyclooxygenase-2 via the NF-κB p65 transcription factor in human vascular endothelial cells. *Circulation.* **94**:2392.

Schneider C, Boeglin WE, Prusakiewicz JJ, Rowlinson SW, Marnett LJ, Samel N, and Brash AR (2002) Control of prostaglandin stereochemistry at the 15-carbon by cyclooxygenase-1 and -2. A critical role for serine 530 and valine 349. *J Biol Chem* **277:** 478-485.

Schonbeck U, Sukhova GK, Graber P, Coulter S, and Libby P (1999) Augmented expression of cyclooxygenase-2 in human atherosclerotic lesions. *Am J Pathol* **155:** 1281-1291.

Schroer K, Zhu Y, Saunders MA, Deng WG, Xu XM, Meyer-Kirchrath J, Wu KK (2002). Obligatory role of cyclic adenosine monophosphate response element in cyclooxygenase-2 promoter induction and feedback regulation by inflammatory mediators. *Circulation.*; **105**: 2760–2765.

Schwartzman M, Ferreri NR, Carroll MA, Songu-Mize E, and McGiff JC (1985) Renal cytochrome P450-related arachidonate metabolite inhibits (Na+-K+)ATPase. *Nature (Lond)* **314:** 620-622.Sciulli MG, Seta F, Tacconelli S, Capone ML, Ricciotti E, Pistritto G, and Patrignani P (2003) Effects of acetaminophen on constitutive and inducible prostanoid biosynthesis in human blood cells. *Br J Pharmacol* **138:** 634-641.[

Schwarz DS, Hutvagner G, Du T, Xu Z, Aronin N, Zamore PD. 2003. Asymmetry in the assembly of the RNAi enzyme complex. *Cell* **115**:199–208.

Scott GK, Mattie MD, Berger CE, Benz SC, Benz CC (2006). Rapid alteration of microRNA levels by histone deacetylase inhibition. *Cancer Res*; **66**: 1277– 81.

Segi E, Sugimoto Y, Yamasaki A, Aze Y, Oida H, Nishimura T, Murata T, Matsuoka T, Ushikubi F, Hirose M, et al. (1998) Patent ductus arteriosus and neonatal death in prostaglandin receptor EP4-deficient mice. *Biochem Biophys Res Commun* **246:** 7-12.

Selinsky BS, Gupta K, Sharkey CT, and Loll PJ (2001) Structural analysis of NSAID binding by prostaglandin H_2 synthase: time-dependent and time-independent inhibitors elicit identical enzyme carformation. *Biochemistry* **40:** 5172-5180.

Semenza GL (2002). HIF-1 and tumor progression: pathophysiology and therapeutics. *Trends Mol Med*. **8**:S62–7.

Semenza GL (2003). Targeting HIF-1 for cancer therapy. *Nat Rev Cancer.* **3**:721–32.

Semizarov D, Frost L, Sarthy A, Kroeger P, Halbert DN, Fesik SW. 2003. Specificity of short interfering RNA determined through gene expression signatures. *Proc. Natl. Acad. Sci. USA* **100**:6347–52

Sen GL, Blau HM (2005). Argonaute 2/RISC resides in sites of mammalian mRNA decay known as cytoplasmic bodies. *Nat Cell Biol*; **7**: 633– 6.

Shea-Donohue PT, Nompleggi D, Myers L, and Dubois A (1982) A comparison of effects of prostacyclin and the 15(S),15-methyl analogs of PGE_2 and $PGF_{2\alpha}$ on gastric parietal and non-parietal secretion. *Dig Dis Sci* **27:** 17-22.

Sheth U, Parker R (2003). Decapping and decay of messenger RNA occur in cytoplasmic processing bodies. *Science*; **300**: 805– 8.

Sheng H, Shao J, Washington MK, and DuBois RN (2001) Prostaglandin E2 increases growth and motility of colorectal carcinoma cells. *J Biol Chem* **276:** 18075-18081.

Shimokawa T, Kulmacz RJ, DeWitt DL, and Smith WL (1990) Tyrosine 385 of prostaglandin endoperoxide synthase is required for cyclooxygenase catalysis. *J Biol Chem* **265:** 20073-20076.

Shimokawa T and Smith WL (1992) Prostaglandin endoperoxide synthase: the aspirin acetylation region. *J Biol Chem* **267:** 12387-12392.

Shindo H, Tawata M, Aida K, and Onaya T (1991) Clinical efficacy of a stable prostacyclin analog, iloprost, in diabetic neuropathy. *Prostaglandins* **41:** 85-96.

Si ML, Zhu S, Wu H, Lu Z, Wu F, Mo YY (2007). miR-21-mediated tumor growth. *Oncogene.* **26**(19):2799-2803.

Simmons DL, Levy DB, Yannoni Y, and Erikson RL (1989) Identification of a phorbol ester-repressible v-src-inducible gene. *Proc Natl Acad Sci USA* **86:** 1178-1182.

Simmons DL and Wilson JE (2001) Apoptosis induced with non-steroid antiinflammatory drugs, in *Therapeutic Roles of Selective COX-2 inhibitors* (Vane JR and Botting R eds) pp 221-251, William Harvey Press, London.

Simmons DL, Xie W, Chipman JG, and Evett GE (1991) Multiple cyclooxygenases: Cloning of an inducible form, in *Prostaglandins, Leukotrienes, Lipoxins and PAF* (Bailey JM ed) pp 67-78, Plenum Press, New York.

Sinicrope FA, Lemoine M, Xi L, Lynch PM, Cleary KR, Shen Y, Frazier ML (1999). Reduced expression of cyclooxygenase 2 proteins in hereditary nonpolyposis colorectal cancers relative to sporadic cancers. *Gastroenterology*. **117**(2): 350–358.

Sladek K, Dworski R, FitzGerald GA, Buitkus KL, Block FJ, Marney SR Jr, and Sheller JR (1990) Allergen-stimulated release of thromboxane A2 and leukotriene E4 in humans. Effect of indomethacin. *Am Rev Respir Dis* **141:** 1441-1445.

Sledz CA, Holko M, de Veer MJ, Silverman RH, Williams BR. (2003). Activation of the interferon system by short-interfering RNAs. *Nat. Cell Biol.* **5**:834–39.

Smith HS and Baird W (2003) Meloxicam and selective COX-2 inhibitors in the management of pain in the palliative care population. *Am J Hosp Palliat Care* **20:** 297-306.

Smith JA, Amagasu SM, Eglen RM, Hunter JC, and Bley KR (1998b) Characterisation of prostanoid receptor-evoked responses in rat sensory neurones. *Br J Pharmacol* **124:** 513-523.

Smith WL, DeWitt DL, and Garavito RM (2000) Cyclooxygenases: structural, cellular and molecular biology. *Annu Rev Biochem* **69:** 145-182.

Smith WL and Lands WE (1972) Oxygenation of polyunsaturated fatty acids during prostaglandin biosynthesis by sheep vesicular gland. *Biochemistry* **11:** 3276-3285.

Song I and Smith WL (1996) C-terminal Ser/Pro-Thr-Glu-Leu tetrapeptides of prostaglandin endoperoxide H synthase-1 and -2 target the enzymes to the endoplasmic reticulum. *Arch Biochem Biophys* **334:** 67-72.

Sonoki T, Iwanaga E, Mitsuya H, Asou N. Insertion of microRNA-125b-1, a human homologue of lin-4, into a rearranged immunoglobulin heavy chain gene locus in a patient with precursor B-cell acute lymphoblastic leukemia. *Leukemia* 2005; **19**: 2009– 10.

Soutschek J, Akinc A, Bramlage B, Charisse K, Constien R, et al. 2004. Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature* **432**:173–78

Stark A, Brennecke J, Bushati N, Russell RB, Cohen SM. Animal MicroRNAs confer robustness to gene expression and have a significant impact on 3' UTR evolution. *Cell* 2005; **123**: 1133– 46.

Stewart WF, Kawas C, Corrada M, and Metter EJ (1997) Risk of Alzheimer's disease and duration of NSAID use. *Neurology* **48:** 626-632.

Stock JL, Shinjo K, Burkhardt J, Roach M, Taniguchi K, Ishikawa T, Kim HS, Flannery PJ, Coffman TM, McNeish JD, et al. (2001) The prostaglandin E2 EP1 receptor mediates pain perception and regulates blood pressure. *J Clin Investig* **107:** 325-331.

Straus DS, Pascual G, Li M, Welch JS, Ricote M, Hsiang CH, Sengchanthalangsy LL, Ghosh G, and Glass CK (2000) 15-Deoxy-Δ12,14-prostaglandin J2 inhibits multiple steps in the NF-κB signaling pathway. *Proc Natl Acad Sci USA* **97:** 4844-4849.

Su C, Sahlin M, and Oliw EH (1998) A protein radical and ferryl intermediates are generated by linoleate diol synthase, a ferric hemeprotein with dioxygenase and hydroperoxide isomerase activities. *J Biol Chem* **273:** 20744-20751.

Sugimoto Y, Yamasaki A, Segi E, Tsuboi K, Aze Y, Nishimura T, Oida H, Yoshida N, Tanaka T, Katsuyama M, et al. (1997) Failure of parturition in mice lacking the prostaglandin F receptor. *Science (Wash DC)* **277:** 681-683.

Sugito N, Ishiguro H, Kuwabara Y, Kimura M, Mitsui A, Kurehara H, *et al*. (2006). RNASEN regulates cell proliferation and affects survival in esophageal cancer patients. *Clin Cancer Res*; **12**: 7322– 8.

Surh Y.J., Chun K.S., Cha H.H., Han S.S., Keum Y.S., Park K.K. and Lee S.S. (2001). Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: Down-regulation of COX-2 and iNOS through suppression of NF-kappa B activation, *Mutat. Res.* **480/481**: 243–268.

Takeda H, Sonoshita M, Oshima H, Sugihara K, Chulada PC, Langenbach R, Oshima M, and Taketo MM (2003) Cooperation of cyclooxygenase 1 and cyclooxygenase 2 in intestinal polyposis. *Cancer Res* **63:** 4872-4877.

Takeuchi K, Ukawa H, Kato S, Furukawa O, Araki H, Sugimoto Y, Ichikawa A, Ushikubi F, and Narumiya S (1999) Impaired duodenal bicarbonate secretion and mucosal integrity in mice lacking prostaglandin E-receptor subtype EP3. *Gastroenterology* **117:** 1128-1135.

Tanzer A, Stadler PF (2004). Molecular evolution of a microRNA cluster. *J Mol Biol.* **339**(2):327-335.

Tazawa H, Tsuchiya N, Izumiya M, Nakagama H (2007). Tumor-suppressive miR-34a induces senescence-like growth arrest through modulation of the E2F pathway in human colon cancer cells. *Proc Natl Acad Sci U S A*. **104(39)**:15472-7.

Tazawa R, Xu XM, Wu KK, Wang LH (1994). Characterization of the genomic structure, chromosomal location and promoter of human prostaglandin H synthase-2 gene. *Biochem Biophys Res Commun.* **203**: 190–199.

Tetsu, O., and McCormick, F (1999). β-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature (Lond.)*, **398:** 422–426.

Thomas M, Lu JJ, Ge Q, Zhang C, Chen J, Klibanov AM. 2005. Full deacylation of polyethylenimine dramatically boosts its gene delivery efficiency and specificity to mouse lung. *Proc. Natl. Acad. Sci. USA* **102**:5679–84

Thuresson ED, Lakkides KM, Rieke CJ, Sun Y, Wingerd BA, Micielli R, Mulichak AM, Malkowski MG, Garavito RM, and Smith WL (2001) Prostaglandin endoperoxide H synthase-1: the function of cyclooxygenase active site residues in the binding, positioning, and oxygenation of arachidonic acid. *J Biol Chem* **276:** 10347-10358.

Tilley SL, Audoly LP, Hicks EH, Kim HS, Flannery PJ, Coffman TM, and Koller BH (1999) Reproductive failure and reduced blood pressure in mice lacking the EP2 prostaglandin E2 receptor. *J Clin Investig* **103:** 1539-1545.

Timmons L, and Fire A (1998). Specific interference by ingested dsRNA. *Nature* **395**: 854.

Topper JN, Cai J, Falb D, and Gimbrone MA Jr (1996) Identification of vascular endothelial genes differentially responsive to fluid mechanical stimuli: cyclooxygenase-2, manganese superoxide dismutase, and endothelial cell nitric oxide synthase are selectively up-regulated by steady laminar shear stress. *Proc Natl Acad Sci USA* **93:** 10417-10422.

Trifan OC, Smith RM, Thompson BD, and Hla T (1999) Overexpression of cyclooxygenase-2 induces cell cycle arrest. Evidence for a prostaglandin-independent mechanism. *J Biol Chem* **274:** 34141-34147.

Tsuda N, Ishiyama S, Li Y, Ioannides CG, Abbruzzese JL, Chang DZ (2006). Synthetic microRNA designed to target glioma-associated antigen 1 transcription factor inhibits division and induces late apoptosis in pancreatic tumor cells. *Clin Cancer Res*; **12**: 6557– 64.

Tsujii M and DuBois RN (1995) Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. *Cell* **83:** 493-501.

Tsujii M, Kawano S, Tsuji S, Sawaoka H, Hori M, and DuBois RN (1998) Cyclooxygenase regulates angiogenesis induced by colon cancer cells. *Cell* **93:** 705-716.

Tuder RM, Cool CD, Geraci MW, Wang J, Abman SH, Wright L, Badesch D, and Voelkel NF (1999) Prostacyclin synthase expression is decreased in lungs from patients with severe pulmonary hypertension. *Am J Respir Crit Care Med* **159:** 1925-1932.

Tuynman JB, Peppelenbosch MP and Richel DJ (2004) Cox-2 inhibition as a tool to treat and prevent colorectal cancer. *Crit Rev Oncol Hematol* **52:** 81 – 101.

Urade Y, Fujimoto N, Kaneko T, Konishi A, Mizuno N, and Hayaishi O (1987) Postnatal changes in the localisation of prostaglandin D synthetase from neurons to oligodendrocytes in the rat brain. *J Biol Chem* **262:** 15132-15136.

Ushikubi F, Segi E, Sugimoto Y, Murata T, Matsuoka T, Kobayashi T, Hizaki H, Tuboi K, Katsuyama M, Ichikawa A, et al. (1998) Impaired febrile response in mice lacking the prostaglandin E receptor subtype EP3. *Nature (Lond)* **395:** 281-284.

Ushikubi F, Sugimoto Y, Ichikawa A, and Narumiya S (2000) Roles of prostanoids revealed from studies using mice lacking specific prostanoid receptors. *Jpn J Pharmacol* **83:** 279-285.

van der Ouderaa FJ, Buytenhek M, Nugteren DH, and Van Dorp DA (1977) Purification and characterisation of prostaglandin endoperoxide synthetase from sheep vesicular glands. *Biochim Biophys Acta* **487:** 315-333.

Varnum BC, Lim RW, Sukhatme VP, and Herschman HR (1989) Nucleotide sequence of a cDNA encoding TIS11, a message induced in Swiss 3T3 cells by the tumor promoter tetradecanoyl phorbol acetate. *Oncogene* **4:** 119-120.

Ventura A, Meissner A, Dillon CP, McManus M, Sharp PA, et al. 2004. Cre-lox-regulated conditional RNA interference from transgenes. *Proc. Natl. Acad. Sci. USA* **101**:10380–85

Vogiagis D, Glare EM, Misajon A, Brown W, and O'Brien PE (2000) Cyclooxygenase-1 and an alternatively spliced mRNA in the rat stomach: effects of aging and ulcers. *Am J Physiol Gastrointest Liver Physiol* **278:** G820-G827.

Voinnet, O., Baulcombe, D. C. (1997) Systemic signalling in gene silencing. *Nature* **389**,553

Wadleigh DJ, Reddy ST, Kopp E, Ghosh S, Herschman HR (2000). Transcriptional activation of the cyclooxygenase-2 gene in endotoxin-treated RAW 264.7 macrophages. *J Biol Chem.*; **275**: 6259–6266.

Wallace JL, McKnight W, Reuter BK, and Vergnolle N (2000) NSAID-induced gastric damage in rats: Requirements for inhibition of both cyclooxygenase 1 and 2. *Gastroenterology* **119:** 706-714.

Wang CL, Wang BB, Bartha G, Li L, Channa N, Klinger M, *et al*. Activation of an oncogenic microRNA cistron by provirus integration. *Proc Natl Acad Sci U S A* 2006; **103**: 18680– 4.

Wang D, Buchanan FG, Wang H, Dey SK, DuBois RN (2005). Prostaglandin E2 enhances intestinal adenoma growth via activation of the Ras-mitogen-activated protein kinase cascade. *Cancer Res.* **65**:1822–9.

Wang W., Fan J., Yang X., Furer-Galban S., Lopez de Silanes I., von Kobbe C., Guo J., Georas S.N., Foufelle F., Hardie D.G., Carling D. and Gorospe M. (2002). AMP-activated kinase regulates cytoplasmic HuR, *Mol. Cell. Biol.* **22**: 3425–3436.

Warner TD, Giuliano F, Vojnovic I, Bukasa A, Mitchell JA, and Vane JR (1999) Nonsteroidal drug selectivities for cyclooxygenase-1 rather than cyclooxygenase-2 are associated with human gastrointestinal toxicity: a full in vitro analysis. *Proc Natl Acad Sci USA* **96:** 7563-7568.

Wegner M, Cao Z, Rosenfeld MG (1992). Calcium-regulated phosphorylation within the leucine zipper of C/EBP beta. *Science.* **256**: 370–373.

Whittle BJ, Boughton-Smith NK, Moncada S, and Vane JR (1978) Actions of prostacyclin (PGI2) and its product 6-oxo-PGF1α on the rat gastric mucosa in vivo and in vitro. *Prostaglandins* **15:** 955-968.

Whittle BJR and Salmon JA (1983) in *Intestinal Secretion* (Turnberg LA ed) pp 69-73, Smith Kline and French Publications, Welwyn Garden City, Hertfordshire, England, UK.

Williams TJ and Peck MJ (1977) Role of prostaglandin-mediated vasodilatation in inflammation. *Nature (Lond)* **270:** 530-532.

Woo KJ, Lee TJ, Park JW, Kwon TK (2006). Desferrioxamine, an iron chelator, enhances HIF-1alpha accumulation via cyclooxygenase-2 signaling pathway. *Biochem Biophys Res Commun*. **343**(1):8-14.

Wu D., Marko M., Claycombe K., Paulson K.E. and Meydani S.N. (2003). Ceramide-induced and age-associated increase in macrophage COX-2 expression is mediated through up-regulation of NF-kappa B activity, *J. Biol. Chem.* **278**: 10983–10992.

Wu M.H., Wang C.A., Lin C.C., Chen L.C., Chang W.C. and Tsai S.J. (2005). Distinct regulation of cyclooxygenase-2 by interleukin-1beta in normal and endometriotic stromal cells, *J. Clin. Endocrinol. Metab.* **90**: 286–295.

Xi Y, Formentini A, Chien M, Weir DB, Russo JJ, Ju J, *et al*. (2006). Prognostic values of microRNAs in colorectal cancer. *Biomarker Insights* ; **2**: 113– 121.

Xi Y, Shalgi R, Fodstad O, Pilpel Y, Ju J. (2006) Differentially regulated micro-RNAs and actively translated messenger RNA transcripts by tumor suppressor p53 in colon cancer. *Clin Cancer Res.* Apr 1;**12**(7 Pt 1):2014-24.

Xiang S, Fruehauf J, and Li CJ (2006) Short hairpin RNA–expressing bacteria elicit RNA interference in mammals. *Nature Biotechnology* **24**, 697 – 702.

Xie W, Chipman JG, Robertson DL, Erikson RL, and Simmons DL (1991) Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. *Proc Natl Acad Sci USA* **88:** 2692-2696.

Yaksh TL and Svensson C (2001) Role of spinal cyclooxygenases in nociceptive processing, in *Therapeutic Roles of Selective COX-2 Inhibitors* (Vane JR and Botting RM eds) pp 168-190, William Harvey Press, London.

Yamagata K, Andreasson KI, Kaufman WE, Barnes CA, and Worley PF (1993) Expression of a mitogen-inducible cyclooxygenase in brain neurons: regulation by synaptic activity and glucocorticoids. *Neuron* **11:** 371-386.

Yamauchi T, Watanabe M, Kubota T, Hasegawa H, Ishii Y, Endo T, Kabeshima Y, Yorozuya K, Yamamoto K, Mukai M, Kitajima M (2002) Cyclooxygenase-2 expression as a new marker for patients with colorectal cancer. *Dis Colon Rectum* **45:** 98 – 103.

Yanaihara N, Caplen N, Bowman E, Seike M, Kumamoto K, Yi M, *et al*. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. *Cancer Cell* 2006; **9**: 189– 98.

Yekta S, Shih IH, Bartel DP. MicroRNA-directed cleavage of HOXB8 mRNA. *Science* 2004; **304**: 594– 6.

Yi R, Doehle BP, Qin Y, Macara IG, Cullen BR. 2005. Overexpression of exportin 5 enhances RNA interference mediated by short hairpin RNAs and microRNAs. *RNA* **11**:220–26

Yokoyama C and Tanabe T (1989) Cloning of human gene encoding prostaglandin endoperoxide synthase and primary structure of the enzyme. *Biochem Biophys Res Commun* **165:** 888-894.

Yu MK, Mendell JT, Glenn MG, Chen Z, Phillips JD (2006). DNA methylation inhibitors upregulate miRNA expression in patients with chronic lymphocytic leukemia. *Blood*; **108**: Abstract 2257.

Zamore, P. D., Tuschl, T., Sharp, P. A., Bartel, D. P. (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**,25-33

Zhang X, Morham SG, Langenbach R, and Young DA (1999) Malignant transformation and antineoplastic actions of nonsteroidal anti-inflammatory drugs (NSAIDs) on cyclooxygenase-null embryo fibroblasts. *J Exp Med* **190:** 451-459.

Zhang Y, Shaffer A, Portanova J, Seibert K, and Isakson PC (1997) Inhibition of cyclooxygenase-2 rapidly reverses inflammatory hyperalgesia and prostaglandin E_2 production. *J Pharmacol Exp Ther* **283:** 1069-1075.

Zhang YH, Lu J, Elmquist JK, and Saper CB (2003) Specific notes of cyclooxygenase-1 and cyclooxygenase-2 in liopolysaccharide induced fever and Fos expression in rat brain. *J Comp Neurol* **463:** 3-12.

Ziche M, Jones J, and Gullino PM (1982) Role of prostaglandin E1 and copper in angiogenesis. *J Natl Cancer Inst* **69:** 475-482.

Zhu S, Si ML, Wu H, Mo YY (2007). MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1). *J Biol Chem.* **282**(19):14328-14336.

Zhu Y, Saunders MA, Yeh H, Deng WG, Wu KK (2002). Dynamic regulation of cyclooxygenase-2 promoter activity by isoforms of CCAAT/enhancer-binding proteins. *J Biol Chem.* **277**: 6923–6928.

Zimmermann TS, Lee AC, Akinc A, Bramlage B, Bumcrot D, et al. 2006. RNAi-mediated gene silencing in nonhuman primates. *Nature* **441**:111–14.

Zusman RM and Keiser HR (1977) Prostaglandin biosynthesis by rabbit renomedullary interstitial cells in tissue culture. Stimulation by angiotensin II, bradykinin, and arginine vasopressin. *J Clin Investig* **60:** 215-22.