THE DISTINCT ROLE OF CYCLOOXYGENASE-2 IN PROSTATE AND BLADDER CARCINOGENESIS

DISSERTATION

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By

Xingya Wang, M.S.

* * * * *

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Dissertation Committee:

Dr. Russell D. Klein, Co-adviser
Dr. Steve K. Clinton, Co-adviser
Dr. Mark L. Failla,
Dr. Martha A. Belury

Approved by

Co-adviser

The Ohio State University
Nutrition Graduate Program
ABSTRACT

Overexpression of cyclooxygenase-2 (COX-2) and prostaglandin E₂ (PGE₂) have been demonstrated to play a significant role in the tumorigenesis of several human cancers. Inconsistent and controversial reports on the expression and activity of COX-2 in prostate cancer raised the question of whether COX-2 plays a pivotal role in prostate carcinogenesis. In contrast, there is more consistent support for a positive link between COX-2 overexpression and bladder carcinogenesis. However, the exact role of COX-2 overexpression and how changes in gene regulation alter in urinary bladder carcinogenesis has not been defined.

I first examined the effects of COX-2 inhibition on prostate tumorigenesis in the transgenic adenocarcinoma mouse prostate (TRAMP) model by either feeding TRAMP mice a diet containing non-steroidal anti-inflammatory drugs or by genetic disruption of the expression of the COX-2 gene in TRAMP mice. Our results demonstrate that neither NSAIDs nor genetic disruption of COX-2 inhibited tumorigenesis of TRAMP prostate. Interestingly, I found that the expression of COX-1 and COX-2 mRNA, protein, and activity (ability to synthesize PGE₂) dramatically decreased during TRAMP prostate carcinogenesis. These observations suggest a mechanism whereby TRAMP carcinogenesis may be resistant to COX-1/COX-2 changes. I further found that treatment with exogenous PGE₂ modulated MAPK/Erk and Akt phosphorylation, and induced
secretion of Vascular Endothelial Growth Factor (VEGF) through PGE2 receptor EP2-mediated cAMP pathway in prostate cancer cells *in vitro*.

I also used a gene expression array analysis to determine the effects of COX-2 overexpression on gene expression profile in urinary bladder of a COX-2 overexpression transgenic mouse model (BK5.COX-2). My results revealed that genes associated with Immune/Stress Response and Cell Cycle/Proliferation systems were significantly overexpressed in the BK5.COX-2 transgenic mouse bladder. Upregulated Cell Cycle/Proliferation genes included growth factors (Ereg and IGF-1) and cell cycle genes (Mki67, Cdc2a, and Top2a). Relevant downregulated genes included three transforming growth factor (TGF) beta related genes (Tgfb2, Tgfb3, Tgfbi), and the anti-angiogenic gene thrombospondin 2 (Thbs2). The growth factor epiregulin was the most highly induced gene among those validated by qRT-PCR. I further demonstrated that epiregulin mRNA was directly induced by PGE2 treatment in the wild type mouse bladders. In addition, epiregulin increased cell proliferation and activated MAPK/Erk activity in bladder cancer cells.

In summary, my results suggest that COX-2 may not play a significant role during prostate carcinogenesis in the prostate TRAMP model. However, overexpression of COX-2 may play a pivotal role in murine bladder carcinogenesis.
DEDICATION

This dissertation is dedicated to my advisor Dr. Russell D. Klein and his family
IN MEMORIUM (1962-2006)

Dr. Russell D. Klein passed away on December 1, 2006 after a year long battle with leukemia at the James Cancer Hospital. He will be greatly missed by his colleagues and students as a superb scientist, mentor, and gentleman.
ACKNOWLEDGMENTS

I deeply thank my advisor, Dr. Russell D. Klein, for his guidance, encouragement, dedication to excellence in research and teaching, his sacrificial commitment to students. He taught me how to conduct research beyond all my expectations. His faith will always lift me up and encourage me to move on whenever I encounter any difficulties.

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VITA

Sep 25, 1975.................. Born in Lanzhou, P. R. China

1994-1998..................... Bachelor of Science, Beijing Agricultural University

2001-2003..................... Master of Science, The Ohio State University

2003-2007..................... Graduate Research Associate, The Ohio State University
                             Interdisciplinary program of Nutrition (OSUN)

PUBLICATIONS


FIELD OF STUDY

Major Field: The Ohio State University Nutrition Program
TABLE OF CONTENTS

Abstract......................................................................................................................... ii
Dedication..................................................................................................................... iv
In memorium (1962-2006). ......................................................................................... v
Acknowledgements. ...................................................................................................... vi
Vita................................................................................................................................. viii
List of tables................................................................................................................ xi
List of figures................................................................................................................ xii

Chapters:
1  Introduction............................................................................................................. 1
2  Literature review.................................................................................................... 5
   2.1  Prostate and bladder cancer............................................................................ 5
       2.1.1 Etiology and pathogenesis of prostate cancer......................................... 5
       2.1.2 Etiology and pathogenesis of bladder cancer......................................... 7
2.2  Diet and Cancer.................................................................................................. 12
   2.2.1 Dietary factors and carcinogenesis.............................................................. 12
   2.2.2 Dietary factors and prostate cancer............................................................. 13
   2.2.3 Dietary factors and bladder cancer............................................................. 15
   2.2.4 Fatty acids, their metabolites and carcinogenesis....................................... 17
2.3  Cancer chemoprevention.................................................................................... 21
   2.3.1 Prostate cancer chemoprevention............................................................... 23
   2.3.2 Bladder cancer chemoprevention............................................................... 25
   2.3.3 The use of NSAIDs as prostate and bladder cancer chemoprevention...... 26
2.4  Molecular pathway in human prostate and bladder cancer.............................. 27
   2.4.1 Hallmarks of cancer.................................................................................... 27
   2.4.2 Molecular pathway in prostate cancer......................................................... 29
   2.4.3 Molecular pathway in bladder cancer......................................................... 30
2.5  Cyclooxygenase genes and enzymes................................................................. 33
   2.5.1 Isomers of cyclooxygenase....................................................................... 33
   2.5.2 Cellular localization of COX isozymes....................................................... 34
   2.5.3 Regulation and tissue expression of COX isozymes.................................. 36
       2.5.3.1 Tissue expression................................................................................. 36
       2.5.3.2 Regulation of COX gene expression.................................................... 37
2.6  COX-2 and cancer................................................................................................ 39
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>40</td>
</tr>
<tr>
<td>2.2</td>
<td>56</td>
</tr>
<tr>
<td>4.1</td>
<td>111</td>
</tr>
<tr>
<td>4.2</td>
<td>120</td>
</tr>
<tr>
<td>5.1</td>
<td>153</td>
</tr>
<tr>
<td>5.2</td>
<td>158</td>
</tr>
<tr>
<td>5.3</td>
<td>159</td>
</tr>
<tr>
<td>5.4</td>
<td>160</td>
</tr>
</tbody>
</table>

2.1 Cyclooxygenase 2 is overexpressed in several premalignant and malignant conditions.

2.2 Prostaglandin receptor agonists and antagonists.

4.1 Incidence of poorly-differentiated (PD) carcinomas and metastases (Mets) in TRAMP mice fed diets containing cyclooxygenase inhibitors.

4.2 Incidence of poorly-differentiated carcinomas (PD) and metastases (Mets) in TRAMP mice either wild-type (WT) or homozygous mutant (KO) for the COX-2 gene.

5.1 Histopathologic analysis of the phenotype of urinary bladders in BK5.COX2 transgenic mice and wild-type mice at different ages.

5.2 Expression Analysis Systematic Explorer (EASE) analysis of top 100 upregulated genes that associated with Biologic Process ontology.

5.3 Expression Analysis Systematic Explorer (EASE) analysis of top 100 upregulated genes that associated with Molecular Function ontology.

5.4 Expression Analysis Systematic Explorer (EASE) analysis of top 100 downregulated genes.
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Contemporary model of prostate cancer progression</td>
<td>8</td>
</tr>
<tr>
<td>2.2 T staging of bladder cancer</td>
<td>10</td>
</tr>
<tr>
<td>2.3 Dual tract concept of human bladder carcinogenesis</td>
<td>11</td>
</tr>
<tr>
<td>2.4 Age-adjusted prostate cancer death rates per 100,000 population in various nations around the world</td>
<td>14</td>
</tr>
<tr>
<td>2.5 The arachidonic acid cascade</td>
<td>19</td>
</tr>
<tr>
<td>2.6 Multistage carcinogenesis: processes and prevention strategies</td>
<td>22</td>
</tr>
<tr>
<td>2.7 The hallmarks of cancer—six essential pathways underling the molecular mechanisms of tumorigenesis in the world</td>
<td>28</td>
</tr>
<tr>
<td>2.8 Model for bladder cancer progression showing the molecular pathways of tumorigenesis in the world</td>
<td>31</td>
</tr>
<tr>
<td>2.9 Crystallographic structures of ovine COX-1 (left) and murine COX-2 (right) homodimers</td>
<td>35</td>
</tr>
<tr>
<td>2.10 Regulation of cyclooxygenase 2 (COX-2) in cancer</td>
<td>38</td>
</tr>
<tr>
<td>2.11 Possible mechanisms of COX-2-derived PGE$_2$ contributions to tumor development.revention strategies</td>
<td>41</td>
</tr>
<tr>
<td>2.12 Prostaglandin synthesis and actions</td>
<td>50</td>
</tr>
<tr>
<td>3.1 Relative mRNA expression of EP2 and EP4 receptors in PC-3, DU145, LNCaP and PrEC cells determined by qRT-PCR</td>
<td>68</td>
</tr>
<tr>
<td>3.2 PGE$_2$ and the EP2 receptor agonist CAY10399 induce VEGF protein</td>
<td>xii</td>
</tr>
</tbody>
</table>
secretion in human prostate cancer cells.

3.3 PGE₂ and the EP2 receptor selective agonist CAY10399 increases cAMP concentrations in PC-3, DU145 and LNCaP cells.

3.4 PGE₂ at 1 nM (A) and 10 μM (B) induces cAMP production in PC-3 cells in a time-dependent manner.

3.5 PGE₂ and CAY10399 induce phosphorylation of Erk in time-dependent manner in PC-3 cells.

3.6 PGE₂ induces phosphorylation of Akt only in EP2-expressing PC-3 and DU145 cells.

3.7 Comparison of the effects of PGE₂ and CAY10399 at 1 nM or 10 μM on Erk and Akt phosphorylation in PC-3, DU145 and LNCaP cells.

3.8 The MAPK/Erk and Akt signaling pathways are not involved in PGE₂-induced VEGF protein secretion in PC-3 cells.

3.9 VEGF protein secretion is significantly increased by the adenylate cyclase activator forskolin in a dose-dependent manner in PC-3 cells.

3.10 The adenylate cyclase inhibitor 2′5′-dideoxyadenosine significantly blocks PGE₂-induced cAMP production.

3.11 Working model of the major signaling pathways elicited by PGE₂ in human prostate cancer cells.

4.1 Expression of cyclooxygenase enzymes in dorsal prostate (DP) tissue is reduced in TRAMP mice compared to wild-type (WT) mice.

4.2 COX-2 immunohistochemical staining of serial sections from TRAMP dorsal prostate (DP) tissue with prostatic intraepithelial neoplasia (PIN), moderately differentiated carcinoma (MD), and poorly differentiated (PD) tumors.

4.3 Prostaglandin E₂ (PGE₂) levels in dorsal prostate (DP) tissue are reduced in 10 wk old TRAMP mice compared to wild-type (WT) mice.

4.4 Histopathologic criteria used to score prostatic glandular ducts and metastases in tissues from TRAMP tumor studies.

4.5 Kaplan-Meier survival analysis for the incidence of palpable tumors.
4.6 Treatment of TRAMP mice with cyclooxygenase inhibitors does not significantly alter prostate wet weights of prostate lobes. ..............................113

4.7 Treatment of TRAMP mice with cyclooxygenase inhibitors does not significantly alter prostate carcinogenesis. ..................................................115

4.8 PGE$_2$ levels in dorsal prostate (DP) tissue from 10 wk old TRAMP mice fed an AIN-76A semi-purified diet (control), or 500 ppm celecoxib, 1500 ppm celecoxib, or 4 ppm indomethacin for two weeks. ..............................................117

4.9 PGE$_2$ levels are significantly reduced in 10 wk old dorsal prostate (DP) tissues of the COX-2 knockout TRAMP mice (COX-2 KO) compared to COX-2 WT TRAMP mice (COX-2 WT).........................................................119

4.10 Kaplan-Meier survival analysis for the incidence of palpable tumors. .............121

4.11 Wet weights of prostate lobes in COX-2 knockout TRAMP (KO) compared to COX-2 WT TRAMP (WT) mice.sied. .....................................................124

4.12 Incidence of pathologic lesions in the glandular ducts of prostatic lobes from TRAMP mice with the indicated genotypes (COX-2 WT and COX-2 KO).................................................................125

5.1 Expression of cyclooxygenase enzymes and PGE$_2$ synthesis in bladder tissues of 10 wk old BK5.COX2 (Tg) mice compared to wild-type (Wt) mice. ..............150

5.2 Transitional Cell hyperplasia (TCH) in 10 wk old BK5.COX2 transgenic mouse urinary bladder.................................................................154

5.3 Histopathology of mouse urinary bladders from older (20-30 wk) BK5.COX2 mice..........................................................155

5.4 qRT-PCR validation of six genes of interest that were significantly changed in 10 wk old BK5.COX2 transgenic mice (Tg) compared to wild-type (Wt) mice by Gene Array analysis..........................................................162

5.5 Expression of the six validated genes in 30 wk old BK5.COX2 (Tg) transgenic mice and age matched wild-type (Wt) mice by qRT-PCR analysis.....163

5.6 Immunohistochemistry analysis of the proliferation marker Ki67 10 wk old BK5.COX2 (Tg) transgenic mice and wild-type (Wt) mice bladders..........164

5.7 PGE$_2$ at 10μM induces epiregulin mRNA expression in a time-dependent manner in bladder tissues from 10 wk old FVB wild-type mice. .................165
5.8 Recombinant hEpiregulin induces proliferation and Erk phosphorylation in bladder cancer UMUC-3 cells. .................................................................167

A.1 EP2, EP3 and EP4 are expressed in different pattern in examined human prostate cell lines.................................................................206

A.2 Non-specificity of antibody against EP4 receptor that from Cayman Chemical…207

A.3 PGE2- and CAY10399-induced Erk and Akt activation in DU145 cells……..208

A.4 Adenylate cyclase inhibitor 2’5’-dideoxyadenosine significantly blocks 1nM PGE2-induced secretion of VEGF in PC-3 cells. .................................................209

A.5 PGE2 levels in three prostate lobes of 10 wk old wild-type mice. ...............210

A.6 COX-2 immunohistochemical staining of the same section in the dorsal prostate (DP), lateral prostate (LP), and ventral prostate (VP) tissues of a 10 wk old Pb.COX-2 mouse. .................................................................211

A.7 PGE2 levels in three prostate lobes of 10 wk old Pb.COX-2 and wild-type mice..212

A.8 H&E stained section of ventral prostate (VP) from one mouse of a founder line of Pb.COX-2 mouse model.................................................................213

A.9 Expression of COX-1 and COX-2 mRNA are significantly reduced in both anterior prostate (AP) and dorsal prostate (DP) tissues of the 10 wk old conditional PTEN knock mice (Tg) compared to wild-type (WT) mice.).................................214

A.10 Western blot analysis of COX-1 and COX-2 protein levels in seven prostate cell lines .........................................................................................215

A.11 Prostaglandin A2 (PGA2), PGD2, 15-d-PGJ2 and 12-HETE levels in dorsal prostate (DP) tissues of 10 wk old wild-type (WT) and TRAMP mice. .........................216

A.12 Transgene construct of BK5.COX2 mouse model......................................217
CHAPTER 1

INTRODUCTION

Prostate cancer and bladder cancer are the two most common malignancies in the adult male urogenital system. Prostate cancer represents the most common malignancy and the second leading cause of cancer death in US men. Bladder cancer is the fourth most common malignancy in men and the ninth most common type of cancer in women in the US. The identification of new molecular targets for the prevention and treatment of prostate and bladder cancer is a potential means to reduce the development and progression of both malignancies. Cyclooxygenase enzymes, COX-1 and COX-2, catalyze a rate limiting step in the synthesis of prostaglandins from arachidonic acid. COX-1 is generally considered to be constitutively expressed in nearly all tissues and has functions in maintaining tissue homeostasis, whereas COX-2 is inducible by a number of factors (1). Overexpression of COX-2 in several cancers, including colon, breast, lung, bladder, skin and pancreas has been observed in numerous studies (1). Prostaglandins have been reported to induce cell proliferation, enhance angiogenesis, and promote invasion and metastasis of these cancers (2, 3). NSAIDs that inhibit COX activity have received much attention as potential chemopreventive agents for these cancers. The primary mechanism by which NSAIDs are thought to prevent cancer is through inhibition of the synthesis of prostaglandins.
Numerous studies have demonstrated that COX-2 is overexpressed in transitional cell carcinoma (TCC) of human urinary bladder (4-7). The degree of COX-2 expression is significantly correlated with the higher tumor grade and greater depth of invasion of TCC (8-10). A substantial number of studies demonstrated that NSAIDs inhibit the growth of bladder cancer cells in vitro (11-14). We recently demonstrated that forced expression of COX-2, under the control of a keratin 5 (K5) promoter, is sufficient to cause urinary bladder transitional cell hyperplasia (TCH), which progresses to TCC in a small percentage of transgenic mice (3). These results suggest that there is a strong association between COX-2, prostaglandin synthesis and the development and progression of urinary bladder cancer. However, the exact role and mechanisms of action for COX-2 overexpression during bladder carcinogenesis have not been well defined.

In contrast to the above, accumulating studies on the role of COX-2 in prostate carcinogenesis are inconsistent and controversial. Retrospective case-control and prospective cohort epidemiologic analyses examining the risk of prostate cancer in men consuming NSAIDs have provided mixed findings. Results from some studies suggest no protective effect of NSAIDS (15-19), whereas there are several reports of a significant reduction in prostate cancer risk attributable to frequent NSAID use (20-23). Initial reports from several research groups indicated that COX-2 was also overexpressed in human prostate cancers (24-29). More recent studies have found that COX-2 is only minimally expressed in most prostate cancers, and that elevated expression is only observed in areas of chronic inflammation or proliferative inflammatory atrophy (30, 31).

There have been relatively few reported animal studies that have tested the level of COX-2 expression and the effect of NSAIDs on prostate carcinogenesis. The TRAMP
model for prostate cancer is now well established and demonstrates several characteristics that make it advantageous for chemoprevention studies (32, 33). Two groups of investigators recently reported that the COX-2 selective inhibitor celecoxib decreased prostate tumorigenesis in the TRAMP transgenic model (34, 35). Celecoxib, however, has been shown to have multiple non-COX-2 mediated activities that could account for its effect on tumorigenesis in this model (36, 37). Until now, investigations into the role of COX-2 in prostate cancer were largely hindered by the lack of prostate specific COX-2 overexpression and knockout mouse models. The potential for non-COX mediated effects of NSAIDs, combined with the lack of expression of COX-2 in human prostate cancers (27, 30, 31), led us to question whether COX-2 inhibition was actually responsible for the reported chemopreventive activity of celecoxib in the TRAMP model.

In addition, it is well established that PGE\textsubscript{2}, the most common and ubiquitously produced PG produced by COX enzymes, may contribute to tumorigenesis via induction of cell proliferation, angiogenesis, invasion and metastasis (1-3). In particular, PGE\textsubscript{2} has been shown to induce the production of VEGF, a key mediator of angiogenesis in several cancers (38-43). The physiological effects of PGE\textsubscript{2} are mediated by four G protein coupled membrane receptors (EP1, EP2, EP3 and EP4) that are encoded by different genes (44). EP receptors have been implicated in promoting carcinogenesis in different types of tumors, including lung (45), ovarian (46), colorectal (47, 48), breast (49), skin (50, 51), and prostate (52). Unfortunately, the study of the expression of EP receptors in prostate and bladder tissues and their direct role in carcinogenesis of these diseases is limited.
The goal of my dissertation research has been to a) define the role of COX-2 overexpression and PGE\(_2\) production in prostate and bladder cancer tumorigenesis, b) to determine the molecular mechanism involved in prostaglandin pathway and carcinogenesis in prostate and bladder cancers, and c) to determine the molecular targets (biomarkers) of COX-2 overexpression for future chemopreventive studies.
CHAPTER 2

LITERATURE REVIEW

2.1 Prostate and bladder cancer

Prostate cancer and bladder cancer are the two most common malignancies in the adult male urogenital system. Prostate cancer is the most commonly diagnosed cancer in men in the US aside from skin cancer and accounts for about 30% of new cancer cases annually. It is the second leading cause of cancer death in US men, after lung cancer. An estimated 218,890 new cases will occur in the US during 2007, and about 27,050 men will die from the disease this year (53). Bladder cancer is the fourth most common malignancy in men and the ninth most common type of cancer in women in the US. An estimated 67,160 new cases are expected to occur in the US during 2007, and about 13,750 deaths will occur this year (53). Worldwide, both prostate and bladder cancers are significant health problems, and health care costs are great for both the individual and society.

2.1.1 Etiology and pathogenesis of prostate cancer

Despite the clinical importance of prostate cancer, the multiple interacting mechanisms underlying the development and progression of this disease remain speculative (54). There are several genetic and environmental factors that contribute to
the development of prostate cancer. Age is the most significant risk factor for prostate cancer. The disease is rare in men under age 45, but incidence rates rise rapidly with age. As the proportion of elderly men continues to increase in the US population, this major public health problem is expected to worsen. Men with a family history of prostate cancer are at increased risk for this disease. African-American men have significantly higher incidence rates than Caucasians, and mortality rates among Africa-American men are twice as high as those of Caucasians (55). Other factors such as androgen levels, diet and lifestyle factors have been proposed to be directly involved in the development of prostate cancer (56, 57). More recently, chronic inflammation has been linked to the development of prostate cancer by several lines of evidence (30). For example, evidence from epidemiologic studies have established an association between prostatitis and sexually transmitted infections, which all cause chronic inflammation, perhaps leading to increased prostate cancer risk (58, 59). Genetic studies have suggested that polymorphisms and mutations in genes regulating the inflammatory process play an important role in the increased risk of prostate cancer (59, 60). De Marzo et al. proposed that chronic inflammation that caused by infection, dietary carcinogens, hormonal imbalances and other factors can lead to early steps in prostate carcinogenesis, which is termed as proliferative inflammatory atrophy (PIA) (61). Lesions of PIA contains activated inflammatory cells and proliferation epithelial cells, which may serve as precursors of prostatic intraepithelial neoplasia (PIN) lesions and prostatic carcinoma (59). However, the exact mechanism by which inflammation influences the development and progression of prostate cancer has not yet been firmly established.
The progression of prostate cancer is characterized by the gradual loss of regulation of cell growth within the prostate epithelium due to accumulating genetic defects. PIN is thought to be the precursor of invasive prostate cancer. PIN is characterized by cellular proliferation and is associated with progressive abnormalities in phenotype and genotype that are intermediate between normal prostatic epithelium and dysplasia. PIN is generally divided into two grades, i.e., low grade and high grade. High-grade PIN (HGPIN) will further develop into superficial cancers, and finally to invasive carcinoma. Clinical studies suggest that PIN predates carcinoma by 10 years or more, and clinically significant cancer may not occur for another 3-15 years (62).

Although the mechanism underlying the development and progression of this disease remain elusive (54), recent scientific discoveries have led to better understanding of the molecular and genetic changes associated with prostate cancer carcinogenesis (63). Figure 2.1 shows the contemporary model of prostate cancer progression. The genetic and molecular changes associated with prostate carcinogenesis will be discussed in detail below.

2.1.2 Etiology and pathogenesis of bladder cancer

There are several genetic and environmental factors that contribute to the development of bladder cancer. Age is one of the most significant risk factor for bladder cancer. As for prostate cancer, bladder cancer will also become a greater health concern with the increasing proportion of elderly in the US. Caucasians are about twice as likely to develop bladder cancer as African Americans. However, the mortality rates are similar.
Figure 2.1 Contemporary model of prostate cancer progression. Genetic predisposition, oxidative damage and inflammatory changes are associated with earliest steps of prostate cancer development (63).
for the two populations (64). The greatest environmental risk factor for bladder cancer is cigarette smoking (65). Occupational exposures to chemical carcinogens and bladder infections have also been associated with the occurrence of this disease (65). In addition, dietary factors have been related to the risk of developing bladder cancer (66), which will be discussed in detail below. More recently, the role of inflammation in the development and progression of bladder cancer has been increasingly recognized (67, 68). Inflammation can be directly or indirectly caused by smoking, chronic irritation, or infection.

Transitional cell carcinoma (TCC), also known as urothelial carcinoma (UC), is the most common histologic type of bladder cancer, accounting for about 90% of all bladder cancers (66). At presentation, 80% of bladder cancers (TCC) are “superficial” and involve low-grade papillary tumors in that they are confined in the epithelium without invading into the surrounding muscle, or known as Ta and T1 tumors (69, 70) (Figure 2.2). The remaining superficial cancers are known as carcinoma in situ (CIS) that involve non-papillary growth. Invasive cancers extend into or through muscle layers, and are called T2-T4 tumors (69). Figure 2.3 shows the two pathways of human bladder carcinogenesis, which are distinct but also overlap each other (71). The pathway to development of invasive TCC seems to start with dysplasia, progressing to CIS, followed by invasion of the lamina propria (72). Despite the high percentage of the papillary pathway in bladder cancer, the most important clinical challenges of bladder cancer are recurrence and progression. Although several advances have been made in the treatment of superficial bladder cancer, the recurrence rate reaches as high as 70% (67).
Figure 2.2 T staging of bladder cancer. Tis, carcinoma in situ; Ta, tumor involves mucosa only; T1, tumor invades through lamina propria; T2a, tumor invades into superficial muscle; T2b, tumor invades into deep muscle; T3a, tumor microscopically invades perivesical tissue; T3b, tumor macroscopically invades perivesical tissue and forms extravesical mass; T4a, tumour invading prostate/uterus/vagina; T4b, tumour fixed to pelvic wall (69).
Figure 2.3 Dual tract concept of human bladder carcinogenesis (71).
2.2 Diet and Cancer

2.2.1 Dietary factors and carcinogenesis

In a 1981 landmark review on the causes of cancer in US, Doll and Peto estimated that about 35% of deaths from cancer might be due to, and thus preventable, by changing in diet (73, 74). Thereafter, many investigations including epidemiological observations, clinical trials, animal and in vitro cell experiments have addressed the potential role of diet. The effects of factors such as total fat, dietary fat, total energy intake, red meat, fruits and vegetables, phytochemicals, vitamins and minerals, on the incidence and progression of cancers have been examined. Although these studies have generated mixed findings, epidemiological evidence continues to support the general conclusion of Doll and Peto in 1981 (74). Numerous studies have examined the mechanisms underlying the initiation and promotion or prevention of human cancers. The hypothesis that dietary factors may affect the risk of cancer is supported by their biologic properties. Some dietary components are carcinogens and cause DNA mutations and others are growth factor stimulators, or antioxidants. A number of nutrients prevent DNA damage, support DNA repair, suppress the expression of oncogenes, or affect hormone levels, the immune system and inflammatory responses (75, 76). More recently, the role of dietary factors in the modification of epigenetics during carcinogenesis, such as alterations in histone acetylation and methylation, have gained increased attention (77).
2.2.2 Dietary factors and prostate cancer

Epidemiological evidence suggests that the incidence and mortality of prostate cancer is considerably lower in Asian populations compared to those in North America and northwest Europe (78-80). Figure 2.4 shows the age-adjusted death rates from prostate cancer per 100,000 population in various countries (81). There is at least a 5-fold difference in prostate cancer mortality between western and Asian populations. Interestingly, migration studies indicate that a marked increase in prostate cancer incidence occurs in the migrants to the United States from low-risk geographical areas who abandon their traditional culture and dietary patterns (82, 83). Shimizu et al. found that Asian men who immigrate at young ages have the same risk as American men, suggesting that the earlier in life are migrants arrive to US, the more closely their risk approaches that of American men (84). Another intriguing fact is that although the incidence and mortality of prostate cancer varies greatly among populations, the incidence of pre-cancerous lesions is similar (85). Early autopsy studies reveal that the incidence of latent carcinoma of the prostate is the same in men of all races worldwide (86). By age 50, 15 to 30% of men have cancer cells present within the prostate gland regardless of where they reside. In high risk areas, these cancer cells will undergo promotion and progression with aging and cause permanent lesions. However, in low risk areas these cancer cells tend to remain dormant and do not undergo promotion and progression to a clinically significant life-threatening malignant state. These associations indicate that environmental factors and lifestyle, such as dietary factors, are directly involved in the development of prostate cancer.
Figure 2.4 Age-adjusted prostate cancer death rates per 100,000 population in various nations around the world (81).
Numerous studies have demonstrated that diets high in fat, particularly animal fats, can promote prostate cancer (87-90). High intake of red meat is also associated with the increased prostate cancer risk (91). Recently, 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP), a heterocyclic amine, has been identified as a dietary carcinogen (92). Studies from Bogen and Keating demonstrate that higher intake of PhIP among African-Americans may partially explain the increase in prostate cancer incidence in this group (93). Excessive energy intake which results in altered hormone status has been linked to increased prostate cancer risk (94). Insulin-like growth factor-1 (IGF-1) has been demonstrated to play a key role in prostate carcinogenesis upon changes in energy intake. In contrast, diets rich in fruits, vegetables and legumes is associated with a reduced risk of prostate cancer (89, 95, 96). Dietary factors, including lycopene, vitamins E and D, isoflavones, selenium and zinc, have been identified in epidemiological, animal and cellular studies to be associated with the prevention of prostate cancer (56, 57, 94, 97).

2.2.3 Dietary factors and bladder cancer

Evidence from numerous studies suggests that diet may also play an important role in the development and progression of bladder cancer. Like organs in the GI tract, the bladder is unique in that many compounds contained in foods and their metabolites, including carcinogens, are excreted through the urinary tract (66). Therefore, dietary compounds and their metabolites could directly interact with the epithelial cells of the bladder to elicit either carcinogenic or anti-carcinogenic effects on the urinary bladder. Although there have been fewer studies on the role of fat intake in bladder carcinogenesis compared to prostate carcinogenesis, a positive link between fat and bladder cancer has
been suggested in some studies. A meta-analysis of 38 studies found a significant association between bladder cancer and high fat intake with a RR of 1.4 (95% CI 1.2-1.6) after adjusting for smoking (98). A multi-center, case control study from Spain demonstrated that men with the highest intake of saturated fat had a significant increased risk of bladder cancer with a relative risk of 2.25 (99). Other studies have reported this association and suggested a dose response relationship between fat intake and bladder cancer incidence (100). However, no significant association was found between meat consumption (RR=1.0) and bladder cancer incidence in this meta-analysis. More recently, results from two large prospective cohort studies, the Health Professionals Follow-UP study (HPFS) and the Nurse’s Health Study (NHS), demonstrate that meat consumption, especially bacon, was associated with an elevated risk of bladder cancer (101). The high contents of nitrosamines and/or heterocyclic amines (such as PhIP) in bacon were speculated to be responsible for its association with increased risk of bladder cancer. In contrast, consumption of fruits and vegetables has been associated with a reduced risk of bladder cancer in many epidemiological studies (102, 103). Evidence from studies on the possible role of micronutrients, including vitamin A, carotenoids, vitamin E and vitamin C in bladder carcinogenesis, is inconsistent and controversial (66). However, selenium does seem to have an inverse association with the risk of bladder cancer (66).
2.2.4 Fatty acids, their metabolites and carcinogenesis

Epidemiological, case-control studies, and cohort studies have examined the correlation between fat intake and the development of human cancers, such as prostate and breast cancer. Although some studies suggest a positive link between fat intake and increased risk of prostate cancer, other studies failed to find such a relationship (104-107). A recent case-control study even suggested that obesity is inversely associated with prostate cancer risk in middle-aged men (108). Despite the inconsistency of such findings, studies generally suggest that the type of dietary fat rather than the amount of total fat intake may play a more important role in the development of prostate cancer. Accumulating evidence from both epidemiological and cohort studies suggests that intake of fish oil that are rich in n-3 polyunsaturated fatty acids eicosapentaenoic (EPA) and docosahexaenoic (DHA), are associated with a lower prostate cancer risk (109-111). In contrast, high intake of the n-6 polyunsaturated fatty acids (PUFA), including α-linoleic, linoleic, and arachidonic acid (AA), may promote prostate tumorigenesis. An early study demonstrated that high intake of red meat, which is the major source of AA, is associated with increased prostate cancer risk (91). In general, these studies suggest that higher n-3/n-6 PUFA ratio is inversely associated with prostate cancer incidence and progression. However, at present, the mechanisms through which dietary fats modulate carcinogenesis of prostate and other cancers are not fully elucidated.

A common feature of most of the proposed mechanisms by which dietary fats modulate carcinogenesis is through the regulation of eicosanoid production and their downstream signaling pathways (112-114). It has been proposed that n-3 PUFA may suppress eicosanoid biosynthesis derived from AA, resulting in altered immune response
to cancer cells and modulation of inflammation, cell proliferation, apoptosis, metastasis, and angiogenesis (114).

The majority of the AA from diet or synthesized from linoleic acid is incorporated into the secondary position of the membrane phospholipids. Phospholipases, such as PLA$_2$, release AA from membrane phospholipids. Upon release, AA serves as substrate for cyclooxygenase (COX-1 and COX-2), lipoxygenase (LOX 5, 12, 15), or cytochrome P450 monoxygenase (MOX) to produce eicosanoids (111). Figure 2.5 shows the AA cascade (115). Both n-3 and n-6 fatty acids can be substrates for production of human eicosanoids. The eicosanoids include prostaglandins (PG), prostacyclins (PGI$_2$), thromboxanes (TX), leukotrienes (LT), and hydroxyl acids. Eicosanoids influence numerous biologic processes, including platelet aggregation, inflammation, hemorrhage, vasoconstriction and vasodilation, blood pressure, and tumorigenesis (116). Greater concentrations of n-3 fatty acids EPA and DHA are able to replace AA in membrane phospholipids. Aronson et al. found that short-term dietary intervention in men with prostate cancer leads to a significant increase in the n-3/n-6 fatty acid ratios in plasma and adipose tissue (117). The n-6 AA derived- eicosanoid is more mitogenic and more potent than n-3 PUFA derived- eicosanoids. In addition, eicosanoids derived from n-3 fatty acids have fewer inflammatory properties than those derived from n-6 fatty acids (112, 118). The substrate utilized by COXs is critically important for prostaglandin effects on cell function (119). Supplementation of the diet with n-3 fish oils has been shown to decrease production of TNF, IL-1, IL-6 by mononuclear cell (120). As such, changes in the ratio of dietary n-3/n-6 fatty acids could have the potential to impact the generation of
Figure 2.5 The arachidonic acid cascade (121).
inflammatory eicosanoids and to contribute to the inflammatory component associated with chronic diseases such as prostate cancer and bladder cancer.

One additional function of fatty acids is to regulate expression of genes involved in metabolism, as well as cell growth and proliferation. Both n-3 and n-6 fatty acids have been shown to regulate the activity of several transcription factors, including PPARs, LXR, NFκB, c/EBPβ, and Ets-1 (122). However, whether fatty acids interact with these genes by directly binding or indirectly, such as through regulation of COX-2 and prostaglandin pathways, is still unknown.

Proposed mechanisms by which individual fatty acids control specific cell functions and gene expression allows us to envision how the type and quantity of ingested dietary fat ingested may contribute to development of prostate and bladder cancer. They also offer insights for identifying novel preventative and therapeutic strategies for such cancers.
2.3 Cancer chemoprevention

Cancer chemoprevention refers to the use of natural and/or synthetic substances to reduce the risk of developing cancer or lessen the likelihood that cancer will recur. Many cancers have a latency period of 10-20 years, which provides sufficient time for preventative strategies. Cancer chemoprevention is commonly divided into three categories, i.e., primary, secondary, and tertiary. Primary prevention involves interventions designed to help at-risk individuals delay or prevent the development of a target disease (123). Secondary prevention involves strategies developed to provide treatment of premalignant lesions with the goal of prevention progression to frank cancer. Tertiary prevention involves the treatments of patients to prevent further functional loss and the development of secondary primary cancers. In a classic view, carcinogenesis involves the accumulation of genetic and epigenetic alterations in a multi-step process characterized by the occurrence of initiation, promotion, and progression (124) (Figure 2.6). Cancer chemoprevention can interfere with all three stages through modifying carcinogen activation, modulating DNA repair processes, scavenging reactive oxygen species, altering expression of genes involved in cell growth and cell death, inhibiting tumor angiogenesis and invasions, and regulating inflammatory processes (124).
Figure 2.6 Multistage carcinogenesis: processes and prevention strategies(124).
2.3.1 Prostate cancer chemoprevention

The discovery of prostate-specific antigen (PSA), a protein produced by prostate tissue, has significantly improved the early detection of prostate cancer (125). Advances have been made in both the diagnosis and treatment of prostate cancer, yet it is still the second leading cause of cancer mortality in men in the US. The features of prostate cancer—high prevalence and long latency suggest an opportunity for chemoprevention (57).

At present, compounds that have been considered for chemoprevention of prostate cancer include lycopene, beta-carotene, vitamin E, selenium, vitamin D, soy isoflavones, 5-alpha-reductase inhibitors (finasteride and dutasteride), and NSAIDs in both clinical and preclinical studies. In particular, several large-scale, randomized, population-based studies have suggested a benefit of vitamin E or selenium in the prevention of prostate cancer. One large-scale randomized placebo-controlled trial, the Alpha-Tocopherol Beta-Carotene Cancer Prevention Trial (ATBC), found a statistically significant 32% reduction in prostate cancer incidence and a 41% lower mortality in those receiving alpha-tocopherol upon secondary analysis (126). The strongest evidence for a protective effect of selenium for prostate cancer comes from the Nutritional Prevention of Cancer Trial, a randomized placebo-controlled study of oral selenized yeast in patients with non-melanoma skin cancer. In this study, the incidence of prostate cancer was reduced in the selenium group by two thirds compared to the placebo after 4.5 years of follow-up, and the incidence was further reduced with longer follow-up (126). The findings from these two trials along with other clinical and pre-clinical evidence promoted the use of selenium and vitamin E in the large-scale phase III, randomized Selenium and Vitamin E.
Cancer Prevention Trial (SELECT) involving 35,534 men to test their efficacy in the prevention of prostate cancer (127, 128). Unlike trials with selenium and vitamin E reported so far in which prostate cancer was a secondary end point, clinical diagnosis of prostate cancer is the primary end point of SELECT (125). However, the final results will not be available until 2013.

Since the observation that castration was effective in reducing the symptoms of metastatic cancer in the 1940s by Huggins and Hodges (129), the role of testosterone and dihydrotestosterone (DHT) in prostate carcinogenesis has been well established. Testosterone is converted to the active form DHT by the membrane-bound enzyme, steroid 5-alpha reductase. Therefore, the 5-alpha reductase inhibitors have been extensively studied for chemoprevention of prostate cancer. The Prostate Cancer Prevention Trial (PCPT) that involved 18,882 men was the largest population based, randomized phase III clinical trial to evaluate the efficacy of finasteride, the type 2 isoenzyme of 5-alpha reductase, in the prevention of prostate cancer (130). There was a 24.8 % reduction of prostate cancer incidence in the finasteride group compared to the placebo group, however, a large number of higher-graded cancers were noted among men randomized to finasteride (104, 131). Although enthusiasm for the widespread use of finasteride has been tempered, future studies are still warranted to definitively examine the chemopreventive activity of 5-alpha reductase inhibitors in prostate carcinogenesis. The ongoing The Reduction by Dutasteride (another 5-alpha reductase inhibitor) of Prostate Cancer Events (REDUCE) trial may help in clarifying this association (131).
2.3.2 Bladder cancer chemoprevention

Although several advances have also been made in the treatment of superficial bladder cancer, recurrence rate can be as high as 70% (67), and approximately 10% to 30% of cases will progress to invasive cancer and potentially lethal (132). In addition, bladder cancer is an expensive cancer to survey and treat due to the need for frequent interval cystourethroscopy, urine cytology and radiological evaluations (133). Therefore, strategies for preventing bladder cancer are needed to reduce both the development and recurrence of this disease. Bladder cancer is an attractive target for prevention efforts in part because renal excretion of certain agents enables prolonged exposure to high concentrations of drugs or metabolites in the urine (134). At present, Bacillus Calmett Guerin (BCG) is considered the most effective chemopreventative and chemotherapeutic agent against recurrence of superficial bladder cancer (123, 135-137). However, BCG has been associated with high rates of side effects and development of resistance after prolonged usage (123, 138).

Chemoprevention of bladder cancer through dietary intervention has been proposed based upon many studies. Test foods and compounds have included intakes of fruits and vegetable (139, 140), alpha-carotene and beta-carotene (139), Vitamin A (141), Vitamin C (141, 142) and pyridoxine (Vitamin B6) (141, 143). However, results from these studies are inconsistent and demonstrate conflicting findings (144). Vitamin A and its analogues (retinoids) have been extensively studied for their chemopreventive activity against bladder cancer (133) and demonstrated promising results. However, large-scale trials evaluating bladder cancer prevention are rare (134). Prospective randomized trials with a large sample size, longer follow-up, and an extended duration of treatment are
needed to clarify the association between micronutrients and bladder cancer protection (132, 134).

2.3. 3 The use of NSAIDs for prostate and bladder cancer chemoprevention

The COXs inhibitors, NSAIDs, have been studied for their chemopreventive effects against prostate and bladder cancers. In general, clinical trials are very limited for NSAIDs, which may be due to the risk of side effects related to NSAIDs. Evidence from both epidemiologic and pre-clinical studies demonstrates conflicting results regarding the role of COX-2 in prostate cancer carcinogenesis, but demonstrates a much stronger association of COX-2 with bladder carcinogenesis. A more detailed review on COX-2 expression and evidence regarding NSAIDs and cancer chemoprevention in both the prostate and bladder cancer will be discussed in the later part of this review. The use of COX-2 selective inhibitors has recently been associated with an increased risk for cardiovascular heart disease (145). It is therefore essential to carefully examine the biologic basis for targeting COX-2 for the prevention of prostate and bladder cancers.

In summary, prostate and bladder cancers are good targets for chemoprevention. Although some agents have demonstrated promising effects, new chemopreventive agents against these two diseases need to be developed and validated. Before identifying any effective agents for chemoprevention, the molecular biology of these two diseases should be understood.
2.4 Molecular pathways in human prostate and bladder cancer

2.4.1 Hallmarks of cancer

The process by which normal cells become progressively transformed to malignancy requires the acquisition and accumulation of mutations which arise as a consequence of damage to the genome. It has become increasingly apparent that the six essential pathways underling the molecular mechanisms of tumorigenesis described by Hanahan and Weinberg are altered during carcinogenesis. These include 1) self-sufficiency in growth signals, 2) insensitivity to growth-inhibitory signals, 3) evasion of apoptosis, 4) limitless replication, 5) sustained angiogenesis, and 6) tissue invasion and metastasis (146) (Figure 2.7). Genetic instability is a critical factor and which plays a key role in driving the above six essential pathways. These six “Hallmarks” are believed to be common to most or all human tumors, albeit through different mechanistic strategies (146). An improved understanding of the genetic and molecular changes during prostate and bladder cancer development and progression is helping us to identify appropriate targets for chemoprevention.
Figure 2.7 The hallmarks of cancer- six essential pathways underling the molecular mechanisms of tumorigenesis (146).
2.4.2 Molecular pathway in prostate cancer

Prostate cancer contains many somatic genomic alterations, including point mutations, deletions, amplifications, chromosomal rearrangements, polymorphisms, and changes in DNA methylation (147). However, unlike carcinomas such as colon and pancreas where specific oncogenes such as k-ras or tumor suppressor genes such as p53 are mutated at a very high frequency, gene mutations reported thus far in prostate cancer appear quite heterogeneous. In addition, genetic alterations appear to accumulate with prostate cancer progression. Genes altered during prostate carcinogenesis include phosphatase and tensin homologue (PTEN), BRCA2, NKX3.1, androgen receptor (AR), telomerase, kruppel-like factor 6 (KLF6), P27, alpha-Methlacul-CoA racemase (AMACR), and Rb (147). Epigenetic events including DNA methylation of many genes have been identified and include ras association domain family protein 1, isoform A (RASSF1A), GSTP1, and COX-2 (63). The genetic instability leading to mutation in a diverse array of genes have been associated with prostate cancer carcinogenesis.

Many of the molecular changes described above have been identified in numerous studies, and appeared to be potential targets for prostate cancer chemoprevention (148). Alterations in several signaling pathways have been identified to be associated with the promotion of prostate cancer growth. High levels of epidermal growth factor receptor (EGFR), insulin-like growth factor receptor type-1 (IGF-1R), and transforming growth factor α (TGFα) have been found in prostate cancer and result in the increased proliferation of prostate cancer cells. The signal transduction and activators of transcription (STATs) signaling, the beta-catenin signaling and the Toll-like receptor (TLR) signaling have also been found to be activated and overexpressed in human
prostate cancers and all promote cell growth and survival. Many molecules involved in cell cycle regulation including CDKs, INK4 (CDK inhibitor), Rb, E2F, and telomerase were deregulated in prostate cancer. Cell survival and apoptosis related molecules including NF-Kb, TNF-α, Bcl-2 and inhibitor of apoptosis protein (IAP) have been identified in prostate cancer. Vascular endothelial growth factor (VEGF) plays an important role during angiogenesis and promotes tumor growth. Overexpression of VEGF and the matrix metalloproteinases (MMPs) that are involved in metastasis and invasion have also been identified in human prostate cancer.

2.4.3 Molecular pathway in bladder cancer

The most common genetic alterations in low grade papillary transitional cell carcinoma (TCC) of bladder cancer are the loss of heterozygosity of part of all of chromosome 9 and mutations in the fibroblast growth factor receptor 3 (FGFR3). As described earlier (Figure 2.2 and 2.3), the pathway to development of invasive TCC seems to start with dysplasia, progresses to carcinoma in situ (CIS), and is followed by invasion of the lamina propria (72). The most frequent genetic alteration in dysplasia and CIS is mutation of the tumor suppressor gene p53, followed by loss of heterozygosity of chromosome 9. The dyregulation of tumor suppressor genes pRb and p53 has been reported to be associated with bladder cancer cell proliferation, resistance to apoptosis, cell cycle progression and invasion (149, 150). Increased genomic instability and aberrant methylation of cytosine residues in DNA has been shown to be correlated with increased tumor invasion and progression. Figure 2.8 shows the model for bladder cancer progression involving genetic and molecular changes.
Figure 2.8 Model for bladder cancer progression showing the molecular pathways of tumorigenesis (72).
Similar to prostate cancer, many molecular changes in the six described pathways have been identified and represent potential targets for bladder cancer chemoprevention (72, 149). The first important group of molecules are growth factors and their receptors. Numerous studies have demonstrated that members of the epidermal growth factor family are overexpressed in bladder carcinoma, including EGF (151-153), TGF\(\alpha\) (152-154), epiregulin (155, 156), and others. EGFR is also associated with the stage and grade of bladder cancer development and progression (150). Pathologic biomarkers of proliferation, such as Ki67 and proliferating cell nuclear antigen (PCNA), are increased with bladder cancer progression (157-159). In contrast, signaling molecules that inhibit growth, such as TGF\(\beta\) (160, 161), CDK inhibitors P15 and P16 (153) are downregulated in TCC. Insulin growth factor 1 (IGF-1) plays a role in proliferation and survival, which has been reported to be upregulated in patients of bladder cancer (149, 162).

Overexpression of VEGF is significantly associated with early recurrence, progression to high stage and grade of urinary bladder cancer (151, 163-165). In contrast, the anti-angiogenic molecule, thrombospondin-1 has been reported to be downregulated during the development of bladder cancer (166, 167). In addition, numerous studies have demonstrated that COX-2 is overexpressed in human TCC of urinary bladder, and this may affect many of the above pathways (4-7, 168).
2.5 Cyclooxygenase genes and enzymes

2.5.1 Isozymes of cyclooxygenase

Cyclooxygenase, also known as prostaglandin H synthase (PGHS) or prostaglandin endoperoxide synthase, is the enzyme that catalyzes the rate-limiting step in prostaglandin synthesis by converting arachidonic acid into prostaglandin H$_2$ (121). PGH$_2$ is then converted to a series of different PGs dependent on the presence of the cell-specific PG synthase enzymes (47, 169). Two isoforms of cyclooxygenase have been identified, i.e., COX-1 and COX-2. Cyclooxygenase and its enzymatic activity was first identified in 1976 by two different groups (121). The gene structure of COX-1 was identified in 1988 (170-172), and subsequently COX-2 (115, 173, 174). The human gene encoding the COX-1 enzyme is located on chromosome 9 (9q32-0q33.3), contains 11 exons and is 40kb; its mRNA is approximately 2.8kb (175). The gene encoding COX-2 is located on chromosomal 1 (1q25.2-25.3), containing 10 exons and encompasses 7.5 kb with a 4.5 kb transcript (1). Despite the difference in genomic structure and transcript size, COX-1 possesses approximately 60% amino acid identity with COX-2 (115). Both COX enzymes have about 600 amino acids with the calculated molecular weight of the nascent polypeptide 68 kDa and the mature protein 70-80 kDa as a result of glycosylation (176). More recently, a variant of COX-1 named as COX-3, has been identified in the brain of dog (177).
2.5.2 Cellular localization of COX isozymes

The structures of the four domains of COX-1 and COX-2 predict that both enzymes are located in the lumen of the nuclear envelope and endoplasmic reticulum. The amino-terminal signal peptides on the nascent COX-1 and COX-2 polypeptides direct COX enzymes into the lumen of the endoplasmic reticulum (115). Both COX-1 and COX-2 form dimers via hydrophobic interactions, hydrogen bonding, and salt bridges between the dimerization domains of each monomer. Three disulfide bonds links the dimerization domains together, and 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 and ER which have redox states that are significantly more oxidized than cytosol. COX isozymes also contain a tandem series of four amphipathic helices which create a hydrophobic surface that penetrates into the upper portion of the luminal side of the hydrophobic core of the lipid bilayer of the lumen of the ER and nuclear envelope. The catalytic domain of the COX isozymes which contains two distinct enzymatic active sites (e.g. peroxidase active site and cyclooxygenase active site) and processes the KDEL sequence that acts as a signal for retention of proteins in the ER (115). Figure 2.9 shows the crystallographic structures of ovine COX-1 and murine COX-2. Several laboratories determined that COX-1 and COX-2 traffic within the nucleus following a variety of stimuli (121). Morita et al. suggest that COX-1 is predominantly localized to the ER, while COX-2 is localized to both ER and nuclear membrane (178).
Figure 2.9 Crystallographic structures of ovine COX-1 (left) and murine COX-2 (right) homodimers. The crystallographic structure of ovine COX-1 was taken from Protein Data Bank file 1PRH and the murine COX-2 structure was from file COX5 of the same source. Functional domains: 1) membrane binding domain (yellow); 2) dimerization domain (green); catalytic domain (blue) heme (red). The open cleft of the peroxidase active site is observable at the top of each monomer (121).
2.5.3 Regulation and tissue expression of COX isozymes

2.5.3.1 Tissue expression

Despite their structural similarities, the regulation and expression pattern of COX-1 and COX-2 isomers are different (179). Although there are some exceptions, COX-1 is generally considered as constitutively expressed in many tissues, whereas COX-2 is an inducible isoform whose expression is stimulated by growth factors, hormone, cytokines and tumor promoters (1, 121). COX-2 is up-regulated in many epithelial cancers and its prostaglandin products (PGs) are proposed to increase proliferation, enhance angiogenesis, inhibit apoptosis, and induce invasion in several tissues (1, 121). RT-PCR analysis revealed that the COX-1 and COX-2 mRNA were expressed in all human tissues examined including lung, uterus, testis, brain, pancreas, kidney, liver, thymus, prostate, mammary gland, stomach and small intestine (180). In this study, the highest levels of COX mRNA were detected in the prostate with approximately equal levels of COX-1 and COX-2 transcripts present. High levels of COX-2 also were observed whereas COX-1 mRNA levels were about 2-fold lower in lung. An intermediate level of expression of both COX-1 and COX-2 mRNA was observed in the mammary gland, stomach, small intestine, and uterus. The lowest levels of COX-1 and COX-2 mRNA were observed in the testis, pancreas, kidney, liver, thymus, and brain (180). Although protein of COX-2 is considered to be absent in most tissues under normal conditions, COX-2 protein is highly expressed in normal prostate (180), kidney and brain (181). Extremely high levels of PGs are found in semen as a product of prostate and seminal vesicles. The constitutive expression of COX-2 in these tissues is likely to be important in maintaining normal homeostasis and function (181, 182).
2.5.3.2 Regulation of COX expression

Many factors have been shown to upregulate COX-2 expression at the transcriptional and post-transcriptional levels, including activator protein 1 (AP-1), nuclear factor interleukin-6 (NF-IL6), nuclear factor κB (NF-κB), nuclear factor of activated T cells (NFAT) and polyomavirus enhancer activator 3 (PEA3) (183) (Figure 2.10). In contrast, few negative effectors have been reported. Wild-type p53 has been shown to inhibit COX-2 transcription by competing with TATA-binding proteins for binding to COX-2 gene (184). Another interesting observation is that the CpG island upstream of the COX-2 gene is methylated in greater than 85% of primary and metastatic prostate cancers and in all the 7 tested prostate cancer cells lines, but not in normal tissues (185). Although COX-2 is frequently overexpressed in colorectal cancer, evidence suggests that COX-2 expression is reduced in colorectal cancer with a defective mismatch repair system (186). This phenomenon is commonly associated with hereditary nonpolyposis colorectal cancer (HNPCC). Interestingly, the lack of tumor COX-2 expression in HNPCC is strongly associated with COX-2 promotor hypermethylation. Although traditionally assumed that COX-1 is constitutively expressed and involved in the maintenance of tissue homeostasis, this view has been challenged by studies with COX-1 knockout mice. Disruption of the COX-1 gene is as effective as COX-2 gene disruption in imparting resistance to chemically induced skin tumors (187) and intestinal polyps in Min mice (188). Furthermore, COX-1 expression has been shown to be elevated in renal, cervical, and ovarian cancers and to regulate angiogenic growth factors (189-191).
Figure 2.10 Regulation of cyclooxygenase 2 (COX-2) in cancer (183).
2.6 COX-2 and Cancer

Early evidence for a potential role of COX-2 in human carcinogenesis was reported in 1994, when the mRNA for COX-2 was found to be markedly elevated in colorectal carcinomas (192). Subsequently, numerous studies have established a positive link between COX-2 overexpression and tumorigenesis of several human cancers. Table 2.1 lists the premalignant and malignant tissues that have been identified to have COX-2 overexpression. Evidence from genetic studies suggests that overexpression of COX-2 is sufficient to enhance tumorigenesis in animal models and inhibition of the COX-2 pathway results in a reduction in tumor incidence and progression (1, 2). For example, transgenic mice overexpressing COX-2 developed mammary gland hyperplasia, dysplasia and metastatic tumors (193). COX-2 overexpression also was able to induced hyperplasia and dysplasia in skin (194) and bladder (3) in transgenic mouse models. In contrast, knocking out COX-2 significantly reduced the development of intestinal tumors (188) and skin papillomas (187) in mouse models.

Several mechanisms have been proposed for the role of COX-2 in tumorigenesis. COX-2 may promote angiogenesis by increasing the production of angiogenic factors, such as VEGF, PDGF, bFGF, and TGFβ (195-198). Other mechanisms include the inhibition of apoptosis, increased invasiveness/motility, and modulation of inflammation and immune responses (199). Figure 2.11 summarizes the possible mechanisms of COX-2 in tumorigenesis.
Table 2.1 Cyclooxygenase 2 is overexpressed in several premalignant and malignant conditions (183).

<table>
<thead>
<tr>
<th>Organ</th>
<th>Premalignancy</th>
<th>Malignancy</th>
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<tbody>
<tr>
<td>Colon</td>
<td>Adenoma</td>
<td>Adenocarcinoma</td>
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<tr>
<td>Stomach</td>
<td>Metaplasia</td>
<td>Adenocarcinoma</td>
</tr>
<tr>
<td>Esophagus</td>
<td>Barrett’s esophagus</td>
<td>Adenocarcinoma, squamous cell carcinoma</td>
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<tr>
<td>Liver</td>
<td>Chronic hepatitis</td>
<td>Hepatocellular carcinoma</td>
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<tr>
<td>Biliary system</td>
<td>Bile duct hyperplasia</td>
<td>Cholangiocarcinoma</td>
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<td>Pancreas</td>
<td>Pancreatic intraepithelial neoplasia</td>
<td>Adenocarcinoma</td>
</tr>
<tr>
<td>Head and neck</td>
<td>leukoplakia</td>
<td>Squamous cell carcinoma</td>
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<tr>
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<td>Atypical adenomatous hyperplasia</td>
<td>Adenocarcinoma, squamous cell carcinoma</td>
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<tr>
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<td>Squamous cell carcinoma or adenocarcinoma of cervix, endometrial carcinoma</td>
</tr>
<tr>
<td>Skin</td>
<td>Actinic keratoses</td>
<td>Squamous cell carcinoma</td>
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Figure 2.11 Possible mechanisms of COX-2-derived PGE$_2$ contributions to tumor development. In epithelial tumors of the mammary gland, COX-2-derived PGE$_2$ may stimulate proangiogenic factors such as vascular endothelial growth factor, which promotes tumor-associated angiogenesis. Solid malignancies are made up of multiple types of cells that produce signals that work in both a paracrine and autocrine manner as depicted (199).
2.6.1 COX-2 and prostate cancer

Although there is a strong correlation between COX-2 and the carcinogenesis of several cancers, available data regarding the expression of COX-2 in prostate cancer and its role in prostate carcinogenesis have not been consistent. In humans, the use of NSAIDs has been associated with a decreased risk of developing prostate cancer. Retrospective case-control and prospective cohort epidemiologic analyses examining the risk of prostate cancer relative to NSAID use have resulted in mixed findings. While results from some studies indicate no protective effect of NSAIDS (15-19), there are several reports of a significant reduction in prostate cancer risk attributable to frequent NSAID use (20-23).

Initial reports from several research groups indicated that COX-2 was also overexpressed in human prostate cancers (24-29). However, there are a number of discrepancies in these studies. These include differences in the cell types found to express COX-2 within prostate tissue, expression in normal or benign tissues, and the relationship of level of expression to grade or stage of prostate cancer (31, 200). To address this issue, Zha and coworkers conducted a very comprehensive and well-controlled analysis of COX-2 expression in PC tissues (31). Tissues from a total of 144 PC patients were analyzed, far more than in any similar study. In contrast to previous results, the authors found only scattered expression of COX-2 in less than 1% of cells, and no difference between cancer and normal cells as determined by immunohistochemistry. The investigators found an increased COX-2 expression only in areas of inflammation, which was in agreement with Wang et al. (30), that COX-2 upregulation is only associated with chronic inflammation in benign prostate hyperplasia. Zha et al. further performed
immunoblotting and qRT-PCR analyses on a subset of frozen tissues and found that mean mRNA levels of COX-2 were higher in normal prostate tissues compared to malignant prostate tissues (31). Similarly, Shappell et al. (27) found that COX-2 expression was more often reduced in prostate tumors compared to benign tissues by using well-controlled immunohistochemistry and in situ hybridization analysis. The observed reduction was significant in Gleason score 5 and 6 tumors (27). Previous studies in general suggest that the expression of COX-2 is either low or non-detectable in the normal prostate tissue. However, this notion is challenged by observation that prostate expresses the highest level of both COX-2 and COX-1 among the various tissues examined (180). A recent study by Wagner et al. found that both normal and the benign prostate tissues have either focal or rare COX-2 staining, whereas the adenocarcinoma tissues were uniformly negative for COX-2 expression (201).

There have been relatively few animal studies that have tested the level of COX-2 expression and the effect of NSAIDs on prostate carcinogenesis. Two groups of investigators recently reported that the COX-2 selective inhibitor celecoxib decreased prostate tumorigenesis in the TRAMP transgenic model (34, 35). Celecoxib, however, has been shown to have multiple COX-2 independent activities that could account for its effect on tumorigenesis in this model (36, 37). Until now, investigations into the role of COX-2 in prostate cancer were largely hindered by the lack of prostate specific COX-2 overexpression and knockout mouse models.

In contrast, numerous in vitro studies have examined the expression and role of COX-2 in prostate normal or cancer cell lines. The majority of the in vitro studies examined the effects of NSAIDs on prostate cancer inhibition. Only studies that
determined the biological role of COX inhibitors and the expression of COX in cells are reviewed. In general, these studies found that the expression of COX-2 was either very low or non-detectable in prostate cancer cell lines PC-3, DU145, LNCaP and TSU (31, 182, 201-203). In addition, the growth of PC-3 and DU145 was resistant to the treatment of COX-2 selective inhibitor (201). Interestingly, Subbarayan et al. demonstrated that in contrast to the low mRNA and protein levels of COX-2 in prostate cancer cells, the basal level of COX-2 expression was high in the normal PrEC cells (182). Another study found that COX-2 protein expression was high in the normal PrEC and the benign BPH-1 cells, but low in LNCaP and PC-3 cells (202).

Collectively, the above data suggest that the expression of COX-2 may actually be reduced during late stages of prostate carcinogenesis.

2.6.2 COX-2 and bladder cancer

In contrast to prostate cancer, numerous studies have demonstrated that COX-2 is overexpressed in TCC of human urinary bladder (4-7). The degree of COX-2 expression is significantly correlated with the tumor grade and depth of invasion (T stage) of TCC (8-10). Increased COX-2 expression has also been reported in rat and canine models of bladder cancer (204, 205). Previous study by Klein et al. have shown that forced expression of COX-2, under the control of a keratin 5 promoter, is sufficient to cause mouse urinary bladder transitional cell hyperplasia (TCH) in an age-dependent manner (3). They further observed that TCH was strongly associated with increased Ki67 staining, induced inflammation, lymphocytes and macrophage infiltration in COX-2 overexpression transgenic (K5.COX-2) mouse bladders. Additionally, TCC was observed
in approximately 10% of the K5.COX-2 transgenic mice with increased vascular proliferation and VEGF expression (3). The level of prostaglandin E₂ was significantly increased in the bladder of BK5.COX-2 mice, as has been reported to be elevated in patients with bladder cancer (4, 206).

COX inhibitors have been shown to induce remission of chemically induced bladder tumors in rodents (207, 208), naturally occurring invasive TCC in dogs (209, 210), and bladder carcinoma in an orthotopic mouse model (211). Smakman et al. found that selective COX-2 inhibitor, NS-398, abrogated PGE₂ secretion and inhibited proliferation of human bladder cancer T24 cells (211). They further observed that in vivo administration of NS-398 reduced the outgrowth of experimental orthotopic T24 bladder carcinoma. A substantial number of studies demonstrated that NSAIDs inhibited growth, induced apoptosis, and arrested cell cycling of bladder cancer cells in vitro (11-14, 212).

Despite the strong evidence of the association between COX-2 and bladder cancer from numerous clinicopathologic studies and the consistent results from in vivo and in vitro studies, the epidemiological and clinical data on NSAIDs and bladder cancer are very limited. At present, only one case control study which involved 1514 bladder cancer cases and an equal number of controls has been reported. In this study, Castelao et al. reported that NSAIDs users had almost 20% decreased risk for urinary bladder cancer (213). Both phase I and phase II clinical trials using NSAIDs for bladder cancer chemoprevention are underway at present. Dr. Jaye Viner of the NCI Division of Cancer Prevention has stated, “NSAIDs are arguably the most promising chemopreventive agents for epithelial cancers, such as bladder cancer”. More prevention trials that targeting COX/PGs pathway are expected to be carried out in the near future.
These studies suggest that there is an association between COX-2, prostaglandin synthesis and the development and progression of urinary bladder cancer. However, the exact role and mechanisms of COX-2 overexpression during bladder carcinogenesis have not been well defined.

2.7 Family of PGs an their role in cancer

2.7.1 Family of PGs

Prostaglandins (PGs) are the most abundant lipid-derived eicosanoids generated by sequential metabolism of AA by the COX enzymes and PG synthase enzymes. Upon released from membrane by PLA2, AA is converted to an unstable PG endoperoxide intermediate PGG2 and then PGH2 in reactions catalyzed by the rate-limiting enzyme COX (47). PGH2 is then converted to a series of different PGs that is dependent on the presence of the cell-specific PG synthase enzymes (47, 169). At present, five PGs have been identified, including PGE2, PGD2, PGF2α, PGI2, and tromboxane A2 (169) (Figure 2.5). Among these PGs, PGE2 is the most common and ubiquitously produced metabolite and acts locally in an autocrine and paracrine manner to elicit a wide range of physiological responses (44). PGE2 was first extracted from semen, prostate, and seminal vesicles by Goldblatt and von Euler in the 1930s and were shown to lower blood pressure and cause smooth muscle contraction (121). It is now well established that PGE2 is important in control of gastric secretion, renal function, smooth muscle contraction and relaxation (depending on the tissue), and immunity (214). In addition, PGE2 has been implicated in a broad array of diseases including cancer, inflammation, cardiovascular disease and hypertension (44). PGE2 may contribute to tumor progression by inducing
cell proliferation, angiogenesis, invasion and metastasis (2, 3). Also, PGE₂ has been reported to participate in these complex processes by stimulating vascular endothelial growth factor (VEGF) secretion, cell migration and matrix metalloproteinase-2 (MMP-2) expression and activation (34, 215).

2.7.2 PGE₂ and prostate cancer

Human prostate epithelial cells synthesize PGs, particularly PGE₂. However, the exact role of PGE₂ in prostate carcinogenesis remains unknown. Although several studies have assumed that PGE₂ levels are increased in prostate cancer tissues and in vitro cultured prostate cancer cells, the level of PGE₂ was not determined in any of these studies (24, 216-218). Collectively, these studies suggested that PGE₂ could mediate signaling pathways involved in increased cell proliferation, promotion of angiogenesis, and inhibition of apoptosis in prostate cancer cells. A potential mechanism for the effects of PGE₂ on angiogenesis is through the induction of VEGF which has been shown to be upregulated by COX-2 activity and PGE₂ treatment in PC-3 ML prostate cancer cells (219).

An in vitro study demonstrated that PGE₂ increased prostate PC-3 cell growth and the mRNA level of COX-2 (218). The investigators further found that flurbiprofen inhibited PGE₂-induced cell proliferation, and COX-2 up-regulation in PC-3 cells. Another in vitro study reported by Liu et al. demonstrated that PGE₂ significantly increased hypoxia-inducible factor-1α (HIF-1α) expression, a transcriptional activator of VEGF, particularly in the nucleus (220). They also demonstrated that two selective COX-2 inhibitors, meloxicam and NS398, were both able to decrease HIF-1α levels and
nuclear localization. However, two investigators who actually measured PGE$_2$ levels in prostate found that PGE$_2$ production is low or non-detectable in prostate benign and tumor tissues (27), and in PC-3 and DU145 cells (201).

Taken together, the exact role and direct molecular mechanisms involved in the tumor stimulating effects of PGE$_2$ in the prostate remain largely unknown. Given the fact that both COX expression and activity might diminish during prostate carcinogenesis, it is essential to elucidate the contribution of the upstream of PG pathway (the COX expression) to prostate carcinogenesis. Moreover, COX inhibitors have been criticized for causing gastrointestinal side effects and other potential risks, which is a major obstacle for large-scale application to the prevention of prostate cancer. Therefore, targeting down-stream signaling pathways of PGE$_2$ may be an attractive new strategy for prevention and therapy of prostate cancer.

2.7.3 PGE$_2$ and bladder cancer

Elevated COX-2 expression and PGE$_2$ has been reported in patients with urinary tract infections and with bladder cancer when compared with age matched controls (168). A study from Badawi et al. found that higher COX-2 expression and PGE$_2$ synthesis were associated with patients with bladder cancer who are smokers compared with nonsmokers (206). These results suggest that elevated COX-2 expression and PGE$_2$ production may be the mechanisms by which cigarette smoking and infection influence the development of human urinary bladder cancer. Shi et al. determined that both the mRNA level of COX-2 and the concentration of PGE$_2$ were significantly increased in the TCC of Wistar rats induced by terephthalic acid (TPA) (221). Increased secretion of PGE$_2$ has been
observed in the bladder cancer cell lines including TSGH8301, TCC 8702, RT4, TCC8701, TCC9101, HT1376 and T24 compared to normal urothelial cells (222). Although compelling evidence suggests that PGE₂ secretion is increased in bladder cancer, studies directly examining the role of PGE₂ in bladder carcinogenesis are lacking. Additional studies are needed to better understand the role of COX-2 and PGE₂ in bladder carcinogenesis. As for prostate cancer, targeting down-stream signaling pathways of PGE₂ may represent novel strategies for prevention and therapy of bladder cancer.

2.8 Mechanism of PGE₂ action

2.8.1 PG receptors

The physiological effects of PGs are mediated primarily by G-protein-coupled receptors, a family of rhodopsin-like seven transmembrane spanning receptors (44). There are at least nine known PG receptor forms found in human (169). PG receptors are designated by the letter “P” and a prefix of “D”, “E”, “F”, “I” and “T” according to their preference for PGD₂, PGE₂, PGF₂α, PGI₂, and thromboxane A₂ respectively (121). Four of the receptor subtypes bind PGE₂ (EP1-EP4), two bind PGD₂ (DP1 and DP2), and FP, IP, and TP binds to PGF₂α, PGI₂, and tromboxane A2 respectively (169) (Figure 2.12). The four PGE₂ receptors are encoded by different genes.
Figure 2.11 Prostaglandin synthesis and actions (169).
EP1 is coupled to phospholipase C, generating two second messengers inositol trisphosphate, which is involved in the liberation of intracellular calcium (Ca$^{2+}$), and diacylglycerol, an activator of protein kinase C (220, 223). EP2 and EP4 are coupled to adenylated cyclase and generate cAMP that activates the protein kinase A signaling pathways. The EP3 receptor is known to have multiple splice variants in humans (at least nine). The signaling of EP3 is more complex, with a wide range of action from inhibiting cAMP to increasing Ca$^{2+}$ and inositol trisphosphate (169). The exact physiologic roles PG receptors play in normal and pathologic settings are determined by multiple factors including cellular context, receptor expression profile, ligand affinity, and different coupling to signal transduction pathways (44).

Most PGE$_2$ receptors are localized at the plasma membrane, although some are found situated at the nuclear envelope. Bhattacharya et al. found that EP4 and a splice variant of EP3 receptors are clearly visualized in the nuclear envelope of HEK 239 cells stably overexpressing these receptors (224). However, the function of these receptors in the nuclear envelope are not clear. Interestingly, nuclear peroxisome proliferators activated receptors $\delta$ and $\gamma$ (PPAR$\delta$/PPAR$\gamma$) are receptors for PGJ$_2$ and cyclopentenone PGs, such as 15-d-PGJ$_2$ (a metabolite of PGD$_2$), respectively (47). PGE$_2$ has been shown to increase angiogenesis and inhibit apoptosis through the classical plasma membrane EP receptors (121). In contrast, accumulating evidence suggest that 15d-PGJ$_2$ is able to induce apoptosis and inhibit angiogenesis upon activating PPAR$\gamma$ (121, 225). A recent study from Subbarayan et al. suggests that prostate cancer PC-3 cells express significantly more PPAR$\gamma$ than normal prostate epithelial cells (226). They further demonstrated that the PPAR$\gamma$ ligand 15-d-PGJ$_2$ is able to induce cell death in PC-3 cells.
expressing PPARγ. These findings suggest that the nuclear-acting PG (15-d-PGJ2) and PPARγ may be involved in negative regulation of prostate cancer cell growth. However, the implication of these results and the molecular mechanisms involved need further investigation.

2.8.2 EP receptor and cancer

Several studies have demonstrated that EP receptors (individually or in combination) are implicated in promoting carcinogenesis in different types of tumors, including lung (45), ovarian (46), colorectal (47), colon (48), and breast (49). Spinella et al. found that EP2 and EP4 are involved in up-regulating VEGF, MMP and ovarian cell invasiveness upon endothelin-1 stimulation, a key mediator in ovarian tumorigenesis (46). A study from Miyata et al. suggests that only EP4 receptor is co-expressed with COX-2 in transitional cell carcinoma of the upper urinary tract (227). Interestingly, Shoji et al. found that the mRNA level of both EP1 and EP2 receptors increased in colon cancer tissue, but not the EP4 receptor, which is constitutively expressed in normal and cancer tissues (48). However, they also found that EP3 receptor mRNA level is significantly decreased in colon cancer tissue compared to normal tissue. They proposed that EP3 may play a key role in the suppression of colon cancer cell growth and its downregulation may enhance colon carcinogenesis at a later stage. Results from these studies suggest that EP receptors modulate tumorigenesis in a tissue-specific manner.

The use of genetically modified EP receptor knock out mouse models have greatly enhanced our understanding of their potential functions in tumorigenesis. A study from Yang et al. found that EP2(-/-) mice that had undergone isograft injection of MC26
or Lewis lung carcinoma cells exhibited significant reduction in tumor growth and prolonged lifespan as compared with the wild type mice (228). Knockout of EP4 receptor, but not the EP2 receptor, decreased the formation of aberrant crypt foci in animals treated with the colon carcinogen azoxymethane (229). A recent study from Kawamori et al. demonstrated that the EP1(-/-) mice significantly reduced colon cancer incidence and tumor volume, suggesting that EP1 receptor may also play an important role in development and progression of colon cancer (230).

Despite many studies demonstrating that PGE₂ and its downstream EP receptors are important in tumorigenesis, the actual molecular targets of activated EP receptors remain illusive. It has been demonstrated that PGE₂ increases VEGF secretion via the cAMP-protein kinase A (PKA) signaling pathway in a EP2- and EP4- dependent manner (223). However, the relative contribution of EP2 verse EP4 receptors is not well defined. In addition to increased VEGF secretion, EP2 receptor has also been demonstrated to inhibit glycogen synthase kinase-3 (GSK-3) by a PKA-dependent mechanism (223). However, EP4 preferentially uses a phosphatidylinositol 3-kinase (PI3K)-dependent pathway involving Akt/protein kinase B and activating MAPK/Erk signaling in HEK cells (231, 232). Buchanan et al. found that PGE₂ is able to induce migration and invasion of colorectal carcinoma cells, which is partially due to the rapid transactivation and phosphorylation of the epidermal growth factor receptor (EGFR) and Akt activation (233). Although the investigators did not implicate EP receptor in their study, these results suggest that EP receptors may be involved in EGFR activation and one of its downstream signaling events, the Akt pathway. Fukuda and co-workers found that PGE₂ induce the up-regulation of VEGF mRNA level in HCT116 human colon carcinoma cells
They further demonstrated that EP1 is the key receptor contributing to VEGF up-regulation through mediating multiple signaling pathways, including ERK, Akt and HIF-1. These results suggest that EP receptors are able to target a variety of molecules involved in several signaling pathways, including cell proliferation, apoptosis, invasion and angiogenesis. These findings also suggest that there are important differences in the signaling potential of the EP receptors, which should be the primary consideration for developing new cancer chemopreventive strategies through targeting EP receptors and their down-stream effectors. The development and use of selective EP receptor agonists and antagonists have greatly facilitated our understanding about the role and mechanisms for COX-2 and PGE₂ in human carcinogenesis. Table 2.2 lists the current developed EP receptor agonist and antagonists.

Unfortunately, the study of the expression of EP receptors in both prostate and bladder tissues and their direct role in tumorigenesis has been limited. So far, there are only two in vitro studies that determined the expression of EP receptors and molecular pathways in prostate cancer cells. Chen et al. found that treatment of PC-3 cells with arachidonic acid remarkably increased PGE₂ secretion, which leads to the induction of c-fos in these cells (52). They further determined the expression of the four EP receptors in PC-3 cells together with two other prostate cancer cell lines (DU145, LNCaP) and a human normal prostate isolate (PrEC) by RT-PCR. Results show that only EP2 and EP4 are expressed in the examined prostate cells, but not EP1 and EP3 receptors. Liu et al. reported that EP2, EP3 and EP4 are expressed in prostate cancer PC ML cells, and EP2 and EP4 receptors are involved in PGE₂-induced HIF-1α in these cells (42). Similarly, there is only one study showing that the expression of EP4 receptor was upregulated in
patients with TCC (227). Taken together, results from these studies are insufficient to make any definitive conclusions regarding the role PGE₂ and its EP receptors play in prostate and bladder carcinogenesis.

In summary, prostate and bladder cancers are significant health problems for middle-aged and elderly population of the United States. Therefore, prostate and bladder cancer chemoprevention through drugs, such as NSAIDs, and dietary intervention, is an important issue. However, before we could use any agents to prevent these two diseases, the molecular targets and their modes of action need to be validated. Although there is a strong association between COX-2/PGE₂ synthesis pathway and bladder cancer carcinogenesis, the molecular mechanisms and gene regulation upon COX-2 overexpression in bladder cancer have not been defined. In contrast, whether targeting COX-2/PGE₂ pathway would be an effective chemopreventive strategy for prostate cancer needs to be critically examined. Recently, COX inhibitors have been criticized for causing gastrointestinal side effects and other potential risks, such as heart disease. Therefore, inhibition of downstream effectors of PGE₂, such as EP receptors, has the potential to be efficacies for prevention and therapy of prostate and bladder cancer. However, both the expression profile of EP receptors and their role in PGE₂-elicited carcinogenic effects need to be determined in these two cancers.
<table>
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<tr>
<th>PG Receptor</th>
<th>Agonist</th>
<th>Company</th>
<th>Antagonist</th>
<th>Company</th>
<th>Natural Ligand</th>
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</thead>
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<td>EP1</td>
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<td>Ono Pharm. Jap.</td>
<td>SC 19220</td>
<td>Cayman</td>
<td>PGE$_2$</td>
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<td></td>
<td></td>
<td>ONO-8713</td>
<td>Ono Pharm. Jap.</td>
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<tr>
<td>EP2</td>
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<td>Cayman</td>
<td>None</td>
<td>None</td>
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<tr>
<td></td>
<td>CAY10399</td>
<td>Cayman</td>
<td></td>
<td></td>
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<tr>
<td>EP3</td>
<td>Sulprostone</td>
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<td>ONO-AE3-240</td>
<td>Ono Pharm. Jap.</td>
<td>PGE$_2$</td>
</tr>
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</table>

**Table 2.2** Prostaglandin receptor agonists and antagonists.
CHAPTER 3

PROSTAGLANDIN E2 INDUCES VESICULAR ENDOTHELIAL GROWTH FACTORS SECRETION IN PROSTATE CANCER CELLS THROUGH EP2 RECEPTOR MEDIATED PATHWAY

Xingya Wang\textsuperscript{1} and Russell D. Klein\textsuperscript{1,2,3}

\textsuperscript{1}The Ohio State University, Interdisciplinary Ph.D. Program in Nutrition (OSUN)
\textsuperscript{2}the Ohio State University Comprehensive Cancer Center, Molecular Carcinogenesis and Chemoprevention Program
\textsuperscript{3}Department of Human Nutrition,
325 Campbell Hall, 1787 Neil Avenue, Columbus, OH 43210

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3.1 ABSTRACT

Prostaglandin E₂ (PGE₂) has been shown to induce expression of vascular endothelial growth factor (VEGF) and other signaling molecules in several cancers. PGE₂ elicits its functions through four G-protein coupled membrane receptors (EP1-4). In this study, we investigated the role of EP receptors in PGE₂-induced molecular events in prostate cancer cells. qRT-PCR analysis revealed that PC-3 cells express a substantially higher level of EP2 and moderately higher EP4 than DU145 and LNCaP cells. LNCaP cells had virtually no detectable EP2 mRNA. EP1 and EP3 mRNAs were not detected in these cells. Treatment of prostate cancer cells with PGE₂ (1 nM -10 µM) increased both VEGF secretion and cAMP production. Levels of induction in PC-3 cells were greater than in DU145 and LNCaP cells. The selective EP2 agonist CAY10399 also significantly increased VEGF secretion and cAMP production in PC-3 cells, but not in DU145 and LNCaP cells. Moreover, PGE₂ and CAY10399 increased MAPK/Erk and Akt phosphorylation in PC-3 and DU145 cells, but not in LNCaP cells. However, neither the MAPK/Erk inhibitor U0126 nor the PI3K/Akt inhibitor LY294002 abolished PGE₂-induced VEGF secretion in PC-3 cells. We further demonstrated that the adenylate cyclase activator forskolin and the cAMP anologue 8-bromo-cAMP mimicked the effects of PGE₂ on VEGF secretion in PC-3 cells. Meanwhile, the adenylate cyclase inhibitor 2’5’-dideoxyadenosine, at concentrations that inhibited PGE₂-induced cAMP, significantly blocked PGE₂-induced VEGF secretion in PC-3 cells. We conclude that PGE₂-induced VEGF secretion in prostate cancer cells is mediated through EP2, and possibly EP4, -dependent cAMP signaling pathways.
3.2 INTRODUCTION

Prostaglandins (PGs) are produced from fatty acids released from membrane lipids by the rate-limiting cyclooxygenases (COX-1 and COX-2) and have been implicated in a broad array of diseases including cancer. At present, five PGs have been identified, including PGE\(_2\), PGD\(_2\), PGF\(_{2\alpha}\), PGI\(_2\), and thromboxane A\(_2\) (169). PGE\(_2\) is the most common and ubiquitously produced PG, which acts locally in an autocrine and over short distances in a paracrine manner to elicit a wide range of physiological functions, including controlling of gastric secretion, renal function and other processes. Extremely high levels of PGs are found in semen as a product of prostate and seminal vesicles (235). PGE\(_2\) is the most abundant PG in prostate. Although the exact physiological function of PGE\(_2\) in prostate is still unknown, it is likely to be important in maintaining normal homeostasis and function of the prostate (182).

It is well established that PGE\(_2\) may contribute to tumorigenesis via induction of cell proliferation, angiogenesis, invasion and metastasis in several cancers, such as colon, lung, breast, skin, and bladder (1-3). In particular, PGE\(_2\) has been shown to induce the production of VEGF, which is a key mediator of angiogenesis in these cancers (38-43, 236). In these studies, either elevated PGE\(_2\) levels have been found in cancer compared to normal conditions or direct role of PGE\(_2\) in tumorigenesis of these cancers have been determined. On the contrast, there is no definitive evidence suggest that PGE\(_2\) plays a key role in prostate carcinogenesis at present. Despite the lack of direct evidence, it has been proposed that the association between PGE\(_2\) and prostate carcinogenesis is supported by results from the use of COX inhibitors in epidemiological (21, 23), \textit{in vivo} (34, 35), and
in vitro studies (218, 220). The majority of these studies have focused on COX inhibitors in regulating prostate carcinogenesis. Available data suggest that PGE₂ may mediate signaling pathways that promote cell proliferation (52, 219), invasion (217), and angiogenesis (42) in prostate cancer cells. However, the direct role and exact molecular mechanisms involved in the carcinogenic effects of PGE₂ in prostate cancer development and progression have received limited attention. Moreover, COX inhibitors are criticized for causing unwanted side effects, which is a major obstacle for widespread use in the prevention of prostate cancer. Therefore, targeting down-stream signaling pathways of PGE₂, if it plays a role in prostate carcinogenesis, may represent an attractive new strategy for prostate cancer prevention and therapy.

The physiological effects of PGE₂ are mediated by four G protein coupled membrane receptors (EP1, EP2, EP3 and EP4), which are encoded by different genes (44). EP1 is coupled to phospholipase C to generate the second messengers inositol trisphosphate that is involved in the liberation of intracellular calcium (Ca²⁺) and diacylglycerol, an activator of protein kinase C (PKC) (47). EP2 and EP4 are coupled to adenylate cyclase and generate cAMP that activates the protein kinase A (PKA) signaling pathways (223). The signaling of EP3 is more complex, with a wide range of actions from inhibition of cAMP to increases in Ca²⁺ and inositol trisphosphate (169). This is due to the presence of multiple splice variants of EP3 receptor. The exact physiologic roles PG receptors play in normal and pathologic settings are determined by multiple factors including cellular type, receptor expression profile, ligand affinity, and coupling to different signal transduction pathways (44). EP receptors have been implicated in promoting carcinogenesis in different types of tumors, including lung (45), ovarian (46),
colorectal (47, 48), breast (49), and skin (50, 51). The use of genetically modified EP receptor knock-out mouse models has greatly enhanced our understanding of their potential functions in tumorigenesis. Despite many studies demonstrating that PGE\textsubscript{2} and its downstream EP receptors are important in tumorigenesis, the molecular targets of activated EP receptors remain illusive. Accumulating evidence demonstrates that EP receptors (individual or in combination) are able to target a variety of signaling molecules in many cancer models. These include VEGF upregulation by PKA-dependent (223) and hypoxia-inducible factor-\textalpha (HIF-1\textalpha)-dependent (234) pathways, epidermal growth factor receptor (EGFR) (233), c-fos (52), PI3K/Akt (231), Erk (232), and beta-catenin (237). Due to the complexity of PGE\textsubscript{2} and its EP receptors induced signaling system, their effects need to be determined in specific cell types.

Unfortunately, the study of the expression of EP receptors in prostate tissue and their direct role in prostate carcinogenesis is limited. To our knowledge, there are only two in vitro studies that are concerned with the expression of EP receptors and their potential role in prostate cancer cells (42, 52). Chen et al. found that EP2 and EP4 are predominantly expressed in three prostate cancer cells, PC-3, DU145 and LNCaP (52). They further demonstrated that both EP2 and EP4 receptors were involved in PGE\textsubscript{2}-induced c-fos mRNA expression through PKA signaling pathways in PC-3 cells. Another study reported that EP2, EP3 and EP4 are expressed in prostate cancer PC-3 ML cells, but only EP2 and EP4 are involved in PGE\textsubscript{2}-induced HIF-1\textalpha in these cells (42). However, results from these two in vitro studies are insufficient for making definitive conclusions about the roles of PGE\textsubscript{2} and its EP receptors in prostate carcinogenesis.
In the present study, we investigated the expression profile of the EP receptors and their role in PGE2-induced signaling events in several prostate cancer cell lines. We found that EP2 and EP4, but not EP1 or EP3 receptors, are predominantly expressed in human prostate epithelial cells. We further demonstrated that PC-3 cells express a substantially higher level of EP2 and a moderately higher level of EP4 as compared to DU145 and LNCaP cells. We report that PGE2 induces VEGF protein secretion in prostate cancer cells, which is primarily mediated by the EP2/cAMP-dependent signaling pathway, but not through MAPK/Erk and Akt signaling pathways. In addition, the activation of both MAPK/Erk and Akt are also correlated with the expression level of the EP2 receptor in prostate cancer cells. However, the role of EP4 could not be ruled out at present. Our study provides evidence that the EP2 receptor is primarily responsible for PGE2-induced molecular events in prostate cancer cells. Assuming PGE2 plays a role in prostate carcinogenesis, the EP2 receptor may serve as a potentially useful target for prostate cancer chemoprevention and therapy.

3.3 MATERIALS AND METHODS

3.3.1 Materials

PGE2 and EP2 receptor agonist CAY10399, indomethacin and polyclonal EP1, EP2, EP3 and EP4 antibodies were purchased from Cayman Chemical (Ann Arbor, MI). Forskolin, 8-bromo-cAMP and 2’5’-dideoxyadenosine were purchased from Calbiochem (San Diego, CA). MAPK/Erk kinase inhibitor U0126 and PI3K/Akt inhibitor LY294002 were from Cell Signaling (Beverly, MA). The Quantikine human VEGF ELISA kit was purchased from R&D Systems (Minneapolis, MN). The Direct Cyclic AMP enzyme
immunoassay (EIA) kit was purchased from Assay Design (Ann Arbor, MI). Phospho- and total Erk, Akt (ser 473) polyclonal antibodies, and anti-Rabbit HRP-linked IgG were purchased from Cell Signaling (Beverly, MA). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA). RNeasy Mini RNA extraction kit was purchased from Qiagen (Valencia, CA). The iScript cDNA synthesis kit was purchased from Bio-Rad (Hercules, CA). Taqman® Gene Expression Assay kits for all primers: EP1 (Hs00168752-m1), EP2 (Hs00168754-m1), EP3 (Hs00168755-m1), EP4 (Hs00168761-m1), VEGF (Hs00173626-m1) and GAPDH (Hs99999905-m1), as well as Taqman® Universal PCR Mastermix were purchased from Applied Biosystems (Foster City, CA). BCA protein Assay kit and RadioImmuno Precipitation Assay (RIPA) cell lysis buffer were purchased from Pierce (Rockford, IL).

3.3.2 Cell culture

Human prostate cancer cell lines PC-3, DU145 and LNCaP were purchased from the American Type Culture Collection. PC-3 and DU145 cells were cultured in Dulbecco’s modified Eagle’s medium (GIBCO) containing 10% fetal bovine serum (FBS). LNCaP cells were cultured in RPMI-1640 medium (GIBCO) containing 10% FBS. The PrEC normal human prostate epithelial cells were purchased from Clonetics (San Diego, CA), and incubated in the PrECM medium (Clonetics). All cells were maintained in a humidified atmosphere with 5% CO₂ at 37°C. For the experiments, cells were seeded at 2 × 10⁵ cells/well in six-well plates, and incubated 24 h in serum containing medium. Cells were then serum-starved overnight followed by treatment with different reagents.
3.3.3 RNA extraction and Real-Time PCR (qRT-PCR)

For EP receptor expression assay, the prostate cancer cells (PC-3, DU145, LNCaP) and the primary PrEC cells were seeded in 10 cm dishes and incubated in their respective medium. When cells reached 70% confluence, they were rinsed with phosphate buffer saline (PBS) twice and RNA isolation was performed. Total RNA from cells was isolated by TRIzol reagent and further purified by RNeasy Mini kit as described by Qiagen. Both the quantity and quality of total RNA were analyzed by the Agilent Bioanalyzer 2100 system. Total RNA (1 µg) was reverse transcribed with iScript cDNA synthesis kit. qRT-PCR was performed to determine the expression of EP receptors using a 1:10 dilution of cDNA with Taqman gene expression primers and ABI mastermix according to the manufacturer’s instructions on an iCycler IQ Real-Time PCR detection system (Bio-Rad). GAPDH was used as the reference gene for all samples.

3.3.4 VEGF measurement by ELISA

Cells were seeded at 2 x 10^5 cells/well in 6-well plates and allowed to attach for 24 h. Medium was removed, cells were washed twice with PBS, and cultures were incubated in serum-free medium for 16 h prior to treatment. Cells were treated with 1 nM -10 µM PGE₂ (and/or CAY10399) in serum-free medium containing 1% bovine serum albumin for 24 h. PGE₂ and CAY10399 were dissolved in absolute ethanol. To examine VEGF induction by the cAMP analogue 8-bromo-cAMP and forskolin, PC-3 cells were treated with 500 nM to 50 µM 8-bromo-cAMP and 100 nM to 10 µM foslkin for 24 h. Both forskolin and 8-bromo-cAMP were solubilized in DMSO. For inhibitor studies, cells were pretreated with the respective inhibitors at indicated working concentrations
for 1 h in serum-free medium prior to adding PGE₂. U0126, LY294002, and 2’5’-dideoxyadenosine were solubilized in DMSO. For all experiments, the amounts of solvent were normalized among the treatments and controls. After treatment, medium was collected to measure the concentration of secreted VEGF₁₆₅ subtype by Quantikine human VEGF ELISA kit (R&D Systems). Cells were washed with PBS and lysed with RIPA lysis buffer. Protein concentrations in the medium and cell lysates were determined by the bicinchoninic acid method (BCA) according to the manufacturer’s instruction (Pierce).

3.3.5 Cyclic AMP measurement

Cells were seeded in 6-well plates as described above. For dose-response experiments, the same doses of PGE₂ and CAY10399 for VEGF assay were used in serum free medium and cells were collected after 30 min. For the time course experiments, cells were treated with 0, 1 nM or 10 µM PGE₂ for 0, 15, 30 min, and 1, 2, 4, 8 and 24 h. After treatment, cells were washed once with PBS before lysing with 0.5 ml 0.1 M HCl and Triton X-100 (0.1%) for 15 min. Cell lysates were subsequently collected and subjected to cAMP EIA assay according to the manufacturer’s instruction (Assay Design). Protein concentrations in the cell lysates were determined by the BCA assay according to the manufacturer’s instruction (Pierce).

3.3.6 Immunoblotting

Cells were seeded in 6-well plates as described above. After appropriate treatment, growth medium was removed, cells were washed twice with PBS, and 60 µl of 1x NEB
buffer (62.5 mM Tris-Base (pH6.8), 2 % SDS, 10 % Glycerol, 50 mM DTT, 0.1% bromophenol blue) was added to each well. Cells lysates were collected, denatured for 5 min at 100°C, and centrifuged at 13,000 rpm for 5 min at 4°C. Approximately 20 µl of each respective supernant was loaded onto a 10% SDS-polyacrylamide gel and electrophoresed at 170 V for about 1 h. Separated proteins were transferred onto a PVDF membrane at 100 V for 30 min on ice. After transfer, membranes were blocked with 5% non-fat dry milk in 1x TBST (50 mM Tris pH7.5, 150 mM NaCl, 0.1% Tween-20) at room temperature for 1 h. Phosphorylated and total Erk1/2 or Akt (serine 473) were determined by western blot using the indicated antibodies according to the manufacturer’s instructions (Cell Signaling). EP receptor western blotting was carried out according to manufacturer’s instructions (Cayman Chemical) using antibody dilutions of 1:333 (EP1), 1:1000 (EP2), 1:500 (EP3), 1:750 (EP4) and 1:2000 (anti-Rb HRP) in 5% non-fat dry milk/TBS with 0.1% Tween-20.

3.3.7 Statistical Analysis

All the experiments were repeated at least three times, and data are presented as means ± standard deviation (SD). Comparisons among multiple groups were performed by one-way analysis of variance (ANOVA) with Tukey’s post-hoc comparisons. A p-value of <0.05 was considered statistically significant. All analyses were performed using SPSS 13.0 software (Chicago, IL).
3.4 RESULTS

3.4.1 EP2 and EP4 receptors are predominantly expressed in prostate cells

The expression of all the four EP receptors in prostate cells was examined by qRT-PCR and immunoblotting analysis. The mRNA of both the EP2 and EP4 receptors, but not EP1 or EP3 receptors, were detected in prostate cells. PC-3 cells express a substantially higher level of EP2 than DU145 and PrEC cells (Figure 3.1 A). However, EP2 mRNA was not detected in LNCaP cells. PC-3 cells also express higher level of EP4 mRNA than DU145, LNCaP and PrEC cells (Figure 3.1 B). We also determined the expression of the four EP receptors in four other prostate cell lines, viz., RWPE-1, RWPE-2, PWR-1E and 22RV1. Again, EP2 and EP4 receptor mRNA were present in these cell lines, whereas EP1 and EP3 were not detected. However, results from immunoblotting did not correlate with results from qRT-PCR (Figure A.1-see appendix). Further experiments demonstrated that the sensitivity and specificity of EP receptor antibodies from Cayman Chemical and Alpha Diagnostic (San Antonio, TX) were unreliable. For example, we found that EP4 binds to unknown antigens in the serum-containing medium (Figure A.2). Results from qRT-PCR suggest that PGE2-elicited physiological functions in prostate cells are primarily mediated by EP2 and EP4 receptors.

3.4.2 VEGF secretion upon PGE2 treatment is correlated with the presence of EP2 receptor

To determine the effects of PGE2 on angiogenic pathways in prostate cells, we examined whether treatment with PGE2 altered expression of VEGF in three prostate cancer cell lines (PC-3, DU145 and LNCaP). The basal level of PGE2 in PC-3, DU145
Figure 3.1 Relative mRNA expression of EP2 and EP4 receptors in PC-3, DU145, LNCaP and PrEC cells determined by qRT-PCR. A, PC-3 cells express a substantially higher level of EP2 receptor mRNA than DU145 and PrEC cells. No EP2 mRNA was detected in LNCaP cells. B, PC-3 cells express a higher level of EP4 receptor mRNA than DU145, LNCaP and PrEC cells. GAPDH was used as the reference gene for all samples. Experiments were repeated three times in triplicate. Results are represented as average expression relative to lowest expressing sample; error bars indicate the 95% CI.
and LNCaP cell culture medium before treatment were 19.5 pmol/L, 16.6 pmol/L and 22.4 pmol/L, respectively. Due to the low basal levels of PGE2 in these cells compared to the concentrations we used in this study, the COX inhibitor indomethacin was not added before our treatment with PGE2 in subsequent experiments. There was a significant increase of VEGF protein secretion compared to controls in all three cell lines after 24 h incubation with concentrations of PGE2 ranging from 1 nM to 10 µM in serum-free medium (Figure 3.2 A). The response of PC-3 cells to each dose of PGE2 significantly exceeded that of DU145 and LNCaP cells. Although the relative increase in VEGF secretion by PGE2 in PC-3 cells was greatest, the basal level of VEGF secretion was much lower on average (45 pg/ml) as compared to DU145 and LNCaP cells (e.g. 1650 pg/ml and 825 pg/ml on average, respectively). VEGF mRNA was not altered by PGE2 treatment in the three prostate cancer cells. To determine whether the EP2 receptor was critical for PGE2-induced VEGF secretion in prostate cancer cells, PC-3 cells were treated with an EP2 selective agonist, CAY10399, using the same concentrations as for PGE2 treatment. Stimulation of the EP2 receptor by CAY10399 significantly increased VEGF secretion in PC-3 cells, although the extent of stimulation was less than that for cells treated with equivalent concentration of PGE2 (Figure 3.2 B). However, there were no additive or synergistic effects of combined PGE2 and CAY10399 on VEGF protein secretion in PC-3 cells (data not shown). Treatment with CAY10399 slightly, but not significantly, increased VEGF secretion in DU145 and LNCaP cells (data not shown). In addition, VEGF secretion was not significantly increased in the non-cancerous prostate cell line RWPE-1 and its transformed tumorigenic RWPE-2 counterpart cells upon PGE2 treatment (data not shown). For all the above experiments, there were no observed
Figure 3.2 PGE\textsubscript{2} and the EP2 receptor agonist CAY10399 induce VEGF protein secretion in human prostate cancer cells. **A**, Percent increase of VEGF secretion with PGE\textsubscript{2} treatment (0 to 10 \(\mu\)M) in PC-3, DU145 and LNCaP cells. Except for LNCaP cells treated with 1 nM PGE\textsubscript{2}, all treatments significantly increased VEGF secretion compared to control (p <0.05, n=4). **B**, The EP2 receptor selective agonist CAY10399 increases VEGF secretion to a lesser extent than equivalent concentrations of PGE\textsubscript{2} (1 nM to 10 \(\mu\)M) in PC-3 cells (p <0.05 at concentrations above 100 nM). VEGF was determined by ELISA. All experiments were repeated at least three times in duplicate. Data are represented as mean ± SD. A p-value of <0.05 is considered as significant.
differences in cell proliferation and protein concentrations (in either the cell lysates or incubation medium) associated with PGE2 treatment after 24 h in serum free medium. Collectively, these data suggest that PGE2 induces VEGF secretion in prostate cancer cell lines posttranscriptionally by a process mediated primarily by EP2 and possibly EP4 receptors.

3.4.3 PGE2-induced cAMP production is correlated with the expression level of EP2 receptor

Since EP2 and EP4 receptors are coupled to an increase in cAMP, the effect of PGE2 on intracellular cAMP regulation was examined. As shown in Figure 3A, incubation with concentrations of PGE2 above 10 nM for 30 min significantly increased cAMP concentration in a dose-dependent manner in PC-3 cells. Similarly, CAY10399 (above 100 nM) significantly, but to a much lesser extent than PGE2, increased cAMP concentration in PC-3 cells (Figure 3.3A). PGE2 treatment also increased cAMP concentration in DU145 and LNCaP cells, although the extent of the increase was much less than that in the PC-3 cells (Figure 3.3B). LNCaP cells which only express low levels of EP4 had the least induction of cAMP upon PGE2 treatment. These data suggest that the EP2 receptor is the most important receptor for bulk stimulation of intracellular cAMP production in prostate cancer cells. We also determined the rate of increase in cAMP levels upon stimulation with low (1 nM) and high (10 μM) concentrations of PGE2 in PC-3 cells (Figure 3.4). Both concentrations of PGE2 increased cAMP concentration within 15 min and maximum concentrations were reached by 1 or 2 h. The increase of cAMP concentration by either 1 nM or 10 μM of PGE2 remained significantly elevated
Figure 3.3 PGE$_2$ and the EP2 receptor selective agonist CAY10399 increases cAMP concentrations in PC-3, DU145 and LNCaP cells. **A**, Treatment of PC-3 cells with PGE$_2$ and CAY10399 for 30 min dose-dependently increases intracellular cAMP production in PC-3 cells (p < 0.05, n=3). **B**, PGE$_2$ treatment increases cAMP production in DU145 and LNCaP cells, although the extent of the induction was much less than that for the PC-3 cells (p < 0.05, n=3).
**Figure 3.4** PGE2 at 1 nM (A) and 10 µM (B) induces cAMP production in PC-3 cells in a time-dependent manner. PC-3 cells were treated with either 1 nM or 10 µM PGE2 in serum-free medium and cell lysates were collected at indicated times to quantify cAMP concentrations by enzyme immunoassay (EIA). All experiments were repeated three times in duplicate. Data are represented as mean ± SD. A p-value of <0.05 is considered as significant.
compared to controls even after 24 h incubation. These results suggest that PGE2-elicited down-stream signaling events are constitutively activated in PC-3 cells.

3.4.4 EP2 and possibly EP4 receptors mediate PGE2-induced activation of ERK1/2 and Akt

To determine the effects of PGE2 on Erk and Akt activation and the involvement of EP receptors, prostate cancer cells (PC-3, DU145 and LNCaP) were treated with either PGE2 or CAY10399 at the indicated doses and times. To eliminate the influence of growth factors on Erk phosphorylation, cells were serum-starved overnight before treatments. After serum starvation, the basal levels of phospho-Erk were nearly undetectable in PC-3 and LNCaP cells. However, phosphorylation of Erk remained high in serum-starved DU145 cells. The phosphorylation of Erk in PC-3 cells was significantly upregulated within 15 min by either 10 µM PGE2 or CAY10399 (Figure 3.5 A). The maximum stimulation was reached at 15 min or 30 min by treating with PGE2 or CAY10399, respectively, and Erk phosphorylation declined to basal level after 2 h. Figure 3.5 B shows the dose-response of Erk activation in PC-3 cells upon treatment with concentrations of PGE2 or CAY10399 ranging from 1 nM to 10 µM for 15 min. Treatment of PC-3 cells with PGE2 induced the phosphorylation of Erk in an inverse dose-dependent manner, whereas activation elicited by CAY10399 was dose independent (Figure 3.5 B). The dose- and time-dependent patterns of Erk phosphorylation by either PGE2 or CAY10399 in DU145 cells were similar to that observed in PC-3 cells (Figure A.3 A and B). In contrast, Erk activation in LNCaP cells, which only express relatively low levels of EP4 receptor mRNA, was less robust in response to PGE2.
Figure 3.5  A, PGE$_2$ and CAY10399 induce phosphorylation of Erk in time-dependent manner in PC-3 cells. Cells were treated with either 10 µM PGE$_2$ or CAY10399 for 15 to 240 min. Total and phosphorylated Erk in cell lysates were analyzed by immunoblotting.

B, PGE$_2$ induces phosphorylation of Erk in an inverse dose-dependent manner in PC-3 cells. Cells were treated for 15 min with concentrations of PGE$_2$ and CAY10399 ranging from 1 nM to 10 µM followed by immunoblotting.
treatment. A noticeable increase of phospho-Erk in LNCaP cells was only observed after extended exposure of the film in the dark room.

After serum starvation, the basal level of phospho-Akt was very low in DU145 cells, moderately low in PC-3 cells, but high in LNCaP cells. Phospho-Akt was activated by 10 µM PGE2 within 15 min in PC-3 cells, within 60 min in DU145 cells, but was not further increased in LNCaP cells (Figure 3.6). The time-dependent pattern of Akt phosphorylation elicited by CAY10399 in DU145 cells was similar to that observed in PGE2-treated cells (Figure A.3 C). Akt phosphorylation by PGE2 remained elevated in DU145 cells after 12 h treatment (Figure A.3 C). Similarly, the effects of concentrations of PGE2 or CAY10399 on Akt phosphorylation in PC-3 and DU145 cells were similar to Erk activation in these cells. Figure 3.7 summarizes and compares the dose-response of Erk and Akt phosphorylation in all the three cell lines upon treatment with PGE2 or CAY10399 (1 nM or 10 µM). Collectively, these results suggest that PGE2-induced Erk and Akt phosphorylation in prostate cancer cells is mediated primarily by the EP2 receptor.

### 3.4.5 PGE2-induced VEGF secretion is not dependent on Erk and Akt signaling pathways

Based on the above results, we decided to focus on PC-3 cells to determine the mechanisms involved in the increase of VEGF protein secretion upon PGE2 treatment. We first examined the role of Erk and Akt signaling pathways in PGE2-induced VEGF secretion in prostate cancer cells by treating PC-3 cells with either the MAPK/Erk
Figure 3.6 PGE$_2$ induces phosphorylation of Akt only in EP2-expressing PC-3 and DU145 cells. Cells were treated with either 10 µM PGE$_2$ or CAY10399 for 15 to 240 min. Cell lysates were analyzed by immunoblotting for total and phosphorylated Akt.
Figure 3.7 Comparison of the effects of PGE\(_2\) and CAY10399 at 1 nM or 10 µM on Erk and Akt phosphorylation in PC-3, DU145 and LNCaP cells. Cells were treated with either PGE\(_2\) or CAY10399 in serum free medium for 15 min and 60 min to determine the activation of Erk (A) and Akt (B), respectively. All experiments were repeated at least three times, and only one representative blot is shown for each experiment.
inhibitor U0126 or the PI3K/Akt inhibitor LY294002. Pretreatment of PC-3 cells with U0126 (≥ 1 µM) or LY294002 (≥ 0.625 µM) for 1 h significantly blocked 10 µM PGE2-induced phosphorylation of Erk and Akt, respectively (Figure 3.8 A). Based on these data, 1 µM U0126 and 0.625 µM LY290024 were used for subsequent inhibitor experiments. However, inhibition of either MAPK or PI3K/Akt signaling did not attenuate PGE2-induced VEGF secretion in PC-3 cells (Figure 3.8 B). Interestingly, U0126 at 1 µM alone increases VEGF secretion, and U0126 in combination with 10 µM PGE2 significantly increased VEGF secretion for unknown reasons. These data suggest that both MAPK/Erk and Akt signaling pathways are not involved in PGE2-induced VEGF secretion in prostate cancer cells.

3.4.6 PGE2-induced VEGF secretion is dependent on cAMP mediated signaling events

To further determine the mechanism involved in PGE2-induced VEGF secretion in PC-3 cells, the effects of the cAMP analogue 8-bromo-cAMP and the adenylate cyclase activator forskolin on VEGF secretion were examined. As shown in Figure 3.9 A and B, forskolin and 8-bromo-cAMP significantly increased VEGF secretion in PC-3 cells. Next we investigated the effects of the adenylate cyclase inhibitor 2’5’-dideoxyadenosine on PGE2-induced VEGF secretion in PC-3 cells. Treatment of PC-3 cells with 2’5’-dideoxyadenosine at 500 µM and 5 mM alone for 1.5 h did not alter basal levels of cAMP in PC-3 cells (Figure 3.10 A). However, pretreatment of these cells with 500 µM and 5 mM 2’5’-dideoxyadenosine for 1 h followed by 10 µM PGE2 stimulation for an additional 30 min significantly blocked 10 µM PGE2-induced increase in cAMP by
96% and 98%, respectively (Figure 3.10 A). Similarly, 2’5’-dideoxyadenosine alone had no effects on basal level of VEGF secretion in PC-3 cells after 24 h incubation (Figure 3.10 B). However, 500 µM and 5 mM 2’5’-dideoxyadenosine significantly inhibited 10 µM PGE₂-induced VEGF secretion from PC-3 cells by 46% and 68%, respectively (Figure 3.10 B). We also determined that treatment of PC-3 cells with 50 µM 2’5’-dideoxyadenosine completely blocked 1 nM PGE₂-induced VEGF protein secretion (Figure A.4). These results suggest that PGE₂-induced VEGF secretion in prostate cancer cells is mediated through cAMP-dependent signaling pathways.
Figure 3.8 The MAPK/Erk and Akt signaling pathways are not involved in PGE2-induced VEGF protein secretion in PC-3 cells. A, MAPK/Erk and Akt pathway specific inhibitors block PGE2-induced activation of Erk and Akt, respectively. PC-3 cells were pretreated with U0126 (0.1 to 100 µM) for 1 h followed by stimulation with 10 µM PGE2 for an additional 15 min. For Akt analysis, PC-3 cells were pretreated with LY294002 (0.625 to 5 µM) for 1 h followed by PGE2 stimulation for an additional 1 h. Cell lysates were analyzed for total and phospho-Erk and Akt by immunoblotting. The experiment was performed three times, and one representative blot is shown. B, Inhibition of MAPK/Erk and Akt signaling pathways failed to block PGE2-induced VEGF secretion in PC-3 cells. PC-3 cells were pretreated with either 1 µM U0126 or 0.625 µM LY294002 for 1 h followed by treatment with 10 µM PGE2 for an additional 24 h. Cell culture media were collected and analyzed for VEGF protein concentration by ELISA. U0, U0126; LY, LY294004; P, PGE2. All experiments were repeated at least three times in duplicate. Data are means ± SD. Bars with different letters are significantly different from each other with a p-value <0.05.
Figure 3.9  A, VEGF protein secretion is significantly increased by the adenylate cyclase activator forskolin in a dose-dependent manner in PC-3 cells (p <0.05). PC-3 cells were treated with either 10 µM PGE2 or various concentrations of forskolin (0.1 to 10 µM) for 24 h in serum-free medium. F, Forskolin. B, VEGF protein secretion is significantly increased by the cAMP analogue 8-bromo-cAMP (p <0.05). PC-3 cells were treated with either 10 µM PGE2 or various concentrations of 8-bromo-cAMP (0.5 to 50 µM) for 24 h in serum-free medium. B, 8-bromo-cAMP.
Figure 3.10  A, The adenylate cyclase inhibitor 2’5’-dideoxyadenosine significantly blocks PGE2-induced cAMP production (p <0.05). PC-3 cells were pretreated with 500 μM and 5 mM 2’5’-dideoxyadenosine for 1 h followed by 10 μM PGE2 stimulation for an additional 30 min. B, Inhibition of cAMP production significantly blocks PGE2-induced secretion of VEGF. PC-3 cells were pretreated with either 500 μM or 5 mM 2’5’-dideoxyadenosine for 1 h followed by 10 μM PGE2 treatment for an additional 24 h in serum-free medium. I, 2’5’-dideoxyadenosine; P, PGE2. All experiments were repeated at least three times in duplicate. Data are means ± SD. Bars with different letters are significantly different from each other with a p-value <0.05.
3.5 DISCUSSION

Numerous studies have demonstrated that PGE₂ and its EP receptors are implicated in promoting carcinogenesis in different types of cancer. PGE₂ binds to the four EP receptors that are coupled to different G proteins and induce a variety of intracellular signaling cascades. Thus, the predominance of a particular EP receptor type may determine the mechanisms of PGE₂-mediated action in a particular cell type. In the present study, we report that EP2 and EP4 mRNA are the predominant receptors expressed in prostate epithelial cells. We further demonstrate that EP2 is expressed in PC-3 cells at a significantly higher level than in DU145 cells. In addition, EP4 expression is moderately higher in PC-3 cells compared to DU145 cells. LNCaP cells express the lowest EP4 and EP2 was non-detectable. Consistent with our results, previous studies by Chen et al. (52) and Liu et al. (42) found that only EP2 and EP4 mRNA were expressed in prostate cancer cells. However, neither of these studies used qRT-PCR to quantitate the expression of the EP receptors in prostate cells. Our study is novel in that we determined the contribution of each EP receptor to PGE₂-elicited signaling events by comparing the three prostate cancer cell lines (PC-3, DU145 and LNCaP) in the subsequent studies.

VEGF is considered to be one of the most important angiogenic factors in tumor angiogenesis (238). There are at least four subtypes of VEGF that are generated by alternative splicing found in human, i.e. VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉, and VEGF₂₀₆ (239). Among these subtypes, VEGF₁₆₅ is the most commonly studied form and is considered to be the strongest signal transducer of all the subtypes (238). Here we report
that PGE$_2$ significantly induced VEGF$_{165}$ protein secretion in PC-3 cells and to a lesser extent in DU145 and LNCaP cells, which correlates with the expression level of EP2 and EP4 receptors in these cells. Both EP2 and EP4 are linked to the generation of cAMP as a second messenger. Indeed, in the present study, we found PGE$_2$ induced a substantially high level of intracellular cAMP in PC-3, but much lower level in DU145 and LNCaP cells. Although EP2 and EP4 are both coupled to Gs proteins and generate cAMP, their activation has markedly distinct impacts on cAMP levels. It was reported that 1 µM PGE$_2$ stimulated cAMP formation about 71 fold in EP2 expressing cells, but only about 10 fold in EP4 expressing cells (232, 240). However, this difference is not due to the distinct ligand affinity for PGE$_2$, since EP4 actually has higher affinity than EP2 for PGE$_2$ (240). EP4, but not EP2, undergoes rapid agonist-induced desensitization and internalization (241, 242). Thus, the differential effects of EP2 and EP4 on intracellular cAMP levels in prostate cancer cells appeared to be a result from differences in PGE$_2$-mediated receptor desensitization and internalization. Consistent with our results, Chang et al. (39) found that PGE$_2$ significantly increased VEGF secretion through cAMP-dependent pathway in mammary tumor cells highly expressing EP2, but not in EP4 expressing cells.

CAY10399, the EP2 selective agonist, has been reported to have both a lower affinity than PGE$_2$ for EP2 receptor ($K_i$: 92nM for CAY10399 vs. $K_i$: 38nM for PGE$_2$) and a lower ability to increase cAMP levels at the same concentrations as PGE$_2$ ($EC_{50}$: 43nM for CAY10399 vs. $EC_{50}$: 2.1nM for PGE$_2$) (243). This may explain why CAY10399 induced increases in VEGF and cAMP in PC-3 cells was less robust compared with PGE$_2$ in our study. The observation that CAY10399 in combination with PGE2 did not enhance the effects of PGE2 on VEGF secretion suggests that either these
two compounds competitively bind to EP2 or EP2 receptor is fully saturated at the concentrations tested. It is interesting that the similar magnitude of increase in cAMP concentration induced by 10µM CAY10399 and 100nM PGE2 also is associated with similar levels of VEGF secreted into medium (Figure 3.2). The observation that CAY10399 significantly increases VEGF secretion and cAMP concentration in PC3 cells, but not in DU145 and LNCaP cells (data not shown), suggests that PGE2-mediated VEGF protein secretion is predominantly mediated by EP2 receptor in prostate cancer cells. However, the observation that VEGF secretion is also slightly but significantly induced by PGE2 (>10 nM) in LNCaP cells that only express EP4 suggests that a role for EP4 cannot be excluded.

Considerable evidence demonstrates that PGE2 is rapidly converted to its inactive metabolite 15-keto-PGE2 and, subsequently, to 13,14-dihydro-15-keto-PGE2 by the enzyme 5-hydroxy prostaglandin dehydrogenase (15-PGDH) (242, 244, 245). Recently, a two-step model of PGE2 metabolic clearance that consisted of sequential PGE2 uptake mediated by a PGE2 transporter followed by cytoplasmic inactivation by 15-PGDH was reported (245). In this study we determined that although PGE2 may be inactivated rapidly after addition to cultures, the down-stream signaling molecule cAMP remains elevated in prostate cancer cells for up to 24 hours compared to non-treatment controls. The possible significance of this chronic elevation of cAMP on the expression of other genes needs further examination.

In addition to inducing VEGF secretion, PGE2 has been reported to activate a variety of other signaling molecules by binding to specific EP receptors coupled to a variety of downstream signaling pathways. Here we report that PGE2 and CAY10399
significantly increased Erk and Akt phosphorylation in EP2 expressing cells (PC-3 and DU145), but not in EP2-null LNCaP cells. Our results suggest that EP2 plays an important role in PGE2-induced activation of the Erk and Akt signaling pathways in prostate cancer cells. We observed a very slight increase of Erk phosphorylation in LNCaP cells after an extended exposure time (data not shown), suggesting that EP4 might play a minor role in PGE2-induced Erk activation in these cells. Studies by others suggested that EP4 is preferentially coupled to Akt and induced Erk activation in a phosphatidylinositol 3-kinase (PI3K)-dependent pathway in HEK cells (232, 246). Mendez and Lapointe also found that PGE2 induces rapid Erk phosphorylation which is mediated through EP4-dependent EGFR transactivation in cardiac myocytes (247). Krysan et al. (248) reported that PGE2-induced Erk activation is mediated by EP1-dependent protein kinase C, but not EGFR signaling pathways in non-small lung cancer cells. We observed that PGE2 induced activation of Erk and Akt is an inverse dose-dependent manner, which is contrary to the dose-response of PGE2 for cAMP production. This observation suggests that cAMP is not likely to be involved in PGE2 induced Erk and Akt activation in prostate cancer cells. Consistent with our results, Mendez and Lapointe reported that PGE2-induced Erk activation occurred through cAMP-independent signaling process (247). However, another study found that cAMP is involved in PGE2-induced transactivation of the EGFR and Erk via EP2-mediated pathways (249). Collectively, these findings suggest that there are important differences in the signaling potential of the EP receptors which are cell-type dependent and such differences need to be considered for developing new cancer chemopreventative strategies that target EP receptors and their down-stream effectors.
Although PGE₂ induces the activation of Erk and Akt activation in PC-3 cells, neither of these two signal pathways seem to be involved in PGE₂-induced secretion of VEGF in these cells based on our inhibitor studies. The observation that PGE₂-induced Erk and Akt activation occurs in an inverse dose-response manner with VEGF secretion further suggests that neither Erk nor Akt is likely to be involved in PGE₂-induced VEGF secretion in prostate cancer cells. Results from Chen et al. (52) and our laboratory suggest that the human primary epithelial PrEC cells express both EP2 and EP4 receptors. The mechanism of the lose of EP2 mRNA expression in LNCaP cells is unknown at present. LNCaP cells responded to PGE₂ stimulation the least for regulating signaling events such as cAMP generation, VEGF upregulation, Erk and Akt activation under experimental conditions. These observations suggest that LNCaP cells may not be an appropriate model for studying the role of PGE₂ and its EP receptor-mediated signal pathways during the carcinogenesis of human prostate cancer, if PGE₂ plays a role.

In our study, both the adenylate cyclase activator forskolin and the cAMP analogue 8-bromo-cAMP significantly induced VEGF secretion in PC-3 cells. We further demonstrated that the adenylate cyclase inhibitor 2′5′-dideoxyadenosine completely and partially blocked 1 nM and 10 µM PGE₂-induced VEGF secretion in PC-3 cells, respectively. These results suggest that PGE₂-induced VEGF secretion is mediated at least partially through cAMP-dependent signaling pathways. Upon generating cAMP by EP2/EP4 coupled Gs protein, cAMP activates protein kinase A and results in the phosphorylation of the cAMP response element binding protein (CREB), a transcription factor that regulate genes whose promoters contain cAMP response element (CRE) (246). In our study, the increase of VEGF protein secretion upon PGE₂ stimulation is not
regulated transcriptionally. In addition, we determined that the PKA inhibitor H89 failed to block PGE₂-induced VEGF secretion by PC-3 cells (data not shown), suggesting that cAMP-mediated PKA activation and the downstream signaling events may not be involved in PGE₂-induced VEGF secretion in these cells. More recently, the exchange protein directly activated by cAMP (Epac) has been found to be another important cAMP effector molecule (250). In future studies, the mechanisms involved in posttranscriptional regulation of VEGF and cAMP-mediated signaling cascades other than PKA (such as Epac) that are responsible for VEGF secretion in prostate cancer cells need to be further addressed.

In conclusion (as shown in Figure 3.11), our study demonstrates that PGE₂-induces VEGF secretion in prostate cancer cells through EP2 and possibly EP4 mediated cAMP-dependent pathways. EP2 is also the major EP receptor that is responsible for PGE₂-induced activation of Erk and Akt in prostate cancer cells. However, a role for EP4 in regulating these processes cannot be ruled out at present. PC-3 cells which express higher levels of both EP2 and EP4 receptors than DU145 and LNCaP cells may serve as a potentially useful and relevant model for the study of the role of PGE₂ in prostate carcinogenesis, if PGE₂ plays a role. Because COX inhibitors may not be useful as chemoprevention agents due to various toxicities associated with long term use, EP receptors, located downstream of PG synthesis in the COX pathway may represent attractive alternative targets for chemoprevention and treatment of prostate cancer, if this pathway is important in prostate carcinogenesis.
Figure 3.11  Working model of the major signaling pathways elicited by PGE$_2$ in human prostate cancer cells. PGE$_2$ synthesized by prostate epithelial cells acts locally in an autocrine, and over short distances, in a paracrine manner by binding to EP2 and EP4 receptors located on prostate epithelial and endothelial cells. EP2 is the primary receptor responsible for the bulk activation of cAMP which mediates VEGF protein secretion in prostate cancer cells. EP2 is also the primary receptor that mediates PGE$_2$-induced phosphorylation of Erk and Akt in prostate cancer cells. CAY10399, the EP2 selective agonist, mimics PGE$_2$-induced signaling pathways in prostate cancer cells. EP4 may play a role in PGE$_2$-induced VEGF secretion and Erk phosphorylation in prostate cancer cells. However, the downstream genes regulated by PGE$_2$ that may contribute to prostate tumorigenesis have not been well defined. The development of new agents that target EP2 receptor may exhibit chemopreventive and chemotherapeutic effects during prostate carcinogenesis.
CHAPTER 4

PROSTATE TUMORIGENESIS IN TRAMP MICE IS NOT INHIBITED BY NSAIDS OR GENETIC DISRUPTION OF CYCLOOXYGENASE-2 EXPRESSION

Xingya Wang¹, Jennifer K.L. Colby³, Peiying Yang⁴, Susan M. Fischer³, Robert A. Newman³, Russell D. Klein¹,²,⁵

¹The Ohio State University, Interdisciplinary Ph.D. Program in Nutrition (OSUN)
²The Ohio State University Comprehensive Cancer Center, Molecular Carcinogenesis and Chemoprevention Program

³The University of Texas M.D. Anderson Cancer Center
Science Park-Research Division
P.O. Box 389, Park Rd. 1C, Smithville, TX 78957

⁴The University of Texas M.D. Anderson Cancer Center
Department of Experimental Therapeutics
8000 El Rio, Houston, TX 77054

⁵The Ohio State University, Department of Human Nutrition
325 Campbell Hall, 1787 Neil Avenue, Columbus, OH 43210

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Overexpression of cyclooxygenase-2 (COX-2) and prostaglandin E₂ (PGE₂) have been demonstrated to play a significant role in the tumorigenesis of colon, lung, breast, bladder, and skin cancer. However, inconsistent and controversial reports on the expression and activity of COX-2 in prostate cancer raised the question of whether COX-2 plays a pivotal role in prostate carcinogenesis. To address this question we examined the effects of COX-2 inhibition on prostate tumorigenesis in the transgenic adenocarcinoma mouse prostate (TRAMP) model. Three wk old TRAMP mice were fed control, celecoxib-, or indomethacin-supplemented diets for 27 weeks. A TRAMP/COX-2 knockout mouse model was also generated to determine the effects of the loss of the COX-2 gene on prostate tumorigenesis in TRAMP mice. These studies demonstrated that neither non-steroidal anti-inflammatory drugs (NSAIDs) nor genetic disruption of COX-2 was inhibitory in terms of tumor and metastases incidence, lobe weight or types of pathological lesions. However, the endogenous levels of PGE₂ were significantly reduced by both approaches in TRAMP prostate tissue compared to control. A careful analysis of wild-type and TRAMP prostate tissues was undertaken for the expression of COX-1 and COX-2 using immunoblotting, qRT-PCR and immunohistochemistry approaches in TRAMP dorsal prostate tissue from mice 10 and 16 wk old, as well as tumor from 30 wk old mice. We found that the expression of both COX-1 and COX-2 dramatically decreased during TRAMP prostate carcinogenesis. In contrast to previous reports, we did not detect overexpression of COX-2 by immunohistochemistry in any prostate tissues from wild-type mice or TRAMP mice. Using the probascin promoter, a COX-2
overexpressing mouse was also generated but failed to show any pathology in any of the prostate lobes. Collectively, our results suggest that COX-2 may not play a tumorigenic role during prostate carcinogenesis in the TRAMP model.

### 3.2 INTRODUCTION

COX-2 expression is elevated in a number of cancer types, and inhibition of COX-2 by NSAIDs has been tested as effective chemoprevention strategy for these cancers, including colon, breast, lung, bladder, and skin. However, studies on the role of COX-2 in prostate carcinogenesis have resulted in mixed findings.

The first discrepancy from available data is whether COX-2 is overexpressed in prostate cancer. Since the first report that COX-2 was overexpressed in human prostate adenocarcinoma compared to the benign prostate tissues by Gupta et al. in 2000 (251), many other investigators also addressed the similar question. In general, results from these studies are inconsistent and controversial. While some studies suggest that COX-2 was overexpressed in human prostate cancers (24-26, 28, 29), other studies indicate that COX-2 was either minimally expressed or even decreased in prostate cancers (27, 30, 31).

The second discrepancy is whether PGE₂ secretion is also increased in human prostate cancers and whether PGE₂ plays a direct role in prostate carcinogenesis. The primary mechanism by which NSAIDs are thought to prevent cancer is through inhibition of the synthesis of PGE₂. Unfortunately, studies that directly measure the level of PGE₂ in prostate cancers are very limited. Although several studies have assumed that PGE₂ levels are increased in prostate cancer tissues and *in vitro* cultured prostate cancer cells,
none of these studies actually determined the level of PGE$_2$ (24, 217, 218, 252). However, two investigators who actually measured PGE$_2$ levels in prostate found that PGE$_2$ production is low or non-detectable in prostate benign and tumor tissues (27), and in prostate cancer PC-3 and DU145 cells (201). In our above *in vitro* study, we also determined that the basal concentrations of PGE$_2$ in prostate cancer cell lines (PC-3, DU145, and LNCaP) were very low. Although stimulation with high concentration of exogenous PGE$_2$ was able to induce VEGF secretion in these cancer cells, PGE$_2$ had no effects on promoting proliferation in these cells. We also determined that both COX-2 and COX-1 proteins were highly expressed in non-tumorigenic prostate cells, but not detectable in all the cancer cell lines that determined by immunoblotting (*Figure A.10*).

The third discrepancy is whether NSAIDs are able to effectively reduce the risk to develop prostate cancer and to inhibit the progression of this disease. Similarly, several retrospective case-control and prospective cohort epidemiologic analyses examining the risk of prostate cancer relative to NSAID use have resulted in mixed findings. While results from some studies indicate no protective effect of NSAIDs (15-19), there are several reports of a significant reduction in prostate cancer risk attributable to frequent NSAID use (20-23). Among studies that suggest a protective effect of NSAIDs against prostate cancer, aspirin but not other NSAIDs was primarily responsible for the decreased prostate cancer risk (21, 253, 254).

However, evidence from chemoprevention clinical trials using NSAIDs to prevent prostate cancer progression has been largely lacking, which is mainly due to the concerns of the cardiovascular toxicity related with the use of selective COX-2 inhibitors (127, 255). At present, there are only two clinical trials that were conducted by the same
investigators to examine the effects of celecoxib on the PSA level in patients with recurrent prostate cancers after definitive radiation therapy or radical prostatectomy (256, 257). Although results from these two trials suggest that celecoxib may affect serum PSA levels in these patients, these studies were limited by small number of patients, short follow-up duration and a single end point (PSA level only).

There have been relatively few animal studies that have tested the level of COX-2 expression and the effect of NSAIDs on prostate carcinogenesis. The TRAMP model for prostate cancer is now well established and demonstrates several characteristics that make it advantageous for chemoprevention studies (32, 33). The pathobiology of the TRAMP model has recently been extensively reviewed (33). Two groups of investigators recently reported that the COX-2 selective inhibitor celecoxib decreased prostate tumorigenesis in the TRAMP transgenic model (34, 35). Celecoxib, however, has been shown to have multiple non-COX-2 mediated activities that could account for its effect on tumorigenesis in this model (36, 37).

Taken together, the exact role and direct molecular mechanisms involved in the tumorsinogenic effects of PGE2 in prostate cancer are still largely unknown. The potential for non-COX mediated effects of NSAIDs, combined with the lack of expression of COX-2 in human prostate cancers (27, 30, 31), led us to question whether COX-2 inhibition was actually responsible for the reported chemopreventive activity of celecoxib in the TRAMP model. In addition, investigations into the role of COX-2 in prostate cancer were largely hindered by the lack of prostate specific COX-2 overexpression and knockout mouse models. To address this question we compared the effect of a non-selective COX inhibitor (indomethacin), a COX-2 selective inhibitor (celecoxib), and
genetic disruption of COX-2 gene expression on prostate carcinogenesis in the TRAMP model. Interestingly, we found that the expression and activity of both COX-1 and COX-2 are dramatically decreased during TRAMP prostate carcinogenesis, and that inhibition of COX activity by either NSAID treatment or by disruption of the COX-2 gene is largely ineffective in preventing prostate cancer in the TRAMP model. We recently generated a transgenic mouse model (Pb.COX-2) with the COX-2 gene under the control of the probasin promoter, which allows for COX-2 overexpression only in prostate tissue. In support of our findings in the TRAMP model, overexpression of COX-2 in mouse prostate tissue had no pro-tumorigenic effects on prostates of Pb.COX-2 mice.

To our knowledge, we are the first laboratory to examine a direct role of COX-2 in prostate carcinogenesis by using genetically modified mouse models that either overexpress or lack expression of COX-2 in the prostate. Our results suggest that COX-2 does not play a tumorigenic role in prostate carcinogenesis in the TRAMP model. The discrepancy of current knowledge on COX-2 in human prostate carcinogenesis needs to be further elucidated in future studies.

4.3 MATERIALS AND METHODS

4.3.1 Materials

PGE₂, polyclonal anti-COX-2 (murine), polyclonal anti-COX-1 (murine), COX-1 (murine) blocking peptide, and goat anti-rabbit IgG horseradish peroxidase (HRP) were purchased from Cayman Chemical (Ann Arbor, MI). Polyclonal anti-rabbit PGHS-2 was purchased from Oxford Biomedical Research (Oxford, MI). Biotin conjugated mouse
anti-COX-2 monoclonal antibody was from BD (Franklin Lakes, NJ). Goat polyclonal anti-COX-2 (c-20), goat polyclonal anti-COX-1 (M-20) and donkey-anti-goat IgG HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-beta-actin was from Abcam (Cambridge, MA). TRIzol reagent and goat serum were purchased from Invitrogen (Carlsbad, CA). RNeasy Mini RNA extraction kit was purchased from Qiagen (Valencia, CA). The iScript cDNA synthesis kit was purchased from Bio-Rad (Hercules, CA). Taqman® Gene Expression Assay kits for all primers (COX-1, COX-2 and 18s), and Taqman® Universal PCR Mastermix were purchased from Applied Biosystems (Foster City, CA). Dako Envision Plus kit (K0411) was purchased from DakoCytomation (Denmark).

4.3.2 Genetically-engineered Mice

C57BL/6J TRAMP mice were originally obtained as a kind gift from Dr. Norman Greenberg (32) and were maintained as a hemizygote colony. TRAMP [C57xFVB] F1 mice were generated by crossing C57BL/6J TRAMP hemizygotes with wild-type FVB/n mice. Male COX-2 knockout (KO) mice (B6;129P2-Ptgs2<sup>2<sub>m1Smi</sub></sup>) were obtained from the Taconic (Hudson, NY) Emerging Models Program by kind permission of Dr. Robert Langenbach (258). Male COX-2(-/-) mice were crossed with female C57BL/6J mice to generate male and female COX-2(+-/-) mice. COX-2(+-/-) mice from this cross were then mated to generate male COX-2(-/-) and COX-2(+-+) mice. Male COX-2(-/-) were then crossed with female hemizygous C57BL/6J TRAMP mice to generate female mice heterozygous for COX-2 and hemizygous for the TRAMP transgene (C+/-;T+/-). To produce mice for the tumor study female C+/-;T+/- mice were crossed with either male
COX-2 -/- or COX-2 +/- mice in order to generate hemizygous TRAMP males wild-type (C +/- :T +/- ) or deficient (C -/- :T +/- ) for COX-2. The Pb.COX-2 transgenic mice were on the FVB background, which overexpress COX-2 under AAR2 probasin promoter (pAAR2Pb) control. We generated several lines of Pb.COX-2 transgenic mice, and only the lines with positive expression of COX-2 in prostate lobes were bred for subsequent studies. All genotyping was done by PCR on DNA isolated from tail snips according to established protocols for the TRAMP transgene (32), the Ptgs2tm1Smi mutation (258), and pAAR2Pb.COX-2 that was developed in our laboratory. All mice were maintained in HEPA-filtered cages according to Institutional Laboratory Animal Care and Use Committee (ILACUC) approved protocols at the Science Park-Research Division, University of Texas, MD Anderson Cancer Center, and at the Ohio State University.

4.3.3 TRAMP Diet Study

Diets were formulated and produced by Research Diets (New Brunswick, NJ) and were based on the AIN-76A semi-purified rodent diet. Celecoxib (LKT Laboratories, St. Paul, MN) at either 500 ppm or 1500 ppm and indomethacin (Sigma, St. Louis, MO) at 4 ppm were added to the diets prior to pelleting. The selected doses of celecoxib and indomethacin were well tolerated by TRAMP mice as reported in a previous study (34). TRAMP mice at 3 wk of age were randomly distributed by weight into experimental and control groups (n=30 per group). The TRAMP mice were fed either AIN-76A control diet or COX-inhibitor supplemented AIN-76A diets until the animals were 30 wk old. Throughout the experiment the animals had access to drinking water and diets ad libitum.
Fresh diets were provided and food intakes were recorded every other day. Body weights were measured weekly.

4.3.4 Tumor Studies

All transgenic mice were weighed and palpated for tumors weekly starting at 12 wk of age. Mice were necropsied when palpable tumors reached 2 cm in diameter, or mice reached 30 wk of age. Mice that died prior to 30 wk from causes unrelated to prostate tumor growth were censored from the analyses. Early necropsies were performed on mice with palpable tumors greater than 2 cm in diameter regardless of age. At necropsy the dorsal (DP), lateral (LP), ventral (VP), and anterior (AP) lobes of the prostate were microdissected into individual lobes whenever possible. Weights of all prostatic lobes, regional lymph nodes (LN) and tumors were recorded. The techniques and protocols for histological dissection of the prostate lobes were in concordance with the 2001 Bar Harbor Pathology Workshop (259). Mice were examined for any grossly observable metastatic tumors. The prostate lobes, LN, tumor tissues, and sections of the lung and liver were fixed in 10% buffered formalin and then paraffin-embedded for histopathological and immunohistochemical examination or were flash-frozen in liquid nitrogen for determination of PGE₂.

4.3.5 Histopathology

The histopathology of embedded tissues from all mice was assessed by scoring 4-micron thick sections stained with hematoxylin and eosin (H&E). All sections were coded, blinded and randomized prior to independent analysis by RDK and XW. Prostatic
lobes, including DP, LP, VP, and AP, were assessed according to published criteria that have been well established for the TRAMP model (33). Only lobes that were fully dissectible were included in the analysis. All glands in a given section were classified as either normal, prostatic intraepithelial neoplasia (PIN), well-differentiated carcinoma (WD), moderately-differentiated carcinoma (MD), or poorly-differentiated carcinoma (PD). The phylloides-like lesions (Phyl) described by Kaplan-Lefko et al. (33) were also examined and recorded. An additional classification of Atrophy as described by Shappell et al (259) was also included in the analysis of the mouse prostate tissues. The numbers of glandular ducts exhibiting each grade were determined for each section, as well as the total number of glandular ducts present. For each pair of lobes, the percent of the tissue that was normal, PIN, WD, MD, PD, Phyl, and Atrophy was determined. These data was used to score each pair of prostatic lobes for the percentage of each pathological grade present and these scores were averaged for all mice in a group. LN, lungs, and liver were assessed for metastases by scoring H&E sections for the presence or absence of apparent metastatic lesions.

4.3.6 Immunohistochemistry

Paraffin-embedded specimens were cut into 4 micron thick sections, deparaffinized, rehydrated, antigen retrieved in citrate buffer (pH 6.0), and blocked for endogenous peroxidase with H₂O₂ (Dako k0411 kit). The specimens were incubated in 5% goat serum for 10 min to block non-specific binding. Primary polyclonal antibodies against COX-1 and COX-2 (both from Cayman Chemical) were used at a dilution of 1:500 and applied to sections for 1 h. The specimens were subsequently washed in
phosphate-buffered saline (PBS) and treated with secondary antibody (Dako k0411 kit) for 30 min. To visualize the antibody, 3,3’-diaminobenzidine tetrahydrochloride (DAB, Dako k0411 kit) was incubated for 7 min. All immunohistochemical stainings for COX-1 and COX-2 were performed in an OptiMax Automated Cell Staining System (BioGenex, San Ramon, CA). Finally, the slides were dehydrated and mounted. Vas-deferens tissue from wild-type mice was used as positive control for COX-1 and COX-2, while vas-deferens from a COX-2 knockout mouse served as a negative control for COX-2. There was no negative control for COX-1, however, a COX-1 blocking peptide was used as control for the specificity of COX-1 staining.

4.3.7 Immunoblotting

Total proteins were collected from frozen tissue by standard methods. Approximately 40 µg protein was electrophoresed on a 10% SDS-polyacrylamide gel followed by standard immunoblotting procedures. Four commercial sources of the primary antibodies against COX-2 were analyzed at the following dilutions: 1:500 (Santa Cruz), 1:750 (Cayman), 1:250 (BD) and 1:500 (Oxford). Two sources of the COX-1 antibodies were analyzed at dilutions of 1:500 for the Santa Cruz antibody and 1:1000 for the Cayman Chemical antibody. The secondary antibody was goat anti-rabbit IgG HRP for COX-1 (Cayman) and COX-2 (Cayman, BD and Oxford). The donkey anti-goat IgG HRP was used for COX-1 and COX-2 antibodies from Santa Cruz. Beta-actin was used as a loading control. Cell lysates from a wild type mouse vas-deferens was used as positive control for COX-1 and COX-2 immunoblotting.
4.3.8 RNA Extraction and Real-Time PCR (qRT-PCR)

Total RNA was isolated by TRIzol reagent and further purified by RNeasy Mini kit as described by Qiagen. Both the quantity and quality of total RNA were analyzed by the Agilent Bioanalyzer 2100 system. Total RNA was reverse transcribed with an iScript cDNA synthesis kit. qRT-PCR was performed to determine the expression of COX-1 and COX-2 using a 1:10 dilution of cDNA with Taqman gene expression primers and ABI mastermix according to the manufacturer’s instructions on an iCycler IQ qRT-PCR detection system (Bio-Rad). 18s RNA was used as the reference gene for all samples.

4.3.9 Quantitation of PGE₂

PGE₂ was measured on DP and PD tumors using an LC/MS/MS system as described by Kempen et al. (260). For endogenous measurements snap-frozen tissues were homogenized followed by extraction with hexane: ethyl acetate (1:1) and total protein was determined on the homogenate. LC/MS/MS analyses were performed using a Quattro Ultima tandem mass spectrometer (Micromass, Beverly, MA) equipped with an Agilent HP 1100 binary pump HPLC inlet. PGE₂ was separated from other eicosanoids using a YMC ODS-AQ 2.0 × 100-mm column with a solvent system of 10 mM ammonium acetate/methanol and a flow rate of 0.3 ml/min. PGE₂ was detected using electrospray negative ionization and multiple reaction monitoring (MRM) and the result was expressed as nanograms of PGE2 per mg protein.
4.3.10 Statistical Analysis

Data are presented as means ± standard deviation (SD). Inter-group comparisons of prostate cancer incidence were made using Fisher’s exact test (two sided). Survival curves were compared using Kaplan-Meier analysis, and the difference between groups was estimated using the log-ranked test. Comparisons among multiple groups were performed by one-way analysis of variance (ANOVA). A p-value of < 0.05 was considered statistically significant. All analyses were performed using SPSS 13.0 software (Chicago, IL).

4.4 RESULTS

4.4.1 Cyclooxygenase expression and activity in TRAMP prostates

We first determined the level of expression and activity of COX enzymes in the prostate tissue of wild-type (WT) and TRAMP transgenic mice. As shown in the immunoblot in Figure 4.1 A, both COX-1 and COX-2 proteins are expressed in DP tissue from WT mice as determined by commercial antibodies from Santa Cruz Biotechnology. Interestingly, the expression of both COX proteins was dramatically reduced in TRAMP DP tissue as early as 10 wk of age. COX protein expression remained at a reduced level at 16 wk of age and was further reduced in tissue from PD tumors. The commercial antibody against COX-2 from Cayman Chemical detected the expression of COX-2 in both the WT and TRAMP prostate tissue, although there was high background binding. The COX-2 antibody from BD only detected COX-2 expression in the positive control, while the COX-2 antibody from Oxford Biomedical Research failed to produce any signal. The Cayman Chemical polyclonal COX-1 antibody worked as well as the
Figure 4.1 Expression of cyclooxygenase enzymes in dorsal prostate (DP) tissue is reduced in TRAMP mice compared to wild-type (WT) mice. A, Western blot analysis of COX-1 and COX-2 protein levels in DP tissue from WT (10 wk), TRAMP DP (10 and 16 wk), and from histological confirmed poorly-differentiated tumor tissue (PD). Mouse vas deferens tissue was used as positive control for COX (+). Tissue lysates from two different mice were analyzed for each tissue type and images are representative of two independent experiments. B, Quantitative RT-PCR of COX-1 and COX-2 mRNA levels in WT (10 wk) and TRAMP DP tissues (10 and 16 wk), and PD tumor. Each bar represents the mean COX-1 or COX-2 mRNA expression (error bars are 95% confidence intervals) of five animals relative to a common calibrator sample generated by pooling the dorsal, ventral, and lateral prostates of a single wild-type mouse. 18s RNA levels were used to normalize the expression of individual samples. Data points with an asterisk are significantly different from other data points in the same panel (p < 0.05).
COX-1 antibody from Santa Cruz. To further confirm the results obtained by immunoblot analysis, we performed real-time RT-PCR to quantify relative expression levels of COX mRNA in WT and TRAMP prostate tissues (Figure 4.1 B). The relative expression levels of COX mRNA in DP and PD tissues correlated extremely well with protein levels. TRAMP DP tissue expressed significantly less COX mRNA at 10 wk and 16 wk of age and expression was further decreased in PD tumor tissue. Since most studies reporting COX-2 over-expression were determined by immunohistochemistry methods (24-26, 28, 29, 261), we also undertook a careful analysis of COX-1 and COX-2 expressions in WT and TRAMP prostate tissues using validated methods. The COX-2 immunohistochemical staining protocol was optimized to consistently produce strong staining of positive control tissue (vas deferens) from WT mouse or prostate lobes from the Pb.COX-2 mouse, while not staining negative control vas deferens from COX-2 knockout mouse (Figure 4.2). We did not, however, detect COX-2 protein expression in prostate tissues from WT or TRAMP mice at any stage of pathology (Figure 4.2). These results suggest that COX-2 antibody detected COX-2 staining in the positive control tissues but was not sensitive enough to detect the relatively low expression in prostate tissues. We also found that commercially available COX-2 antibodies produce strong non-specific epithelial and stromal staining in both the TRAMP and COX-2 null prostate tissues if immunohistochemical conditions are not carefully controlled. Strong COX-1 protein expression was initially observed in both WT and TRAMP prostate tissues under conditions lacking a negative control. However, use of the COX-1 blocking peptide demonstrated that COX-1 staining in prostate tissue is non-specific. We found two
Figure 4.2 COX-2 immunohistochemical staining of serial sections from TRAMP dorsal prostate (DP) tissue with prostatic intraepithelial neoplasia (PIN), moderately-differentiated carcinoma (MD), and poorly differentiated (PD) tumors. Vas deferens (Vas-D) from WT mouse served as positive control, while vas deferens from a COX-2 knockout mouse served as negative control.
additional bands migrating at ~ 110 and ~ 40 kDa in immunoblots, suggesting cross reactivity of this antibody with non-COX-1 antigens.

To further determine the activity of COX enzymes in the TRAMP mice, we measured the endogenous PGE₂ level in DP from 10 wk old WT and TRAMP mice using LC/MS/MS. In the WT mice, DP had the highest level of PGE₂ as compared to other lobes (Figure A.5). In correlation with the expression of COX enzymes, PGE₂ levels were significantly lower in DP from TRAMP mice compared to WT littermates (Figure 4.3). PGE₂ levels remained significantly lower in TRAMP PD tumors than in DP from WT mice (Figure 4.9). Collectively, the above results suggest that both the expression and the activity of COX enzymes are significantly decreased in the TRAMP prostate tissue compared to the WT littermates.

4.4.2 Tumorigenesis and histopathological evaluation of TRAMP mice

We characterized the histopathology of TRAMP mice (chow diet-fed) by collecting prostate tissues at different ages (6 to 35 wk). The results correlated well with the pathological changes previously described (33). Briefly, histological PIN and early carcinoma was present in 100% of TRAMP prostates by 8 wk of age, which progresses to PD with distant site metastasis by 16 to 32 wk of age. Metastatic sites included the pelvic lymph nodes, liver and lung. In very rare cases, metastases were found in the kidney and adrenal glands. The WD and MD were observed at all ages between 8 to 32 wk old. We scored prostatic glandular ducts and metastases in TRAMP prostate tissues for the tumor studies according to the histopathologic criteria described previously (33). The
Figure 4.3 Prostaglandin E₂ (PGE₂) levels in dorsal prostate (DP) tissue are reduced in 10 wk old TRAMP mice compared to wild-type (WT) mice. Tissues were analyzed for PGE₂ by LC/MS/MS. Bars represent the mean from at least five mice (error bars are 95% confidence intervals). * p < 0.05.
representative H&E stained sections of DP (Figure 4.4 A), LP (Figure 4.4 B), VP (Figure 4.4 C), and AP (Figure 4.4 D) tissues from TRAMP mice and sections of TRAMP lung (Figure 4.4 E) and liver (Figure 4.4 F) tissue with micro-metastases are shown.

4.4.3 Effects of celecoxib and indomethacin on the incidence of poorly-differentiated tumors and metastases in TRAMP mice

To determine the effects of cyclooxygenase inhibition on prostate tumorigenesis, we fed TRAMP mice either control diet (AIN-76A) or diets containing celecoxib at 500 ppm and 1500 ppm, or indomethacin at 4 ppm. All animals (30 per group) were fed diets starting at weaning (3 wk old) and continued for 27 weeks unless mice were necropsied or died prior to 30 wk of age. Mice that died prior to 30 wk of age without a PD carcinoma or that had primary tumors of non-prostatic origin were excluded from the analysis. There were no significant differences in body weights or food intake for animals that received either control or experimental diets (data not shown). As summarized in Table 4.1, feeding TRAMP mice NSAID diets did not reduce the incidence of tumor and metastases in LN, liver and lung compared to TRAMP mice fed the control diet. By 30 wk, approximately 60 % of TRAMP mice developed palpable tumors (PD) in control- (15/26), 500 ppm celecoxib- (18/28), and 1500 ppm celecoxib- (15/25) fed groups. Although there was a slight reduction in the incidence of PD tumors (12/26) and LN metastases (13/26) in mice fed 4 ppm indomethacin, this difference was not significant statistically. There were no significant differences in the size and weight of the PD
Figure 4.4  Histopathologic criteria used to score prostatic glandular ducts and metastases in tissues from TRAMP tumor studies. Representative H&E stained sections of dorsal (A), lateral (B), ventral (C), and anterior (D) prostate tissue from TRAMP mice as well as sections of TRAMP lung (E) and liver (F) tissue with micro-metastases are shown. For sections from each lobe glandular ducts with histological features of normal ducts (N), prostatic intraepithelial neoplasia (PIN), well-differentiated carcinoma (WD), and moderately-differentiated carcinoma (MD) are indicated if present. Micro-metastases in the lung and the liver are indicated by arrows. Length of the black bar present in each panel is 100 microns.
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Table 4.1  Incidence of poorly-differentiated (PD) carcinomas and metastases (Mets) in TRAMP mice fed diets containing cyclooxygenase inhibitors. *Mice that died prior to 30 weeks of age without a poorly-differentiated carcinoma or had primary tumors of non-prostatic origin were excluded from the analysis.
tumors between groups. Tumor-free survival analysis revealed that NSAID treatment did not prolong the time to development of palpable tumor of TRAMP mice (Figure 4.5).

4.4.4 Effects of NSAIDs on prostate histopathology and PGE$_2$ levels in TRAMP mice

To further evaluate the effects of celecoxib and indomethacin on the development and progression of prostate adenocarcinoma in TRAMP mice, we analyzed the pathologic lesions in the glandular ducts of each individual pair of prostatic lobes from the control and experimental mice. We first analyzed the wet weight of each individual lobe by using lobes that were only fully dissectible upon necropsy, e.g., DP (n = 12 to 16), LP (n = 8 to 12), VP (n= 10 to 13), and AP (n = 13 to 16). Although the average wet weights of AP, LP and DP were lower in the celecoxib-fed groups than in the control and indomethacin treated groups, the differences were not statistically significant (Figure 4.6). However, there was a significant reduction in wet weight of VP from mice fed 1500 ppm of celecoxib (Figure 4.6).

As described above, the pathologic lesions in the glandular ducts of individual prostatic lobes were assessed using published criteria (33). Only lobes clearly identifiable with microscopic analysis were included. Therefore, we analyzed total lobes of DP (n=19 to 26), LP (n=14 to 17), VP (n= 14 to 17) and AP (n=17 to 24). As shown in Figure 4.7, treatment of TRAMP mice with either celecoxib or indomethacin did not significantly alter the distribution of pathologic lesions in the glandular ducts of prostatic lobes. Compared to other lobes, DP had the most severe lesions in the glandular ducts in
Figure 4.5 Kaplan-Meier survival analysis for the incidence of palpable tumors. Treatment of TRAMP mice with cyclooxygenase inhibitors does not significantly alter prostate carcinogenesis. Three wk old TRAMP mice were treated with an AIN-76A semi-purified diet, or the control diet supplemented with 500 ppm celecoxib, 1500 ppm celecoxib, or 4 ppm indomethacin for 27 weeks. Mice that did not complete the entire 27 week treatment period due to causes other than a palpable tumor are indicated as being censored.
Figure 4.6  Treatment of TRAMP mice with cyclooxygenase inhibitors does not significantly alter prostate wet weights of prostate lobes. Three wk old TRAMP mice were fed either AIN-76A semi-purified diet (Control, C) or the control diet supplemented with 500 ppm celecoxib (500 Cele), 1500 ppm celecoxib (1500 Cele), or 4 ppm indomethacin (Indo) for 27 weeks. Wet weights of anterior (AP, n = 13 to 16), dorsal (DP, n = 12 to 16), lateral (LP, n = 8 to 12), and ventral (VP, n = 10 to 13) prostatic lobes dissected from mice treated with the indicated diets. Only lobes that were fully dissectible were included in the analysis. Bars represent means, error bars are 95% confidence intervals (CI), and means within a panel with different letters significantly differ from each other (p < 0.05).
Figure 4.7 Treatment of TRAMP mice with cyclooxygenase inhibitors does not significantly alter prostate carcinogenesis. Three wk old TRAMP mice were fed AIN-
76A semi-purified diet (Control, C) or the control diet supplemented with 500 ppm celecoxib (500 Cele), 1500 ppm celecoxib (1500 Cele), or 4 ppm indomethacin (Indo) for 27 weeks. Incidence of pathologic lesions in the glandular ducts of prostatic lobes from TRAMP mice fed the indicated diets. The percent of normal (Norm) ducts and ducts containing prostatic intraepithelial neoplasia (PIN), well-differentiated carcinoma (WD), moderately-differentiated carcinoma (MD), phylloides-like lesions (Phyl), atrophic lesions (Atrophy), and poorly-differentiated carcinoma (PD) was determined for each lobe. Only clearly identifiable lobes were included in the analysis; AP (n = 17 to 24), DP (n = 19 to 28), LP (n = 14 to 17), VP (n = 14 to 17). Bars represent means and error bars are 95% CI.
that they have higher percentages of WD and MD, but lower percentages of normal and PIN. Interestingly, in correlation with the effects of 1500 ppm celecoxib on the wet weight of VP, celecoxib at 1500 ppm slightly, but not significantly, increased the percentage of PIN and decreased the percentage of WD in VP compared to the TRAMP mice fed control diet. However, these effects could be due to the non-COX effects of celecoxib.

To verify the effectiveness of our feeding regimen on TRAMP prostate tissue, 10 wk old TRAMP mice (n = 5 per group) were fed either AIN-76A diet (control) or the experimental diets (500 ppm, 1500 ppm celecoxib, and 4 ppm indomethacin) for two weeks. We found that celecoxib at 500 ppm has essentially no effects on PGE2 levels in DP tissue compared to the DP of control mice (Figure 4.8). However, PGE2 concentration in the DP tissue was significantly reduced with 1500 ppm celecoxib. Indomethacin had the greatest significant inhibitory effect on the concentration of PGE2 in the DP tissue of TRAMP mice compared to other groups (Figure 4.8). The above data suggest that NSAIDs effectively reduced the activity of the COX enzymes in TRAMP mice, but had very minimal, if any, effect on the inhibition of prostate tumorigenesis in this animal model.
Figure 4.8 PGE₂ levels in dorsal prostate (DP) tissue from 10 wk old TRAMP mice fed AIN-76A semi-purified diet (control), or control diet containing 500 ppm celecoxib, 1500 ppm celecoxib, or 4 ppm indomethacin for two weeks. Tissues were analyzed for PGE₂ by LC/MS/MS. Bars represent the mean obtained from at least five mice (error bars are 95% confidence intervals). Bars with different letters significantly differ from each other (p < 0.05).
4.4.5 Effects of genetic disruption of COX-2 on prostate tumorigenesis in TRAMP mice

We next examined whether disruption of the COX-2 gene in the TRAMP mice could inhibit prostate tumorigenesis by crossing TRAMP transgenic mice with COX-2 KO mice. We generated the TRAMP (+/-) x COX-2 (+/+), as the COX-2 WT (n = 33), and TRAMP (+/-) x COX-2 (-/-) as the COX-2 KO mice (n = 35). Figure 4.9 shows that the endogenous PGE2 level was significantly lower in the DP tissue of COX-2 KO mice compared to the COX-2 WT mice. PGE2 concentrations were further reduced in the PD of TRAMP mice with either COX-2 WT or COX-2 KO (Figure 4.9). Similar to the treatment with celecoxib and indomethacin, there were no significant effects of the loss of COX-2 gene expression on the incidence of PD tumors, LN or liver metastases in TRAMP mice by 30 wk of age (Table 4.2). We observed a significant increase in the percentage of lung metastases in TRAMP x COX-2 KO mice compared to the control littermates, which might be due to the intrinsic variation in the TRAMP model. There were no significant differences in the size and weight of the PD tumors between groups. Tumor-free survival analysis revealed that loss of the COX-2 gene slightly, but not significantly, prolonged the time to development of palpable tumor in the TRAMP (+/-) x COX-2 (-/-) mice compared to the COX-2 positive littermates (Figure 4.10). The lower overall incidence of PD tumors in these mice (as compared to the NSAID study) is likely due to background strain difference as these mice were not crossed with FVB mice.
Figure 4.9  PGE₂ levels are significantly reduced in 10 wk old dorsal prostate (DP) tissues of the COX-2 knockout TRAMP mice (COX-2 KO) compared to COX-2 WT TRAMP mice (COX-2 WT). PGE₂ levels are further reduced in the 30 wk old poorly-differentiated tumors (PD) of both the COX-2 WT and the COX-2 KO mice compared to 10 wk old DP. Tissues were analyzed by LC/MS/MS and bars represent the mean obtained from at least five mice (error bars are 95% confidence intervals). Bars with different letters significantly differ from each other (p < 0.05).
<table>
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<th>n*</th>
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<th>Lymph nodes Mets</th>
<th>Lung Mets</th>
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<td>43%</td>
<td>60%</td>
<td>70%</td>
<td>83%</td>
</tr>
<tr>
<td>COX-2 KO</td>
<td>28</td>
<td>36%</td>
<td>54%</td>
<td>93%</td>
<td>82%</td>
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**Table 4.2**  Incidence of poorly-differentiated carcinomas (PD) and metastases (Mets) in TRAMP mice either wild-type (WT) or homozygous mutant (KO) for the COX-2 gene. *Mice that died prior to 30 weeks of age without a poorly-differentiated (PD) tumor or had primary tumors of non-prostatic origin were excluded from the analysis. †Significantly different from COX-2 wild-type (p = 0.026).
Figure 4.10 Kaplan-Meier survival analysis for the incidence of palpable tumors. Genetic disruption of COX-2 expression dose not prolong the development of poorly-differentiated (PD) tumors. TRAMP mice either wild-type (COX-2 WT, n = 33) or homozygous mutant (COX-2 KO, n = 35) for the COX-2 gene were followed until 30 weeks of age at which time all surviving animals were necropsied. Mice that did not complete the entire 27 week experimental period due to causes other than a palpable tumor are indicated as being censored.
As shown in Figure 4.11, disruption of the COX-2 gene in the TRAMP mice decreased the wet weight of LP in a significant manner, but not the weights of other lobes. We further analyzed the effects of loss of COX-2 on the pathologic lesions in the glandular ducts of individual prostatic lobes. We found that genetic disruption of COX-2 expression only very modestly reduced prostate tumorigenesis in the LP and VP lobes of TRAMP mice (Figure 4.12). Taken together, these data suggest that COX-2 deficiency was not sufficient to inhibit tumorigenesis in the TRAMP model.

4.4.6 Effects of COX-2 overexpression on prostate tumorigenesis

To provide further evidence for a role for COX-2 in prostate tumorigenesis, we generated transgenic mice with COX-2 under the control of a probasin promoter (Pb.COX-2), allowing prostate-specific expression of COX-2. We originally developed more than 10 founder lines, and only two founder lines had positive COX-2 expression as determined by immunohistochemistry and immunoblot. Analysis using immunohistochemistry demonstrated that COX-2 is predominantly expressed in VP, followed by LP and DP (Figure A.6). The expression of COX-2 in AP was at similar levels as in WT control. The synthesis of PGE$_2$ was also significantly increased in the three lobes from the Pb. COX-2 mice than from the WT littermates. PGE$_2$ levels in DP, LP and VP were 31, 37 and 28 ng/mg protein in the Pb.COX-2 mice, respectively, while the concentrations of PGE$_2$ were 15, 2 and 1 ng/mg protein in DP, LP and VP in the WT littermates, respectively (Figure A.7). Consistent with what we observed from the NSAID study, overexpression of COX-2 in the Pb.COX-2 mice failed to induce any of the histopathology seen in the TRAMP model. Interestingly, we found lymphocyte
Figure 4.11 Wet weights of prostate lobes in COX-2 knockout TRAMP (KO) compared to COX-2 WT TRAMP (WT) mice. TRAMP mice either wild-type (n = 33) or homozygous mutant (n = 35) for the COX-2 gene were followed until 30 weeks of age at which time all surviving animals were necropsied. Wet weights of anterior (AP, n = 23 WT, 19 KO), dorsal (DP, n = 23 WT, 22 KO), lateral (LP, n = 18 WT, 16 KO), and ventral (VP, n = 18 WT, 18 KO) prostatic lobes dissected from mice with the indicated genotypes were determined. Only lobes that were fully dissectible were included in the analysis. Bars represent means, error bars are 95% confidence intervals (CI), and KO means within a lobe with an asterisk significantly differ from WT (p < 0.05).
Figure 4.12  Incidence of pathologic lesions in the glandular ducts of prostatic lobes from TRAMP mice with the indicated genotypes (COX-2 WT and COX-2 KO). The
percent of Normal (Norm) ducts and ducts containing prostatic intraepithelial neoplasia (PIN), well-differentiated carcinoma (WD), moderately-differentiated carcinoma (MD), phylloides-like lesions (Phyl), and poorly-differentiated carcinoma (PD) was determined for each lobe. Only clearly identifiable lobes were included in the analysis; the number of lobes assessed for each genotype was the same as that for the wet weight analysis. Bars represent means and error bars are 95% CI. Bars with an asterisk significantly differ from other bars in the same panel (p < 0.05).
infiltration into the VP and the appearance of hyperplasia in a very small percentage of the mice in one of the Pb.COX-2 founder lines (Figure A.8).

4.5 DISCUSSION

Prostate cancer (PC) is a major public health problem in the US which results in significant morbidity and mortality each year. New strategies that target molecular changes in prostate carcinogenesis are needed for both the prevention and treatment of this disease. There is substantial evidence for the upregulation of COX-2 in a number of cancers and its pro-tumorigenic activity in these cancers (1). COX-2 selective inhibitors have been intensely studied for their cancer chemopreventive potential. However, available data regarding the expression of COX-2 in prostate cancer and its role in prostate carcinogenesis have been inconclusive. Recently, the use of COX-2 selective inhibitors has been associated with an increased risk for cardiovascular heart disease (145, 255). The lack of definitive evidence showing a benefit of COX-2 inhibition in PC calls for a more critical examination of the role for COX-2 in prostate carcinogenesis before considering interventions to inhibit COX-2 in PC (201).

In this study, we addressed this question using both pharmacologic and genetic approaches. We first examined the effect of NSAID treatment on prostate tumorigenesis in the TRAMP model. We found that neither celecoxib nor indomethacin was able to inhibit the incidence of tumors or metastases or the progression of pathological lesions. Our results contradict two earlier studies in TRAMP mice in which celecoxib reduced the incidence of PIN, decreased tumor incidence and blocked metastasis (34, 35). However, t
there are marked differences in experiments between our study and those studies. We used the C57/BL6J TRAMP transgenics that were crossed with WT FVB mice, while the TRAMP mice used in the above two published studies were on a pure C57BL/6 background. Background strain has been reported to affect the prostate tumor phenotype in the TRAMP model (262). In addition, due to the stochastic development of PD in the TRAMP model between 16 to 32 wk of age and the occasional idiopathic loss of mice from the study, at least 30 mice per treatment group are needed to provide sufficient power to detect statistical significance between experimental groups. In agreement with our findings, Zeng et al. (263) found that the selective COX-2 inhibitor nimesulide failed to inhibit prostate tumorigenesis in probasin/SV40 T antigen transgenic rats, a model in which carcinogenesis is genetically initiated in a same manner as in the TRAMP model. Until now, only a few animal studies have been carried out to test the effect of NSAIDs on prostate carcinogenesis. Recently, COX-2 selective inhibitors have been reported to have effects that are independent of COX-2 activity that could account for their effects on prostate tumorigenesis (36). Taken together, experiments using pharmacologic approaches do not appear to provide strong evidence that COX-2 plays a pro-tumorigenic role in prostate carcinogenesis.

We next used genetic approaches in which the COX-2 gene was either knocked out or overexpressed in the mouse prostate to definitively determine the role of COX-2 in prostate carcinogenesis. In support of the results from the NSAID study, deletion of COX-2 in the TRAMP mice had essentially no effect on prostate tumorigenesis, although there was a slight inhibitory effect on the severity of the lesions in the LP and VP. Similarly, we did not observe any pathological lesions in the prostates of the Pb.COX-2
mice. In contrast, genetic studies using either transgenic or knockout technology have firmly established a link between COX-2 and tumorigenesis in cancers of colon, mammary, skin and bladder (1). To our knowledge, we are the first laboratory to examine the role of COX-2 in prostate carcinogenesis using genetic approaches. Our results indicate that COX-2 may not play a critical role in prostate carcinogenesis. However, in one of the Pb.COX-2 founder lines we found lymphocyte infiltration into the VP and the appearance of hyperplasia in a small percentage of the mice (Figure A.8). Given the recent evidence that COX-2 may be overexpressed only in areas of inflammation in PC tissues (30, 31), a role for COX-2 in either the initiation or promotion of this disease by eliciting an inflammatory response cannot be ruled out.

Since our results contradict many previously published studies, we performed a careful analysis of the expression and activity of the COXs in the WT and TRAMP mouse prostate tissues. Unexpectedly, both the expression and activity of COX-1 and COX-2 were substantially decreased in the TRAMP prostates compared to the WT littermates. The expression and activity of the COXs were also progressively reduced as the PC progressed. To show that this phenomenon was not unique to the TRAMP model, we examined the expression of COX mRNAs in the AP and DP of 10 wk old prostate specific phosphatase and tensin homolog deleted on chromosome 10 (PTEN) knockout mice (34, 36). Unlike TRAMP mice in which carcinogenesis is driven by the SV40 viral oncogene, prostate carcinogenesis in prostate-specific PTEN knockout mouse is driven by the deregulation of downstream pathways of PTEN (262, 264), in which AP has the most severe phenotype. Similar to our observation in TRAMP mice, COX mRNA levels were significantly reduced in both the AP and DP of conditional PTEN knockout mice.
compared to the WT littermates (Figure A.9). Taken together, the loss of COX expression and its activity during prostate carcinogenesis concurs well with results from our tumor studies and further suggests that COX-2 is unlikely to play a critical role in prostate carcinogenesis.

A review of the literature reveals possible dissimilarities between studies on COX-2 expression in PC, including differences in the cell types found to express COX-2, expression levels in normal vs. benign tissue, and the relationship of level of expression to grade or stage of PC (1, 200). Some studies only compared the expression of COX-2 in the benign versus the malignant tissue but ignored measurement in normal tissue. Most studies used non-quantitative methods (26, 28, 29, 34). Additionally, the majority of the studies that used immunohistochemistry to determine COX-2 expression had neither positive nor negative controls. To address this issue, we carefully optimized immunohistochemical protocols to consistently produce strong staining of positive control tissue, while not staining negative control tissue. Under this condition, we found that the commercial COX-2 antibody could only detect highly expressed COX-2 in positive control tissue, but was not sufficiently sensitive enough to detect the relatively low expression of COX-2 in WT and TRAMP prostate tissues. To address the same issue, Zha and coworkers (31) conducted a very comprehensive and well-controlled analysis of COX-2 expression in human PC tissues. Tissues from a total of 144 PC patients were analyzed, far more than in any similar study. In contrast to previous results, these investigators found only scattered expression of COX-2 in less than 1% of cells, and no difference between cancer and normal cells as determined by immunohistochemistry. They observed increased COX-2 expression only in areas of inflammation, which was in
agreement with Wang et al. (30) who reported that COX-2 upregulation is only associated with chronic inflammation in benign prostate hyperplasia. Zha et al. (31) further performed immunoblotting and qRT-PCR analyses on a subset of frozen tissues. Interestingly, in agreement with our findings, mean mRNA levels of COX-2 were considerably reduced in malignant prostate as compared to normal tissue, suggesting a down-regulation of COX-2 in PC. Similarly, by using well-controlled immunohistochemistry and in situ hybridization analysis, Shappell et al. (27) found that COX-2 expression was more often reduced in prostate tumor compared to benign tissue, and this reduction was significant for Gleason score 5 and 6 tumors. However, the authors did not examine the level of COX-2 expression in normal prostate tissue. Although Gupta et al. (34) found a progressive increase in COX-2 expression in the TRAMP prostate tissue from 8 to 24 wk old, COX-2 expression was lost after 24 wk of age. Thus, there are a substantial number of reports on human and murine tissues that show COX-2 expression diminishes during progression of the lesion.

Although the concept that COX-2 diminishes with the tumorigenesis of PC has not been well acknowledged, results from a substantial number of in vitro studies actually support this concept. Either very low or non-detectable expression of COX-2 in prostate cancer cells PC-3, DU145, LNCaP and TSU has been reported by several investigators (31, 182, 201-203). In addition, growth of PC-3 and DU145 have been found to be resistant to the treatment with a COX-2 selective inhibitor (201). Interestingly, Subbarayan et al. demonstrated that in contrast to the low mRNA and protein levels of COX-2 in prostate cancer cells, the basal level of COX-2 expression was high in the normal PrEC cells (182). Another study found that COX-2 protein expression was high in
the normal PrEC and the benign BPH-1 cells, but low in LNCaP and PC-3 cells (202). We also determined the expression of COX-1 and COX-2 in seven prostate cell lines by immunoblotting. In agreement with results from our in vivo study and the above studies, we found that COX-1 and COX-2 were not detectable in four prostate cancer cell lines, i.e., PC-3, DU145, LNCaP and 22RV1. However, high level of COX-1 and COX-2 expression were observed in the non-tumorigenic prostate PWR-1E, RWPE-1 and its tumorigenic form RWPE-2 cells (Figure A.10).

Although it has been generally assumed that COX-1 is constitutively expressed and involved in the maintenance of tissue homeostasis, this view has been challenged by studies with COX-1 knockout mice. Disruption of the COX-1 gene is as effective as COX-2 gene disruption in imparting resistance to chemically induced skin tumors (187) and intestinal polyps in Min mice (188). Furthermore, COX-1 expression has been shown to be elevated in renal, cervical, and ovarian cancers and to regulate angiogenic growth factors (189-191). Our study also suggests that the expression of COX-1 was progressively lost during prostate tumorigenesis in the TRAMP model. Given the fact that indomethacin failed to inhibit prostate tumorigenesis in TRAMP mice, COX-1 may also not play a role in the development and progression of this disease.

Contrary to the conventional wisdom about COX-1, COX-2 is considered to be absent in most tissue under normal conditions, but rapidly induced by growth factors or cytokines and promotes tumorigenesis in several tissues. However, unlike most epithelial tissues where COX-2 is not expressed under normal conditions, COX-2 is highly expressed in normal prostate (180). Extremely high levels of PGs are found in semen as a product of prostate and seminal vesicles (235). The constitutive expression of COX-2 is
likely to be important in maintaining normal homeostasis and function of the prostate (182). Collectively, strong evidence suggests that the expression of COX-2 and maybe COX-1 (which needs more evidence) is either decreased or lost during prostate carcinogenesis. The loss of COX-1 and COX-2 may be characteristic of the molecular changes during prostate carcinogenesis.

Although the mechanisms underlying the marked decrease of COX mRNA and protein levels during prostate tumorigenesis have yet to be determined, there are a number of possible mechanisms may explain. The down-regulation of COX enzymes could be a result of negative-feed back regulation due to decreased substrate availability due to competition with LOX for arachidonic acid. In vitro evidence that COX-2 mRNA can be upregulated by COX-2 stimulators in prostate cancer cells that only minimally express COX-2 suggests that COX-2 gene was not impaired in prostate cancer cells (31, 182). Although many factors have been shown to upregulate COX-2 expression at the transcriptional and post-transcriptional levels, few inhibitory effectors have been reported. Wild-type p53 has been shown to inhibit COX-2 transcription by competing with TATA-binding proteins for binding to COX-2 gene (184). Although the SV40 large T antigen interacts with p53, p53 activity is actually increased in the TRAMP mice (32). This observation is also supported by the observation of Perez-Stable et al. that p53 was upregulated in a prostate transgenic mouse line driven by SV 40 T antigen (265). Upregulation of the WT p53 in human PC has also been reported. The downregulation of COX-2 in prostate carcinogenesis may result from suppression by p53. Another interesting observation is that the CpG island upstream of the COX-2 gene is methylated in greater than 85% of primary and metastatic prostate cancers and in all the seven tested
prostate cancer cell lines, but not in normal tissues (185). Although COX-2 is frequently overexpressed in colorectal cancer, evidence suggests that COX-2 expression is reduced in colorectal cancer with a defective mismatch repair system (186), a phenomenon commonly associated with hereditary nonpolyposis colorectal cancer (HNPCC). Interestingly, the lack of tumor COX-2 expression in HNPCC is strongly associated with COX-2 promoter hypermethylation. This evidence suggests that the loss of COX-2 expression may due to the hypermethylation of COX-2 in several tumors, including prostate. However, the epigenetic mechanism for COX-1 down-regulation in cancer has not been examined. Regardless of the mechanism, it is not likely that loss of COX expression contributes to the tumorigenesis of PC. Further studies are needed to address this question and the basis for this molecular change.

Upregulation of COX-2 results in increased synthesis of PGE₂. This product is the key mediator of the COX-2 pathway that activates downstream signaling and thus contributes to tumorigenesis by inducing cell proliferation, angiogenesis, invasion and metastasis (3). The exact role of PGE₂ in prostate carcinogenesis, however, is currently unknown. Although several studies have assumed that PGE₂ levels are increased in prostate cancer tissues and in vitro cultured prostate cancer cells, PGE₂ levels were not measured in most studies. We found that PGE₂ levels were significantly reduced in TRAMP prostate tissue compared to wild type littermates. In support of our findings, two investigators found that PGE₂ production is low or non-detectable in human benign prostates and prostate tumor tissues (27), and in PC-3 and DU145 cells (201). However, there are no reports on PGE₂ levels in normal human or mouse prostate tissue.
A substantial amount of evidence suggests that the level of arachidonic acid, precursor of PGE₂, is decreased in prostate malignant tissues compared with non-cancer or benign tissues in human (252, 266-270) and rat (252). It was initially assumed that decreased arachidonic acid in prostate cancer is a result of increased metabolism of arachidonic acid from producing prostaglandins or leukotrienes (267). However, given the evidence that the expression of COX and level of PGE₂ are significantly decreased in prostate cancer, it is not likely that the increased metabolism of arachidonic acid is to produce prostaglandins. Lipoxygenase (LOX) metabolizes arachidonic acid to various hydroxyl-eicosatetraenoic acids (HETE) which have cancer-promoting effects by several mechanisms (268). Indeed, recent evidence suggests that expression of different LOX isomers and their metabolites were increased in the urine and tissue from prostate benign or cancer patients (27, 270, 271). Interestingly, Kelavkar et al. found that the majority of arachidonic acid metabolites are 12-HETE and 15-HETE in TRAMP prostate with increased 12/15-LOX activity, an ortholog of human 15-LOX-1, compared to prostate in WT mice (272). The same investigators further demonstrated that conditional expression of human 15-LOX-1 induces PIN in transgenic mouse model (273). However, the synthesis of prostaglandins in these studies was low or non-detectable. We also found that 12-HETE production was significantly increased in the TRAMP DP compared to the WT DP (Figure A.11). However, COX metabolites other than PGE₂, including PGA₂, PGD₂, and 15-d-PGJ₂, were also decreased in the TRAMP DP (Figure A.11). Collectively, these results suggest that LOX isomers and their metabolites originated from arachidonic acid present in high fat diets may play a significant role in prostate carcinogenesis.
In conclusion, we have demonstrated that neither pharmacological inhibition nor genetic modification of COX-2 had an effect on prostate carcinogenesis. The lack of a pro-tumorigenic role for COX-1 and COX-2 in prostate carcinogenesis is further supported by our observation that both the expression and activity of COX-1 and COX-2 are progressively lost during prostate carcinogenesis. This is the first study to definitively establish the lack of a positive link between COX-2 and the progression of prostate cancer. Given the epidemiological evidence that NSAID use may be protective against PC (20-23) and the evidence that COX-2 may be associated with inflammation processes in PC, a role for COX-2 in the initiation of prostate cancer cannot yet be ruled out. Further work will be required to determine the significance of the loss of COX genes in prostate carcinogenesis, and the role of COX-2, if there is any, in the early stage of prostate cancer development.
CHAPTER 5

OVEREXPRESSION OF CYCLOOXYGENASE-2 (COX-2) IN THE MOUSE URINARY BLADDER INDUCES THE EXPRESSION OF IMMUNE AND CELL PROLIFERATION RELATED GENES

Xingya Wang¹, Jennifer K.L. Colby³, Robert Rengel², Susan M. Fischer³, Steve K. Clinton¹,², Russell D. Klein¹,²,⁴

¹The Ohio State University, Interdisciplinary Ph.D. Program in Nutrition (OSUN)
²the Ohio State University Comprehensive Cancer Center, Molecular Carcinogenesis and Chemoprevention Program
³The University of Texas M.D. Anderson Cancer Center
Science Park-Research Division
P.O. Box 389, Park Rd. 1C, Smithville, TX 78957
⁴Department of Human Nutrition, the Ohio State University
325 Campbell Hall, 1787 Neil Avenue, Columbus, OH 43210

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5.1 ABSTRACT

Increased COX-2 expression has been reported in transitional cell carcinoma (TCC) of the urinary bladder and has been positively correlated with grade and stage. However, the exact role of COX-2/PGs play in bladder carcinogenesis remains unknown. We have recently developed a transgenic mouse model which overexpresses COX-2 under the control of a bovine keratin 5 (BK5) promoter. BK5.COX2 mice had a high incidence of bladder transitional cell hyperplasia (TCH), but a relatively low incidence of bladder neoplasia. We have now conducted microarray gene analysis to determine the effects of COX-2 overexpression on the regulation of gene expression profile in urinary bladder. Total RNA was isolated from whole urinary bladders of 10 wk old BK5.COX2 transgenic mice and wild-type FVB/n mice. Gene array analysis was carried out using Affymetrix 430 2.0 mouse gene chips. Analysis of gene array data by t-test (p<0.05) revealed 2-fold or more changes in 70 of the upregulated genes and 60 of the downregulated genes in bladders from transgenic compared to wild-type mice. Gene set analysis using Expression Analysis Systematic Explorer (EASE; NIH) software revealed that genes associated with Immune/Stress Response and Cell Cycle/Proliferation biologic processes were significantly overexpressed in the top 100 upregulated genes. Relevant downregulated genes included three TGF beta related genes (Tgfb2, Tgfb3, and Tgfbi), and the anti-angiogenic gene thrombospondin 2 (Thbs2). Expression analysis of the growth factors and TGF beta-related genes by qRT-PCR was carried out to validate these findings. The growth factor, epiregulin, was the most highly induced gene among those validated by qRT-PCR in the transgenic bladders compared to the wild-type bladders,
with 3.7 fold and 5.3 fold increase at 10 and 30 wk of age, respectively. Increased staining of Ki67, the proliferation biomarker, was observed in TCH of BK5.COX2 mouse bladder compared to wild type mouse bladder tissues. We then found that PGE2 at concentration in BK5.COX-2 transgenic mouse bladders significantly induced the expression of epiregulin mRNA in bladders from wild-type FVB mice ex vivo, suggesting a direct regulatory effect of PGE2 on epiregulin expression. We further determined that treating bladder cancer UMUC-3 cells with recombinant hEpiregulin increased both cell proliferation and Erk phosphorylation. The results of gene expression analysis indicate that the initial response of the mouse urinary bladder to elevated COX-2 expression includes mounting an inflammatory response and inducing cell proliferation. Epiregulin, the growth factor that associated with cell proliferation, may serve as a novel biomarker and target for future prevention and treatment of bladder cancer.

5.2 INTRODUCTION

Bladder cancer is the fourth most common type of cancer in men and the ninth most common type in women in the US. The morbidity of bladder cancer is increasing due to high rate of recurrence. It is widely accepted that specific molecular targets in bladder carcinogenesis must be identified to decrease both the incidence and mortality of this disease. There is strong evidence that COX-2 is overexpressed in transitional cell carcinoma (TCC) of human urinary bladder (4-7). The degree of COX-2 expression is significantly correlated with the tumor grade and depth of invasion (T stage) of TCC (8-10). Increased COX-2 expression has also been reported in rat and canine models of
bladder cancer (204, 205). A case control study involving 1514 incident bladder cancer cases and an equal number of controls reported that use of NSAIDs decreased the risk for urinary bladder cancer by almost 20% (213). NSAIDs have been shown to inhibit the growth of bladder cancer cells in vitro (11-14, 212). In addition, COX inhibitors also induced remission of chemically induced bladder tumors in rodents (207, 208), naturally occurring invasive TCC in dogs (209, 210), and bladder carcinoma in an orthotopic mouse model (211).

Although the above evidence suggests that there is a strong association between COX-2/prostaglandin synthesis and the development and progression of urinary bladder cancer, the exact role and mechanisms of COX-2 overexpression during bladder carcinogenesis have not been well defined. Overexpression of COX-2 has also been associated with the development of other types of cancer, including lung, colon, mammary, and skin. Evidence from genetic and pharmacologic studies suggest that COX-2 and prostaglandins have tumorigenic effects in these cancers by increasing cell proliferation, enhancing angiogenesis, promoting invasion, and inhibiting apoptosis (1, 3). However, the exact molecular pathways that are regulated by COX-2 overexpression are largely unknown. At present, there has been no study to determine the effects of COX-2 overexpression on gene profiles during tumorigenesis in any type of cancers.

The use of genetically modified mouse models has greatly enhanced our understanding of the potential function of certain genes, such as COX-2, in tumorigenesis. A previous study by Klein et al. has shown that forced expression of COX-2 under the control of a keratin 5 promoter is sufficient to cause mouse (NMRI background) urinary bladder transitional cell hyperplasia (TCH) in an age-dependent manner (3). They further
observed that TCH was strongly associated with increased Ki67 staining, and induced lymphocytes and macrophage infiltration in COX-2 overexpression transgenic K5.COX-2 mouse bladders. We have now developed a transgenic mouse model (BK5.COX2) in which overexpression of COX-2 is driven by bovine keratin 5 (BK5) promoter on FVB background. In this model, a similar but more severe phenotype developed in the urinary bladders compared to the K5.COX-2 mouse model. The level of prostaglandin E2 was significantly increased in the bladder of BK5.COX-2 mice, as has been reported to be elevated in patients with bladder cancer (4, 206). Here, we conducted Affymetrix microarray analysis to determine the effects of COX-2 overexpression on genetic and molecular changes during the early stage of bladder carcinogenesis. We found that genes involved in the Cell Cycle/Proliferation biologic processes and Immune/Stress Response are significantly upregulated. Relevant genes that are downregulated include the TGF beta-related genes and the anti-angiogenic gene thrombospondin 2. We further determined that the growth factor, epiregulin, was the most highly induced gene among those validated by qRT-PCR.

Our study is innovative in that we developed a unique mouse model that provided us an opportunity to study the role of COX-2 and PGs during bladder carcinogenesis. The results clearly demonstrated that COX-2 overexpression and PGE2 overproduction play a tumorigenic effect in bladder cancer through regulating growth/proliferation, extracellular remodeling, and immune responses related genes. This is the first study that determined the regulation of gene expression profile by COX-2 during early stages of carcinogenesis of any type of cancer.
5.3 MATERIALS AND METHODS

5.3.1 Materials

PGE$_2$, PGE$_2$ EIA kit, polyclonal murine COX-1 and COX-2 antibodies, and goat anti-rabbit IgG horseradish peroxidase (HRP) were purchased from Cayman Chemical (Ann Arbor, MI). Phospho- and total- Erk polyclonal antibodies, EGFR, erbB4, anti-Rabbit HRP-linked IgG, and HRP-conjugated Anti-Biotin antibody were purchased from Cell Signaling (Beverly, MA). Polyclonal beta actin antibody was obtained from abCAM (Cambridge, MA). Recombinant hEpiregulin was purchased from R&D Systems (Minneapolis, MN). TRIzol reagent and goat serum were purchased from Invitrogen (Carlsbad, CA). RNeasy Mini RNA extraction kit was purchased from Qiagen (Valencia, CA). The iScript cDNA synthesis kit was purchased from Bio-Rad (Hercules, CA). Taqman® Gene Expression Assay kits for all primers (COX-1, COX-2, Ereg, IGF-1, IGFBP6, TGFBi, TGFB2, TGFB3, GAPDH, and 18s), and Taqman® Universal PCR Mastermix were purchased from Applied Biosystems (Foster City, CA). Dako Envision Plus kit (K0411) was purchased from DakoCytomation (Denmark). BCA protein Assay kit and RadioImmuno Precipitation Assay (RIPA) cell lysis buffer were purchased from Pierce (Rockford, IL). The 3-[4,5-2-yl]-2-, 5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (St. Louis, MO).

5.3.2 Genetically-engineered mice

The BK5.COX2 transgenic mice were generated on the FVB background by pronuclear injection of a bovine keratin 5 (BK5) promoter construct containing the
coding region of the mouse COX-2 gene, as well as a rabbit beta-globin intron and an
SV40 poly-A tail (Figure A.12). Nine founder lines were initially generated and one line
that expressed high level of COX-2 and also produced offspring was used from
subsequent studies (line #7). In order to maintain the line, BK5.COX2 transgenic males
were bred to FVB/n wild type females. Because fertility of female transgenics was
greatly reduced, female offspring only were used for experimental analysis. It was
noticed in early attempts at breeding the mice that many females were undergoing
premature parturition as a result of high PGE2 levels derived from placenta of transgenic
pups. In order to avoid loss of entire litters, premature delivery was prevented by feeding
the dams low levels of 4 ppm indomethacin-supplemented AIN-76 A diet (Research
Diets, St.Paul, MN) several days before delivery. Hemizygous offspring were used for all
subsequent analyses. Both male and female transgenic mice were used for histopathology.
However, only female transgenic mice were used for microarray and other biologic
studies due to the higher rate of reproduction than the male transgenic mice. Initial
genotyping was done by PCR using genomic DNA extracted from tail snips. Primers
used to recognize the COX-2 transgene are as follows: 5’-TCA-AAG-ACA-CTC-AGG-
TAG-AG-3’(forward); 5’-CTT-GAG-TTT-GAA-GTG-GTA-AC-3’(reverse). Since
BK5.COX2 mice were hairless due to defects in hair follicles, transgenic mice were
identified by a sparse hair coat without performing genotyping assay. All mice were
maintained in HEPA-filtered cages according to Institutional Laboratory Animal Care
and Use Committee (ILACUC) approved protocols at the Science Park-Research
Division, University of Texas, MD Anderson Cancer Center, and at the Ohio State
University.
5.3.3 Tissue preparation and histopathology analysis

The bladder tissues were collected at different ages (10, 20, 30 and 40 wk old). The urinary bladders were excised and cut in half and fixed in 10% buffered formalin. The fixed urinary bladders were then positioned with the cut faces oriented in the same direction for paraffin embedding. Four-micron sections were stained with hematoxylin and eosin (H&E) by standard protocols. All bladder sections from different age of mice (about 15 mice/age group) were evaluated according to established published criteria (274, 275).

5.3.4 Immunohistochemistry

Paraffin-embedded specimens were cut into 4 micron thick sections, deparaffinized, rehydrated, antigen retrieved in citrate buffer (pH 6.0), and blocked for endogenous peroxidase with H₂O₂ (Dako k0411 kit). The specimens were incubated in 5% goat serum for 10 min to block non-specific binding. Primary polyclonal antibody against COX-2 were used at a dilution of 1:1000 and applied to sections for 1 h. The specimens were subsequently washed in phosphate-buffered saline (PBS) and treated with secondary antibody (Dako k0411 kit) for 30 min. To visualize the antibody, 3,3’-diaminobenzidine tetrahydrochloride (DAB, Dako k0411 kit) was incubated for 5 min. Finally, the slides were dehydrated and mounted. The mKi67 staining was performed by the pathology core facility of the CCC at OSU.
5.3.5 Immunoblotting

Total proteins were collected from frozen bladder tissues of BK5.COX2 mice or cultured cells by standard methods. Approximately 40 µg protein (from tissue) or 25 µg protein (from cell culture) was electrophoresed on a 10% SDS-polyacrylamide gel followed by standard immunoblotting procedures. The primary antibodies against COX-1 and COX-2 were analyzed at dilution 1:1000. Beta-actin was used as a loading control. Cell lysates from a wild-type mouse vas deferens was used as positive control for COX-1 and COX-2 immunoblotting. Phosphorylated and total Erk1/2 were determined by western blot according to the manufacturer’s instructions (Cell Signaling).

5.3.6 RNA extraction and Real-Time PCR (qRT-PCR)

Total RNA was isolated by TRIzol reagent and further purified by RNeasy Mini kit as described by Qiagen. Both the quantity and quality of total RNA were analyzed by the Agilent Bioanalyzer 2100 system at the core facility of the OSU Comprehensive Cancer Center. Total RNA was reverse transcribed with an iScript cDNA synthesis kit. qRT-PCR was performed to determine the expression of all the examined genes using a 1:10 dilution of cDNA with Taqman gene expression primers and ABI mastermix according to the manufacturer’s instructions on an iCycler IQ qRT-PCR detection system (Bio-Rad). GAPDH and 18s RNA were used as the reference gene for all samples.

5.3.7 Quantitation of PGE₂

PGE₂ levels in the bladder tissues of the BK5.COX2 and wild-type mice were measured using a PGE₂ enzyme-linked-immunosorbent (EIA) kit from Cayman Chemical
(Ann Arbor, MI). Briefly, bladder tissues were snap frozen and pulverized in liquid nitrogen, then purified with methanol on a C-18 column. The eluate was collected and further processed according to the manufacturer’s instructions (Cayman Chemical). Levels of PGE2 were determined using the multiple linear regression program and expressed as ng/mg of tissue. Experiments were performed in duplicate and 5 mice from each genotype were used in individual experiment.

5.3.8 Affymetrix microarray and EASE Analysis

Bladder tissues were excised from 10 wk old wild-type and BK5. COX2 transgenic mice (5 mice/group). Total RNA were extracted and purified as described above. The Affymetrix analysis was performed by the Affymetrix Core Facility at the Columbus Children’s Hospital Medical Center. One Mouse 430 2.0 GeneChip (Affymetrix Inc., Santa Clara, CA) was used for each of 10 samples. The mouse genome 430 2.0 array is a high-density oligonucleotide array comprised of over 45,101 probe sets representing over 40,000 mouse genes. The database for the oligonucleotide in the Mouse 430 2.0 gene chip is available at http://www.affymetrix.com/support/technical/libraryfielsmain'affx. Briefly, 10µg of total RNA was used to synthesize double-stranded cDNA. *In vitro* transcription was performed to produce biotin-labeled cRNA. The biotinylated cRNA was cleaned with an RNeasy Mini Kit (Qiagen), fragmented to 50-200 nucleotides, and hybridized to Affymetrix 430 2.0 arrays. Affymetrix MicroArray Suite was used to scan and quantitate the Genechips. Data were collected for subsequent analysis.
5.3.9 Microarray data and EASE Analysis

Genes differentially expressed in BK5.COX2 mice versus wild-type mice were identified at a p value < 0.05 and fold change (> 2) by two-way analysis of variance. Intensity data were collected from each chip and the top 100 up- and top 100 down-regulated probesets with p <0.05 were then analyzed using Expression Analysis Systematic Explorer (EASE; NIH) (276). EASE analysis allows one to discern particular gene classes associated with biologic processes and molecular functions. The results were segregated into Biological Process, Cellular Component and Molecular Function ontologies. Within each of these groups, themes are sorted according to EASE-score. The probability of over-representation is called the EASE score, which is the upper boundary of the distribution of Jackknife Fisher exact probabilities as determined by EASE. The probesets having EASE scores of less than 0.05 were considered to be significantly over-represented.

5.3.10 Cell culture

Human bladder cancer cell line UMUC-3 was purchased from the American Type Culture Collection. UMUC-3 cells were cultured in RPMI-1640 medium (GIBCO) containing 10% FBS. Cells were maintained in a humidified atmosphere with 5% CO₂ at 37°C. For the experiments, cells were seeded at 2 x 10⁵ cells/well in six-well plates, and incubated 24 h in serum containing medium. Cells were then serum-starved overnight followed by treatment with hEpiregulin.
5.3.11 Cell Proliferation Assay

UMUC-3 bladder cancer cells were plated in 96-well microtiter plates at an initial density of $2 \times 10^4$ cells per well. Cells were treated with hEpiregulin (0-1 ng/ml) and incubated for 48 h. After incubation, cell proliferation was determined by MTT assay according to manufacturer’s instruction (Sigma, MO).

5.3.12 Organ culture experiment

A total of 12 wild-type FVB mice at 10 wk of age were ordered from Taconic (Germantown, NY). Upon arrival, mice were maintained in HEPA-filtered cages overnight with access to diet and water. At necropsy, bladder tissues from all the mice were excised and cut in half. A total of 24 half bladders from the 12 FVB mice were emerged into 24 individual wells in a 96-well plate containing RMPI medium with 10µM PGE$_2$. Tissues were incubated (37°C, 5% CO$_2$) for 0, 1h, 2h, 4h, 6h and 8 h. Four samples (4 wells) were collected at each individual time point. Tissues were snap frozen and total mRNA was extracted as described above. The effects of PGE$_2$ on epiregulin mRNA expression in the FVB mice bladders were determined using qRT-PCR as described above.

5.3.13 Statistical Analysis

Data are presented as means ± standard deviation (SD). Comparisons among multiple groups were performed by one-way analysis of variance (ANOVA) with Tukey’s post-hoc comparisons. A p-value of <0.05 was considered statistically significant. All analyses were performed using SPSS 13.0 software (Chicago, IL).
5.4 RESULTS

5.4.1 The expression and activity of transgene in BK5.COX2 mice urinary bladder

COX-2 expression was detected in bladder tissues of the BK5.COX2 mice as early as 3 wk of age. At 10 wk, increased COX-2 expression was present in the basal epithelial cells with diffusion into superficial epithelial cells of transgenic urinary bladders, whereas no staining was observed in wild-type epithelial cells (Figure 5.1 A). COX2 expression remained high as BK5.COX2 mice aged, whereas there was no change in wild-type mice (data not shown). Results form immunoblot analysis also demonstrate that COX-2 is highly expressed in bladder tissue of BK5.COX2 mice, but not detectable in the wild-type mice (Figure 5.1 B). The level of COX-2 expression varies considerably within transgenic mice at the same age. Interestingly, COX-1 expression was reduced in the urinary bladder of the BK5.COX2 mice compared to the wild-type control (Figure 5.1 B). The relative expression levels of COX mRNA in bladder tissues from transgenic and wild-type mice correlated extremely well with protein levels as determined by immunoblotting. COX-2 mRNA expression increased about 400 fold in the bladder tissue of transgenic mice compared to the control mice, while level of COX-1 mRNA was reduced by 50% in the transgenic mice compared to the wild-type mice (Figure 5.1 C). We also determined the level of PGE2 in the transgenic mice bladder as a result of COX-2 overexpression in these mice. As shown in Figure 5.1 D, the level of PGE2 significantly increased about 100 fold in the BK5.COX2 mouse bladder tissue compared to the PGE2 level in age-matched wild-type bladders.
Figure 5.1 Expression of cyclooxygenase enzymes and PGE₂ synthesis in bladder tissues of 10 wk old BK5.COX2 (Tg) mice compared to wild-type (Wt) mice. A. COX-2 immunohistochemical staining in Tg and Wt mice bladders. B. Western blot analysis of COX-1 and COX-2 protein levels in Tg and Wt mice bladders. Cell lysates of two individual mice from both Wt (1-2) and Tg (3-4) were used. Mouse vas deferens tissue was used as positive control for COX (+). Beta-actin was used as loading control. C. Quantitative RT-PCR of COX-1 and COX-2 mRNA levels in Tg and Wt mice bladder tissues. Each bar represents the mean COX-1 or COX-2 mRNA expression (error bars are 95% confidence intervals) of five animals relative to a common calibrator sample generated by pooling the 3 bladders from wild-type mice. GAPDH mRNA levels were used to normalize the expression of individual samples. Bar with an asterisk is significantly different from other bar in the same panel (p < 0.05). D. PGE₂ levels in Tg and Wt bladder tissues. Tissues were analyzed for PGE₂ by EIA kit. Bars represent the mean from 4 mice, and error bars are 95% confidence intervals. Bar with an asterisk is significantly different from other bar in the same panel (p < 0.05).
Figure 5.1 continued

B

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</table>

C

**COX-2**

![COX-2 Graph](image)

**COX-1**

![COX-1 Graph](image)

D

**PGE2 (ng/mg of tissue)**

![PGE2 Graph](image)
5.4.2 Histopathological evaluation of BK5.COX2 mice

A total of 73 urinary bladders from 10- to 40-wk-old BK5.COX2 transgenic and wild-type FVB mice were examined for pathologic lesions. Histopathologic analysis revealed that 100% incidence of transitional cell hyperplasia in 10 wk old BK5.COX2 transgenic mice (Table 5.1). Only simple (~47%) and papillary (~53%) hyperplasia were observed in 10 wk old transgenic mice. Simple TCH is characterized by an increase in the number of epithelial cell layers compared with control animals, which only have 2-3 layers of transitional cells (Figure 5.2, panel a-c). Papillary TCH is characterized by an increase in the number of epithelial cell layers, papillary extensions into the bladder lumen, and some times thickened lamina propria (Figure 5.2, panel d-f). No TCH was observed in any of the urinary bladders from wild-type mice. As mice aged to over 20 wk old, the pathologic lesions are very heterogeneous. We found 29 out of a total of 35 transgenic mice developed TCH from 20 to 40 wk of age and 6 developed transitional cell neoplasia (Table 5.1). Besides simple (~22%) and papillary (~37%) TCH, we also observed nodular hyperplasia characterized by the downward proliferation into the lamina propria and nests of epithelial cells (Figure 5.3 panel a, b) in mice over 20 wk old. The lamina propria in the transgenic mice was significantly thickened in almost all the transgenic mice as indicated in Figure 5.3 panel a (*). There was a marked increasing lymphocyte infiltration nto lamina propria and more often into muscle (Figure 5.3 panel a, c). The degree of lamina propria thickening appeared to be proportional to the severity of epithelial proliferation. Carcinoma in situ (CIS, 6%) and possible transitional cell carcinoma (TCC, 6%) were observed in a small percentage of the transgenic mice (Figure 5.3 panel d,e). We also observed transitional cell papilloma in about 6% of
Table 5.1 Histopathologic analysis of the phenotype of urinary bladders in BK5.COX2 transgenic mice and wild-type mice at different ages.

<table>
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<th>Genotype</th>
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<th>Transitional Cell Neoplasia*</th>
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<tr>
<td></td>
<td></td>
<td>simple</td>
<td>papillary</td>
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<tr>
<td>Wild type</td>
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<td>0/10 (0)</td>
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<tr>
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<td>1/13 (7)</td>
<td>0/13 (0)</td>
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<tr>
<td>BK5.COX2</td>
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<td>7/15 (47)</td>
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<tr>
<td>BK5.COX2</td>
<td>&gt; 20</td>
<td>8/35 (22)</td>
<td>13/35 (37)</td>
</tr>
</tbody>
</table>

*number of positive/number of total (%)
**Figure 5.2** Transitional Cell hyperplasia (TCH) in 10 wk old BK5.COX2 transgenic mouse urinary bladder. Bladders from wild-type (Wt, a-c) and BK5.COX2 (Tg, d-f) mice were stained with hematoxylin and eosin. Note the thin epithelium (black arrow) in Wt bladders in panel a, b and c. Papillary TCH denoted by papillary extensions into the bladder lumen (d - f)) and thickened hyperplastic epithelium (black arrow in panel d and f).
Figure 5.3 Figure 2. Histopathology of mouse urinary bladders from older (20-30 wk) BK5.COX2 mice. Note the thickened lamina propria (* in a) and increased TCH, very often, the nodular hyperplasia (white arrow in a) that characterized by the downward proliferation into the lamina propria and nests of epithelial cells (a-b). Note the nests of darkly strained inflammatory cells in panel a (black arrow) and panel c at higher magnification. Panel d is an example of carcinoma in situ (CIS, black arrow) in a small percentage of the transgenic mice. Possible transitional cell carcinoma (TCC, panel e) and transitional cell papilloma (f) were observed in a small percentage of the transgenic mice. Both lamina propria invasion (g, black arrow) and muscle invasion (h, black arrow) were observed in sections from TCC.
the total transgenic mice examined (Figure 5.3 panel f). Increased vascular vessels were observed in stromal and lamina propria in many sections from the BK5.COX2 transgenic mice (data not shown). A total of 15 wild-type mice that over 20 wk old was examined as controls and only one mouse developed simple hyperplasia which may due to an incidental lesion (Table 5.1).

5.4.3 Gene regulation profile in BK5.COX2 mice and EASE analysis

The effects of COX-2 overexpression on gene expression profile during bladder carcinogenesis were determined by microarray on 10 wk old BK5.COX2 transgenic and wild-type mice. We chose this age of mice so that we could test the early effects of increased COX-2 expression and elevated prostaglandin levels on gene expression before the induction of substantial TCH has occurred. Analysis of gene array data by t-test (p<0.05) revealed 2270 genes were significantly (p<0.05) upregulated and 1740 genes were significantly downregulated in transgenic compared to wild-type bladders. However, only 70 of the upregulated genes and 60 of the downregulated genes had expression changed by 2-fold or more in transgenic compared to wild-type bladders. The expression of COX-2 was increased 11 fold in the transgenic mice compared to the wild-type mice. Gene set analysis using EASE software revealed that genes associated with Immune/Stress Response, Cell Cycle/Proliferation and Cell Adhesion biologic processes were significantly (EASE score < 0.05) overrepresented in the top 100 upregulated genes as listed in Table 5.2. Examples of upregulated genes in Immune/Stress response biologic processes include immunoglobulin genes (Igj, Igh-4, IgVJ558), tumor suppressor
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<td>histocompatibility 2, complement component factor B</td>
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**Immune-Stress Response**

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**Cell Adhesion**

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**Table 5.2** Expression Analysis Systematic Explorer (EASE) analysis of top 100 upregulated genes that associated with Biologic Process ontology.
genes (Dbccr1) and complement cascade genes (H2-Bf and C4). Upregulated Cell Cycle/Proliferation biologic process genes include growth factors (Ereg and Igf1) and cell cycle genes (Mki67, Cdc2a, Top2a, and Prc1). EASE analysis also revealed that genes associated with Immune Regulatory, Hydrolase/Peptidase Activity, and Oxidoreductase Activity molecular function were significantly overrepresented in the top 100 upregulated genes as listed in Table 5.3. These findings correspond well with the phenotype observed in the bladders of BK5.COX2 mice as described above. Analysis of the top 100 downregulated genes by EASE failed to identify genes associated with any ontologies. However, relevant downregulated genes included three TGF beta related genes (Tgfb2, Tgfb3, Tgfbi), the anti-angiogenic gene thrombospondin 2 (Thbs2), and the insulin-like growth factor binding protein 6 (gfbp6). Examples of downregulated genes are listed in Table 5.4.

5.4.4 qRT-PCR and immunohistochemistry confirmation of microarray findings

We next validated some the genes by qRT-PCR and immunohistochemistry. qRT-PCR analysis confirmed that epiregulin and IGF-1 mRNAs were significantly upregulated, while IGFBP6, TGFbi, TGFb2, and TGFb3 mRNAs were significantly downregulated in 10 wk old transgenic mice bladders compared to the wild-type control (Figure 5.4). We further examined the expression of these genes in 30 wk old transgenic and wild-type mice. Interestingly, the mRNA levels of the three members of TGF beta related genes remained significantly reduced in transgenic compared to wild-type bladders (Figure 5.5). However, the mRNA levels of IGF-1 and IGFBP6 in BK5.COX2
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**Hydrolase/Peptidase Activity**

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<thead>
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<tr>
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<td>Mcm6</td>
<td>minichromosome maintenance deficient 6</td>
<td>1.96</td>
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<tr>
<td>1421921_AT</td>
<td>Serpina3m</td>
<td>serine (or cysteine) proteinase inhibitor, clade A, member 3M</td>
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<tr>
<td>1422760_AT</td>
<td>Padi4</td>
<td>peptidyl arginine deiminase, type IV</td>
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</tr>
<tr>
<td>1426302_AT</td>
<td>Tmprss4</td>
<td>transmembrane protease, serine 4</td>
<td>1.8</td>
</tr>
<tr>
<td>1448136_AT</td>
<td>Enpp2</td>
<td>ectonucleotide pyrophosphatase/phosphodiesterase 2</td>
<td>2.04</td>
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<tr>
<td>1417314_AT</td>
<td>H2-Bf</td>
<td>histocompatibility 2, complement component factor B</td>
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<tr>
<td>1418937_AT</td>
<td>Dio2</td>
<td>deiodinase, iodothyronine, type II</td>
<td>2.32</td>
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<tr>
<td>1419476_AT</td>
<td>Adamdec1</td>
<td>ADAM-like, decysin 1</td>
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<tr>
<td>1419323_AT</td>
<td>Padi1</td>
<td>peptidyl arginine deiminase, type I</td>
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<td>1422868_S_AT</td>
<td>Gda</td>
<td>guanine deaminase</td>
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<td>1425985_S_AT</td>
<td>Masp1</td>
<td>mannan-binding lectin serine protease 1</td>
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<td>1451537_AT</td>
<td>Chi3l1</td>
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**Oxidoreductase Activity**

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<th>Fold Increase</th>
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<tr>
<td>1418264_AT</td>
<td>Solt</td>
<td>SoxLZ/Sox6 leucine zipper binding protein in testis</td>
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<td>1418509_AT</td>
<td>Cbr2</td>
<td>carbonyl reductase 2</td>
<td>1.76</td>
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<td>Dio2</td>
<td>deiodinase, iodothyronine, type II</td>
<td>2.32</td>
</tr>
<tr>
<td>1419618_AT</td>
<td>Bbox1</td>
<td>butyrobetaine (gamma), 2-oxoglutarate dioxygenase 1</td>
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<td>1434437_X_AT</td>
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<td>ribonucleotide reductase M2</td>
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<td>1448792_A_AT</td>
<td>Cyp2f2</td>
<td>cytochrome P450, family 2, subfamily f, polypeptide 2</td>
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**Table 5.3** Expression Analysis Systematic Explorer (EASE) analysis of top 100 upregulated genes that associated with Molecular Function ontology.
### Down-regulated Genes

<table>
<thead>
<tr>
<th>Affymetrix ID</th>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Fold Decrease</th>
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</thead>
<tbody>
<tr>
<td>1417455_AT</td>
<td>Tgfb3</td>
<td>transforming growth factor, beta 3</td>
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<tr>
<td>1415871_At</td>
<td>Tgfb1</td>
<td>transforming growth factor, beta induced</td>
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<tr>
<td>1438303_AT</td>
<td>Tgfb2</td>
<td>transforming growth factor, beta 2</td>
<td>1.2</td>
</tr>
<tr>
<td>1417933_AT</td>
<td>gfbp6</td>
<td>insulin-like growth factor binding protein 6</td>
<td>1.58</td>
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<tr>
<td>1422571_AT</td>
<td>Thbs2</td>
<td>thrombospondin 2</td>
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<tr>
<td>1422324_A_AT</td>
<td>Pthlh</td>
<td>parathyroid hormone-like peptide</td>
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<td>1460230_AT</td>
<td>Syn2</td>
<td>synapsin II</td>
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<tr>
<td>1435459_AT</td>
<td>Fmo2</td>
<td>flavin containing monoxygenase 2</td>
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<tr>
<td>1448162_AT</td>
<td>Vcam1</td>
<td>vascular cell adhesion molecule 1</td>
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<tr>
<td>1449979_A_AT</td>
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<td>sparc/osteonectin, cwcw and kazal-like domains proteoglycan 3</td>
<td>2.08</td>
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<tr>
<td>1451527_AT</td>
<td>Pcolce2</td>
<td>procollagen C-endopeptidase enhancer 2</td>
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<tr>
<td>1425644_AT</td>
<td>Lepr</td>
<td>leptin receptor</td>
<td>1.94</td>
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<td>1449015_AT</td>
<td>Retnla</td>
<td>resistin like alpha</td>
<td>1.88</td>
</tr>
<tr>
<td>1452514_A_AT</td>
<td>kit</td>
<td>kit oncogene</td>
<td>1.86</td>
</tr>
<tr>
<td>1422561_AT</td>
<td>Adamts5</td>
<td>a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 5 (aggrecanase-2)</td>
<td>1.82</td>
</tr>
<tr>
<td>1419492_S_AT</td>
<td>Defb1</td>
<td>defensin beta 1</td>
<td>1.78</td>
</tr>
<tr>
<td>1420796_AT</td>
<td>Ahrr</td>
<td>aryl-hydrocarbon receptor repressor</td>
<td>1.78</td>
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<tr>
<td>1427139_AT</td>
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<td>1437671_X_AT</td>
<td>Prss23</td>
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<td>Cck</td>
<td>cholecystokinin</td>
<td>1.68</td>
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<td>1421006_AT</td>
<td>Col4a6</td>
<td>procollagen, type IV, alpha 6</td>
<td>1.66</td>
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<tr>
<td>1426622_A_AT</td>
<td>Qpct</td>
<td>glutaminyl-peptide cyclotransferase (glutaminyl cyclase)</td>
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<td>1418486_AT</td>
<td>Vnn1</td>
<td>vanin 1</td>
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<td>1456084_X_AT</td>
<td>Fmod</td>
<td>fibromodulin</td>
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<td>Serpinf1</td>
<td>serine (or cysteine) proteinase inhibitor, clade F, member 1</td>
<td>1.58</td>
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<td>1420512_AT</td>
<td>Dkk2</td>
<td>dickkopf homolog 2 (Xenopus laevis)</td>
<td>1.56</td>
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**Table 5.4** Expression Analysis Systematic Explorer (EASE) analysis of top 100 downregulated genes.
bladders were not significantly different from the levels in wild-type bladders (Figure 5.5). Among these genes, epiregulin is the most significantly increased gene in the BK5.COX2 transgenic mice bladders. The mRNA level of epiregulin increased by 3.7 fold in 10 wk old transgenic mice bladders and 5.3 fold in 30 wk old transgenic mice bladders compared to age-matched wild-type bladders.

A subset of tissues sections from 10 wk old BK5.COX2 and wild-type mice bladders were analyzed for expression of the proliferation marker Ki67 by immunohistochemistry. We found very few cells were stained positive for mKi67 in the wild-type mice bladders (Figure 5.6 panel a-c). However, the number of epithelial cells positive for Ki67 is significantly increased in bladders from BK5.COX2 transgenic mice (Figure 5.6 panel d-f).

5.4.5 Epiregulin mRNA induction by PGE2

As shown in Figure 5.1 D, the average concentration of PGE2 in bladder tissues of BK5.COX2 mice was about 17ng/mg of tissue. This concentration (10µM after conversion) was used to treat bladder tissues from FVB wild-type mice to determined whether epiregulin mRNA level could be induced by PGE2 at this concentration under an ex-vivo organ culture condition. We found that 10µM PGE2 induced epiregulin mRNA expression in a time-dependent manner in bladders from 10 wk old wild-type mice (Figure 5.7). The maximum level of epiregulin mRNA was reached at 4 h. The increase of epiregulin mRNA remained significantly elevated compared to controls after 8 h incubation. These results suggest that PGE2 has a direct role in the induction of epiregulin mRNA expression. However, the regulation of epiregulin at protein level by PGE2 was
Figure 5.4 qRT-PCR validation of six genes of interest that were significantly changed in 10 wk old BK5.COX2 transgenic mice (Tg) compared to wild-type (Wt) mice by Gene Array analysis. Note that epiregulin is the most significantly upregulated gene (increased in BK5.COX2 by 3.7 fold, p<0.01). Each bar represents the mean mRNA expression of indicated gene (error bars are 95% confidence intervals) of five animals relative to a common calibrator sample generated by pooling 3 bladders from wild-type mice. GAPDH mRNA levels were used to normalize the expression of individual samples. A p value < 0.05 was considered as significantly different from each other.
Figure 5.5 Expression of the six validated genes in 30 wk old BK5.COX2 (Tg) transgenic mice and age matched wild-type (Wt) mice by qRT-PCR analysis. Note that epiregulin is again the most significantly upregulated gene in the 30 wk old animals (increased in BK5.COX2 by 5.3 fold, p<0.01). Each bar represents the mean mRNA expression of indicated gene (error bars are 95% confidence intervals) of five animals relative to a common calibrator sample generated by pooling 3 bladders from wild-type mice. GAPDH mRNA levels were used to normalize the expression of individual samples. A p value < 0.05 was considered as significantly different from each other.
Figure 5.6 Immunohistochemistry analysis of the proliferation marker mKi67 10 wk old BK5.COX2 (Tg) transgenic mice and wild-type (Wt) mice bladders. Note that the number of epithelial cells positive for mKi67 is significantly increased in bladders from Tg mice (d-f). Whereas, very few cells were stained positive for mKi67 in the wild-type bladder epithelial cells (a-c, arrow in panel c).
Figure 5.7 PGE$_2$ at 10µM induces epiregulin mRNA expression in a time-dependent manner in bladder tissues from 10 wk old FVB wild-type mice. Bladder tissues were excised from mice, cut in half, and then incubated in RPMI medium with 10µM PGE2 for indicated time. Levels of epiregulin mRNA were determined by qRT-PCR. Bars represent means, error bars are 95% confidence intervals (CI), and means with different letters significantly differ from each other (p<0.05, n=4).
not detected at this time due to the non-specificity and insensitivity of the commercial available antibody against epiregulin (Santa Cruz).

5.4.6 Effects of Epiregulin on proliferation and Erk activation in UMCC-3 cells

We next screened five bladder cells line (RT4, HT1376, J82, T24, and UMUC-3) for the effects of recombinant hEpiregulin on cell proliferation in these cells. We found that hEpiregulin has essentially no effects on proliferation in RT4, HT1376, J82, and T24 cells (data not shown). These cells have both high level of COX-2 expression and PGE2 concentration. However, UMUC-3 cells which have very low COX-2 expression and PGE2 production, significantly responded to epiregulin treatment. The recombinant hEpiregulin significantly increased UMUC-3 cell proliferation at 48 h at concentrations ranging from 10pg/ml to 1 ng/ml compared to untreated controls (Figure 5.8 A). In addition, hEpiregulin induced phosphorylation of Erk in a dose- and time-dependent manner in UMUC-3 cells (Figure 5.8 B, C). The phosphorylation of Erk in UMUC-3 cells was significantly upregulated within 15 min by 10 ng/ml hEpiregulin. The maximum stimulation was reached at 15 min and Erk phosphorylation declined but remained high after 4 h (Figure 5.8 C).
Figure 5.8 Recombinant hEpiregulin induces proliferation and Erk phosphorylation in bladder cancer UMUC-3 cells. **A.** hEpiregulin increases cell proliferation in UMUC-3 cells. Cells were treated with hEpiregulin (0, 1pg/ml, 10pg/ml, 100pg/ml and 1ng/ml) for 24 hr, cell proliferation was determined by MTT assay. Bars represent means, error bars are 95% confidence intervals (CI), and means with different letters significantly differ from each other (p<0.05, n=3). **B.** hEpiregulin induces phosphorylation of Erk in a dose-dependent manner in UMUC-3 cells. Cells were treated for 30 min with concentrations of hEpiregulin ranging from 1 pg/ml to 10000 pg/ml followed by immunoblotting. **C.** hEpiregulin at 10ng/ml induces phosphorylation of Erk in time-dependent manner in UMUC-3 cells. Cells were treated with 10 ng/ml hEpiregulin for 15 to 240 min followed by immunoblotting.
4.5 DISCUSSION

COX-2 overexpression and increased PGE$_2$ synthesis have been associated with bladder tumorigenesis by numerous studies (3). We have developed a transgenic mouse model which over expresses COX-2 under the control of a bovine keratin 5 (BK5) promoter. We found that BK5. COX2 mice developed high incidence of urinary bladder TCH and transitional cell neoplasia in a small percentage of the transgenic mice. To determine the molecular pathways elicited by COX-2 overexpression during the early events of bladder carcinogenesis, we conducted DNA microarray to determine the effects of COX-2 and prostaglandin synthesis on regulating gene profile in the urinary bladder of 10 wk old BK5.COX2 mice before the onset of substantial TCH. We found that genes involved in the Cell Cycle/Proliferation and Immune/Stress response biologic processes were significantly upregulated. These findings correspond well with the previously described phenotype observed in the bladders of BK5.COX2 mice.

Mohammed et al. (5) were the first to characterize the expression of COX-2 in normal human urothelium, TCC, and CIS in bladder cancer patients. The investigators observed that COX-2 was not expressed in all normal bladder urothelium examined, although COX-2 was overexpressed in 83% of TCC specimens and 75% of CIS specimens (5). Since then, a substantial number of studies has confirmed these findings, and have correlated the intensity of COX-2 overexpression to tumor grade and stage of this disease (6-10). Evidence for a functional role of COX-2 in bladder cancer is primarily from preclinical studies including genetic and pharmacologic studies. Results from these studies suggest that COX-2 and PGE$_2$ play tumorigenic effects in a number of
cancers through increasing proliferation, enhancing angiogenesis, promoting invasion, and inhibiting apoptosis (3). However, the exact molecular mechanisms involved have not been well studied. DNA microarray allowed us to identify early genetic and molecular changes that lead to superficial and invasive bladder cancer drove by COX-2 overexpression. Many molecular changes have been identified during human bladder carcinogenesis (72, 150). Interestingly, we found that certain genes that are deregulated in the BK5.COX2 urinary mouse bladder model also have been reported by others, such as upregulation of epiregulin, IGF-1 and ki67, and downregulation of members of TGF-beta and thrombospondin families (149, 150). Therefore, the BK5.COX2 model certainly appears to be relevant to human urinary bladder cancer, thereby allowing us to study mechanisms of tumorigenic effects of COX-2/PGs and their downstream pathways.

The development of TCH in the bladder of BK5.COX2 mice was associated with a dramatic increase in proliferating cells in the basal cell layers of the urothelium. Results from gene array analysis supported our original hypothesis that prostaglandin synthesis via COX-2 overexpression induces cell proliferation through induction of growth factors. Both epiregulin and IGF-1 were induced more than 2 fold greater in the bladders of 10 wk old BK5.COX2 transgenic mice compared to bladders from wild-type mice. However, there was no such marked difference in IGF-1 mRNA between transgenic and wild-type mice at 30 wk of age. This suggests that IGF-1 may only play a carcinogenic role upon COX-2 overexpression in the early stage of tumor development in bladder.

Epiregulin is a novel member of the EGF family. It was the most significantly upregulated gene among genes validated by qRT-PCR and remained significantly high in bladders of 30 wk old transgenic mice. This suggests that epiregulin may serve as a key
modulator of cell proliferation as a result of COX-2 overexpression in urinary bladders. Epiregulin binds to ErbB1 (EGFR) and ErbB4 and is a potent mitogen for rat primary hepatocytes (69). A study by Zhu et al. found that epiregulin is highly expressed in pancreatic cancer cells, and recombinant human epiregulin was able to induce the growth of pancreatic cancer cells in a dose-dependent manner (277). Epiregulin binds to various combinations of erbB receptors with low affinity, but transmits a more potent mitogenic signal than EGF (137). Nicholson et al. found that epiregulin is one of the four biomarkers that are consistently and progressively upregulated in a mouse lung metastatic bladder model (155). The mRNA level of epiregulin was also increased in HCV28 bladder cancer cells upon the stimulation with the DNA damage agent VP16 (278). Thogersen et al. reported that epiregulin is the most significantly increased EGF like ligand in human T2-T4 tumors as compared with Ta tumors (154). Together with our finding that epiregulin was highly induced in BK5.COX2 transgenic bladders, these studies suggest that epiregulin may be a potential important biomarker of bladder cancer. We further found that PGE₂ at a concentration equivalent to that in the bladder tissue of BK5.COX2 mice increased epiregulin mRNA in bladders from FVB mice ex-vivo. This suggests that PGE₂ may directly induce the transcription of this gene. We further determined that epiregulin significantly induced cell proliferation and MAPK/Erk phosphorylation in bladder cancer UMUC-3 cells, but not other bladder cell lines examined. UMUC-3 cells are differ from the other cell lines (RT4, HT1376, J82, and T24) in that it has non-detectable expression of COX-2 and low levels of PGE₂ (11, 279). However, ERGF and ErbB4 were not activated in UMUC-3 cells upon treatment with
epiregulin. The different response of UMUC-3 cells to epiregulin will require further investigation.

Although EASE analysis failed to group the top 100 downregulated genes into any gene ontologies, we found that the three members of TGFβ family, i.e., Tgfb2, Tgfb3, and Tgfbi, were significantly downregulated in the bladder of transgenic mice at 10 and 30 wk of age. The TGFβ family is multifunctional cytokines responsible for cell proliferation, differentiation, extracellular matrix synthesis/degradation, and mediation of apoptosis (280). Reduced expression of TGFβ-1 is significantly associated with high-grade of bladder tumors (160, 161, 281). Loss of expression of TGFβ receptors is significantly associated with higher tumor grade and stage, presence of lymph node metastases, progression and reduced survival (138, 159, 160, 281). Conditional deletion of TGFβ Receptor type II is associated with thickened lamina propria and myofibroblast proliferation in Tgfbr2fspko mice bladders (282). One significant observation in the phenotype that developed in the bladder tissue of BK5.COX2 mice is that TCH was strongly associated with thickness of stroma and laminar propria. The downregulation of TGFβ-related genes and together with other genes associated with Cell Adhesion and Hydrolase/Peptidase Activity identified by EASE analysis may play a key role in the remodeling of extracellular matrix compartment of urinary bladder during carcinogenesis driven by COX-2 overexpression.

Another observation in the urinary bladder of BK5.COX2 mice is that a dramatic increase in vascularization was observed in the lamina propria and stroma. Neovascularization depends on an increase in proangiogenic factors relative to antiangiogenic factor. In the K5.COX-2 mouse model, Klein et al. (3) demonstrated that
the proangiogenic factor VEGF was clearly elevated in the urinary bladder of transgenic mice. However, the gene array study did not identify significant dysregulation in genes associated with angiogenesis, except for the downregulation of the anti-angiogenic gene thrombospondin 2. We previously reported that PGE$_2$ induces VEGF secretion in prostate cancer cells by posttranscriptional regulation (283). Immunohistochemical analysis of the expression of angienic factors, such as VEGF in sections of BK5.COX2 bladders merits further investigation.

In the K5.COX-2 mouse model, inflammation was proposed as the causative factor in the development of TCH in these mice upon COX-2 overexpression (3). We also observed significant lymphocyte infiltration in the lamina propria and more often in the muscle area. EASE analysis of the gene array data revealed that a substantial number of genes involved in Immune-Stress Response in biological process and Immune Regulatory in molecular function were significantly upregulated. Although the exact function of these immune response related genes is not clear, it is evident that the initial response of the mouse urinary bladder to elevated COX-2 expression includes mounting an inflammatory response. A better understanding of the mechanisms associated with the observed inflammatory response is expected to provide insights about the etiology of bladder cancer.

The physiological effects of PGE$_2$ are mediated by four G protein coupled membrane receptors, i.e., EP1, EP2, EP3 and EP4. EP receptors have been implicated in promoting carcinogenesis in different types of tumors, including lung (45), ovarian (46), colorectal (47, 48), breast (49), and skin (50, 51). Miyata et al. suggests that only EP4 receptor is co-expressed with COX-2 in TCC of the upper urinary tract (227).
Interestingly, gene array analysis found that EP4 is that only EP receptor that was significantly elevated in the BK5.COX2 transgenic mouse bladders, although it was only induced by 70% compared to the wild-type controls. Because COX-2 selective inhibitors may not be useful as chemoprevention agents due to potential side effects associated with long term use, EP receptors, located downstream of PG synthesis in the COX pathway may represent attractive alternative targets for chemoprevention and treatment of bladder cancer. However, further studies are needed to determine the role of EP receptors (potentially the EP4 receptor) and mechanisms involved in bladder carcinogenesis.

Although bladder TCH developed in mice as young as 10 wk of age in 100% of the transgenic mice, TCC developed only in 6% of the transgenic mice. The total percentage of the transitional cell neoplasia is about 18%. These observations suggest that additional mutations are required for progression form TCH to TCC. The alteration and mutation of tumor suppressor gene p53 has been shown to be significantly associated with bladder cancer tumorigenesis (149, 150). However, crossing the BK5.COX2 mice with p53-/- mice did not enhance the severity of lesions in the urinary bladder of the double crossed transgenic mice (data not shown). The ErbB family, including EGFR, ErbB-2, ErbB-3 and ErbB-4, has been strongly associated with the stage and grade of bladder cancer development and progression (151, 284). Overexpression of EGFR in urothelium has been demonstrated to induce hyperplasia and promotes bladder tumor growth in transgenic mice (285). Crosstalk between COX-2 and EGFR has been established to be associated with carcinogenesis by many studies (286). In future study, we may cross EGFR overexpressing mouse with BK5.COX2 transgenic mouse to determine if the incidence of TCC would be increased. It will also be interesting to learn
whether knocking out EGFR in BK5.COX2 mice prevents the development of TCH. Netherless, the BK5.COX2 transgenic mouse model may serve as a potential useful pre-clinical model for superficial bladder cancer.

In summary, our study provides evidence that COX-2 overexpression and PGE$_2$ overproduction play a tumorigenic effect in bladder cancer through regulating growth/proliferation, extracellular remodeling, and immune responses related genes. The biomarkers of COX-2 overexpression may be useful targets for future prevention studies focusing on bladder cancer. Our study also provides rational for future clinical trials to test the ability of COX-2-selective inhibitor alone or in combination with EGFR inhibitors to prevent the development and progression of bladder cancer.
Prostate and bladder cancers are significant health problems. Identification of new molecular targets may provide effective strategies to reduce the development and progression of these two diseases. The research presented above was to determine whether COX-2/PGE2/EP signaling pathway was valid targets for the prevention and treatment of prostate and bladder cancers. The overall conclusion of my dissertation was that COX-2 may not play a tumorigenic role in prostate cancer progression, but has a pivotal role in bladder carcinogenesis though regulating the expression of specific patterns of genes.

The first study was conduct to examine the expression profile and the role of EP receptors in PGE2-mediated signaling cascades in prostate cancer cells. It was found that EP2 is the predominantly expressed receptor in prostate cancer cell lines, and it also played a key role in mediating PGE2-induced VEGF secretion and activation of Erk and Akt in these cells. However, this kind of work has not been fully addressed in bladder cancer cell lines. We have determined that all the EP receptors may express in both bladder cancer cell lines and mouse bladder tissues. It would be interesting to determine whether PGE2 could induce the similar signal cascades as we observed in prostate cell lines, and to determined which EP receptors are involved in these biologic effects. Due to the unwanted side effects associated with the use of COX inhibitors, the EP receptors
may be novel targets for the prevention and treatment of bladder cancer. Therefore, future studies are needed to determine the contribution of each individual EP receptor in bladder carcinogenesis using genetically modified mouse models.

One of the most significant findings from our second study was that the expression and activity of COX-1 and COX-2 are progressively lost during prostate carcinogenesis and tumor progression. Given the recent evidence that COX-2 may be overexpressed only in areas of inflammation in prostate cancer tissues, a role for COX-2 in either the initiation or promotion of this disease by eliciting an inflammatory response need to be investigated in future. In addition, the mechanisms underlying the marked decrease of COX mRNA and protein levels during prostate tumorigenesis need to be explored. Whether epigenetic regulation, such as gene methylation, plays a role in COX downregulation would be more interesting to study. Although it may not be likely that loss of COX expression contributes to the tumorigenesis of prostate cancer, further work will be required to determine the significance of this phenomenon.

Results from our third study demonstrated that COX-2 overexpression regulates the expression of genes involved in Immune/Stress, Proliferation/Cell Cycle and Extracellular Matrix remodeling in the bladder of BK5.COX2 transgenic mice. However, the exact function of these immune response related genes and many genes associated with other ontologies in bladder carcinogenesis in this model are not clear. It would be very difficult to study each individual gene. A comparison of the molecular changes during bladder carcinogenesis between human bladder cancer and BK5.COX2 mouse model in future studies are needed to further elucidate the etiology and biomarkers of human bladder cancer. We found that the growth factor, epiregulin, may play a key role
in bladder carcinogenesis upon elevated PGE$_2$ production. However, at present, little is known about the localization of epiregulin and which erbB receptors and down-stream signaling cascades are responsible for epiregulin-elicited biologic effects in bladder carcinogenesis. Further studies are needed to address these questions. Upon we get these information, we could target specific erbB receptors as individual or in combination with COX-2/PGE$_2$/EP inhibition to prevent the development and progression of bladder carcinogenesis in pre-clinical and clinical studies. As we know, dietary fatty acids play an important role in the modulation of COX/PG synthesis and would therefore regulate bladder carcinogenesis. Whether dietary intervention through fish oil could inhibit bladder carcinogenesis in BK5.COX2 mouse model warrants evaluation in future studies.
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185


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APPENDIX A

(figures)
Figure A.1  EP2, EP3 and EP4 are expressed in different pattern in examined human prostate cell lines (PWR-1E, RWPE-1, RWPE-2, 22RV1, PC-3, DU145, and LNCaP). Cell lysates from the seven human prostate cell lines were run on 4%-15% SDS-PAGE criterion gel (Bio-Rad) in duplicates. The expression of EP2, EP3 and EP4 in these cell lines were analyzed by immunoblotting. Lysate from mouse kidney was used as positive control. EP1 was not detectable in any of the cell lines and mouse kidney.
Figure A.2  Non-specificity of antibody against EP4 receptor that from Cayman Chemical. **A**, EP4 is only detectable in cell lysates that were not serum-starved. **B**, EP4 binds to unknown antigen in serum-containing medium. 1, cell lysates from serum starved PC-3 cells. 2, cell lysates from PC-3 cells in medium with serum. 3, cells lysates from 2 that added with additional 10% serum-containing medium. 4, serum-containing medium only (no cell lysates).
Figure A.3  PGE₂- and CAY10399-induced Erk and Akt activation in DU145 cells. A, PGE₂ and CAY10399 induce phosphorylation of Erk in time-dependent manner in DU145 cells. Cells were treated with either 10 µM PGE₂ or CAY10399 for 15 to 240 min. Total and phosphorylated Erk in cell lysates were analyzed by immunoblotting. B, The dose-response of PGE₂ and CAY10399 induced phosphorylation of Erk in DU145 cells. Cells were treated for 15 min with concentrations of PGE₂ and CAY10399 ranging from 1 nM to 10 µM. C, Extended time course of phosphorylation of Akt in DU145 cells. Cells were treated with either 10 µM PGE₂ or CAY10399 for 15 to 720 min (12h).
Figure A.4 Adenylate cyclase inhibitor 2′5′-dideoxyadenosine significantly blocks 1nM PGE2-induced secretion of VEGF in PC-3 cells. Cells were pretreated with either 5µM or 50 µM 2′5′-dideoxyadenosine for 1 h followed by 1nM PGE2 treatment for an additional 24 h in serum-free medium. I, 2′5′-dideoxyadenosine; P, PGE2. Data are means ± SD. Bars with different letters are significantly different from each other with a p-value <0.05.
Figure A.5  PGE2 levels in three prostate lobes of 10 wk old wild-type mice. Tissues were analyzed for PGE2 by LC/MS/MS. Bars represent the mean obtained from at least five mice (error bars are 95% confidence intervals). DP (dorsal prostate), LP (lateral prostate), and VP (ventral prostate).
Figure A.6  COX-2 immunohistochemical staining of the same section in the dorsal prostate (DP), lateral prostate (LP), and ventral prostate (VP) tissues of a 10 wk old Pb.COX-2 mouse.
Figure A.7  PGE$_2$ levels in three prostate lobes of 10 wk old Pb.COX-2 and wild-type mice. Tissues were analyzed for PGE$_2$ by LC/MS/MS. Bars represent the mean obtained from at least five mice (error bars are 95% confidence intervals). DP (dorsal prostate), LP (lateral prostate), and VP (ventral prostate).
**Figure A.8** H&E stained section of ventral prostate (VP) from one mouse of a founder line of Pb.COX-2 mouse model. Please note lymphocyte infiltration (white arrow) and the appearance of hyperplasia (yellow arrow).
A: anterior prostate

B: dorsal prostate

**Figure A.9** Expression of COX-1 and COX-2 mRNA are significantly reduced in both anterior prostate (AP) and dorsal prostate (DP) tissues of the 10 wk old conditional PTEN knock mice (Tg) compared to wild-type (WT) mice. Each bar represents the mean COX-1 or COX-2 mRNA (error bars are 95% confidence intervals) of five animals relative to a common calibrator sample generated by pooling the dorsal, ventral, and lateral prostates of a single WT mouse. 18s RNA levels were used to normalize the expression of individual samples. * P < 0.05. A, anterior prostate; B, dorsal prostate.
Figure A.10  Western blot analysis of COX-1 and COX-2 protein levels in seven prostate cell lines (PWR-1E, RWPE-1, RWPE-2, 22RV1, PC-3, DU145, and LNCaP). Mouse vas deferens tissue was used as positive control for COX (+).
Figure A.11 Prostaglandin A$_2$ (PGA$_2$), PGD2, 15-d-PGJ2 and 12-HETE levels in dorsal prostate (DP) tissues of 10 wk old wild-type (WT) and TRAMP mice. Tissues were analyzed for eicosanoids by LC/MS/MS. Bars represent the mean of values obtained from at least five mice (error bars are 95% confidence intervals). * p < 0.05.
Figure A.12 Transgene construct of BK5.COX2 mouse model.

<table>
<thead>
<tr>
<th>5'</th>
<th>Bovine Keratin 5 Promoter</th>
<th>B-globin intron</th>
<th>Murine COX-2 cDNA</th>
<th>SV40-poly A</th>
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