Cyclooxygenase-2 Directly Induces MCF-7 Breast Tumor Cells to Develop into Exponentially Growing, Highly Angiogenic and Regionally Invasive Human Ductal Carcinoma Xenografts

FREDIKA M. ROBERTSON[1], SUSAN R. MALLERY[2,3], VALERIE K. BERGDALL-COSTELL[4], MARK (MING-CHENG) CHENG[5,6], PING PEI[3], JENIFER R. PROSPERI[7] and MAURO FERRARI[1,5,6,8,9]

[1]*The Department of Experimental Therapeutics, The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030;*
[2]*The Ohio State University Comprehensive Cancer Center, Columbus, OH 43210;*
[3]*The Department of Oral Surgery and Pathology, The Ohio State University College of Dentistry, Columbus, OH 43210;*
[4]*The Ohio State University College of Veterinary Medicine, Columbus, OH 43210;*
[5]*Brown Institute of Molecular Medicine;*
[6]*Department of Biomedical Engineering, The University of Texas Health Sciences Center, Houston, TX 77030;*
[7]*The Ohio State University School of Biological Sciences Integrated Biomedical Graduate Program, Columbus, OH 43210;*
[8]*The Department of Bioengineering, Rice University, Houston, TX 77030;*
[9]*The Alliance for NanoHealth, Houston, TX 77030, U.S.A.*

Abstract. *Based on our studies demonstrating first time evidence that the cyclooxygenase-2 (Cox-2) enzyme is abundant within invasive human breast tumors, we developed a clonally derived human breast tumor cell clone designated as MCF-7/Cox-2 Clone 10 by transfection of human Cox-2 cDNA into slow growing, Cox-2 null, non-metastatic MCF-7 human breast tumor cells. The present studies evaluated the biological characteristics of the MCF-7/Cox-2 Clone 10 human breast tumors compared to the characteristics of MCF-7/empty vector control tumors when grown in vivo following injection of $5x10^6$ tumor cells into mammary fat pads of ovariectomized female Crl:Nu-Foxn1^{nu} mice implanted with slow release 17-β estradiol pellets. At 60 days after tumor cell injection, MCF-7/Cox-2 Clone 10 human breast tumors were 4-fold greater ($p<0.01$) in volume than MCF-7/empty vector control tumors. MCF-7/Cox-2 Clone 10 human breast tumor xenografts were highly angiogenic based on histological observation of large-bore blood vessels, which was confirmed by immunohistochemical staining with anti-CD-31 antibody and quantitation of mean vessel density. MCF-7/Cox-2 Clone 10 human breast tumor cells were present within regional lymph nodes adjacent to mammary fat pads with their local invasion confirmed by Western blotting of Cox-2-protein. This unique Cox-2-dependent breast tumor model rapidly produces large, angiogenic, locally invasive human breast tumor xenografts in mammary fat pads of ovariectomized female Crl:Nu-Foxn1^{nu} mice at 42-60 days which recapitulate human breast ductal carcinomas. This unique model may be invaluable as a means to evaluate preclinical safety and efficacy of novel adjuvant therapies for women with metastastic breast cancer including prostanoid receptor antagonists, newly developed anti-angiogenic therapies, as well as other novel approaches for targeting and destruction of human breast tumors and their vasculature.*

Abbreviations: BSA, bovine serum albumin; COX-2, cyclooxygenase-2; cDNA, complementary deoxyribonucleic acid; DMEM/HEPES, Dulbecco's Modified Eagles Medium HEPES Modification; EP, prostaglandin receptor; HRP, horse radish peroxidase; MMP, matrix metalloproteinase; μm, micron; ml, milliliter; mm, millimeter; MMTV, mouse mammary tumor virus; nu, nude; PECAM, platelet endothelial cell adhesion molecule-1; PGE_2, prostaglandin E_2; PVDF, polyvinylidene diflouride; TBST, Tris-buffered Saline with Tween VEGF, vascular endothelial growth factor.

Correspondence to: Fredika M. Robertson, The Department of Experimental Therapeutics, The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030, U.S.A. Tel: +1 713 745 9291, Fax: +1 713 563 8001, e-mail: frobertson@mdanderson.org

Key Words: Cyclooxygenase-2, human breast cancer model, ductal carcinoma, angiogenesis, invasion, MCF-7/Cox-2 Clone 10, xenografts.

We were the first laboratory to report that the cyclooxygenase-2 (Cox-2) enzyme is present in human breast tumors with evidence of invasion (1), which has since been confirmed by a number of other laboratories (2-4). One study, which evaluated 1576 invasive human breast cancers, reported that high levels of Cox-2 protein were associated with histology consistent with ductal carcinomas, with large tumor size and a high proliferative rate, the presence of the Her2/Neu

oncogene, high p53 expression, a high rate of axillary node metastasis, and an overall poor prognosis (5).

A number of studies from our laboratory (6-8) as well as those of others (9-17) have used chemically-induced mammary carcinogenesis models and models that have endogenously high levels of Cox-2 (18-19) to demonstrate a role for Cox-2 and PGE_2 in mammary tumors. In addition to these rodent models of mammary tumorigenesis, there has been one primary Cox-2 driven mammary tumor transgenic mouse model developed using the mouse mammary tumor virus combined with human Cox-2, which has been defined as the MMTV-Cox-2 transgenic mouse (20-22). The MMTV-Cox-2 transgenic model has significantly advanced our knowledge about the central role that Cox-2 and PGE_2 play in mammary tumor development, in resistance to apoptosis, and of the role of PGE_2 in the "angiogenic switch" which activates new blood vessel development that is considered essential to tumor expansion and invasion (21, 22). Recent studies using MMTV-Cox-2 transgenic mice have reported that Cox-2 and PGE_2 regulate the oncogene HER2/Neu, which is found in 33% of human breast tumors and have reported that Cox-2 may be central to the control of production of estrogenic hormones through modulation of the P450 enzyme aromatase (23, 24). Given that Cox-2 is present in over 66% of human tumors regardless of hormone status, these recent studies re-emphasize the importance of Cox-2 in human breast cancer, regardless of the recent issues raised with using selective Cox-2 inhibitors for adjuvant therapies in women with metastatic breast cancer due to cardiovascular side-effects.

While the models of carcinogen-induced mammary tumors, including those that have high endogenous Cox-2, and MMTV-Cox-2 transgenic model of mammary tumorigenesis have been very useful in identifying molecular mechanisms associated with murine mammary tumor development, resistance to apoptosis and angiogenesis, the present studies were focused on developing a model of human breast cancer that recapitulated the biological characteristics of human ductal carcinoma. Once developed and characterized, such a human breast tumor xenograft model may be useful in testing novel targeted therapeutics, and hormonal therapies which cannot be fully evaluated in murine mammary tumors.

We previously described the development and characterization of MCF-7/Cox-2 clonally derived human breast tumor cell lines which were established by stable transfection of human Cox-2 cDNA into the Cox-2 null, estrogen-dependent MCF-7 human breast cancer cell line (25). One of these clonally derived cell lines, designated as MCF-7/Cox-2 Clone 10, produces high levels of PGE_2, which is the product of the Cox-2 enzyme, and proliferates with a 3-fold increased rate of proliferation compared MCF-7/empty vector control breast tumor cells, which is consistent with the decreased turnover time observed. The MCF-7/Cox-2 Clone 10 human breast tumor cells also express mRNA for heparin binding pro-angiogenic growth factors within the vascular endothelial growth factor family (VEGF A) of angiogenic factors including $VEGF_{145}$, $VEGF_{165}$ and $VEGF_{189}$, are invasive across an artificial basement membrane, and grow as large anchorage-independent colonies in soft agar (25).

Our previous studies using samples from invasive human breast tumors were also the first to establish a link between elevated Cox-2 mRNA and increased gene expression for the P450 enzyme aromatase, which is the enzyme that is responsible for production of estrogenic hormones (26). Our recent studies have demonstrated that the MCF-7/Cox-2 Clone 10 breast tumor cells express high levels of mRNA of the P450 aromatase which biotransforms androgenic hormones to estrogen and estradiol, with selective expression of specific promoter regions of the P450 aromatase. We also found that MCF-7/Cox-2 Clone 10 human breast tumor cells use specific promoter regions of the aromatase enzyme during growth of breast tumor cells and simultaneously secrete large amounts of estradiol, the product of the aromatase enzyme (27). These studies also demonstrated that the combination of the aromatase inhibitor, formestane, and the selective Cox-2 inhibitor, celecoxib, effectively blocked MCF-7/Cox-2 Clone 10 proliferation and estradiol production.

While our previous studies have evaluated the *in vitro* characteristics of the MCF-7/Cox-2 Clone 10 human breast tumor cells, the current studies are focused on development of human breast tumors that grow *in vivo* in mammary fat pads of ovariectomized female Crl:Nu-Foxn1^{nu} mice. The present studies evaluate the biological characteristics of MCF-7/Cox-2 Clone 10 human breast tumors that are a direct result of the presence of human cyclooxygenase cDNA. Studies included those to determine the timing of initial development, growth, angiogenesis and invasion of MCF-7/Cox-2 Clone 10 human breast tumors compared to these characteristics in MCF-7/empty vector control tumors. In addition, the histopathology, angiogenesis, mean vessel density and invasion into regional lymph nodes was examined in these two different breast tumor types over a period of 60 days. The present studies describe the biological characteristics of a model of a unique human breast tumor which develops exponentially, is highly angiogenic, are rapidly invasive to adjacent regional lymph nodes and recapitulates human breast ductal carcinoma, effects which are directly attributable to the presence of Cox-2 cDNA.

Materials and Methods

Cell culture conditions. The MCF-7/Cox-2 Clone 10 human breast cells and the MCF-7/empty vector control tumor cell clones were developed in our laboratory as previously described (25). Cells were grown in Dulbecco's modified Eagle's medium HEPES modification (DMEM/HEPES) (Sigma Chemical Co., St. Louis,

MO, USA), 10% heat-inactivated FBS (Invitrogen, Carlsbad, CA, USA), 1% nystatin (Sigma) and 50 mg/ml gentamycin (Invitrogen) supplemented with 0.25 mg/ml Zeocin (Invitrogen). Cells were maintained at 37˚C with 5% CO_2.

Development of human breast tumor xenografts in nude mice. Ovariectomized Crl:Nu-Foxn1^{nu} mice (6-8 weeks of age, Charles River Laboratories, Wilmington, MA, USA) were housed in a barrier-free vivarium space at The Ohio State University which meets the requirements of the American Association for Accreditation of Laboratory Animal Care. The Institutional Animal Care and Use Committee of The Ohio State University approved all protocols prior to beginning the study. Animals had access to food and water *ad libitum* and were kept on a 12 h cycle of light and dark.

Ovariectomized Crl:Nu-Foxn1^{nu} mice were implanted subcutaneously with 0.5 mg, slow release, 17β-estradiol pellets (SE-121, Innovative Research of America, Sarasota, FL, USA) at 24 h prior to tumor cell injection. To implant the estradiol pellets, mice were anesthesized with 5% isoflurane, using an automated anesthesia machine, which was continued throughout the procedure using 2.5% isoflurane introduced *via* a nose cone.

To prepare for tumor cell injection, MCF-7/Cox-2 Clone 10 cells and MCF/empty vector cells were trypsinized, counted using a hemocytometer and resuspended in Matrigel® (Fisher Scientific, Pittsburgh, PA, USA) at a concentration of $2.5x10^7$ cells/ml. Subsequently, 0.2 ml of cell suspension ($5x10^6$ cells) was injected into the right mammary fat pads of ovariectomized Crl:Nu-Foxn1^{nu} mice implanted subcutaneously with 17β-estradiol pellets. Tumors were monitored every other day, measured using digital calipers, weight and tumor sizes with recorded at those times. Tumors were isolated at 42 and 60 days post injection from ovariectomized Crl:Nu-Foxn1^{nu} mice. Upon removal of tumors, tumor volume was calculated using the equation: tumor volume = (length x width^2) / 2. Tumor samples were either fixed in 10% neutral buffered formalin or snap frozen in liquid nitrogen for further analysis.

Histology and immunohistochemistry of CD-31 (PECAM) and quantification of angiogenic blood vessel development in MCF-7/Cox-2 human breast tumor xenografts. Tumor samples fixed in 10% neutral buffered formalin were paraffin embedded and sectioned (3 μm) for either hematoxylin and eosin (H&E) staining for histological evaluation by a pathologist, or were used for immunohistochemical analysis of anti-CD-31 (PECAM) staining. Tissue sections were mounted onto SuperFrost Plus slides (Fisher Scientific), deparaffinized in Histo-Clear (National Diagnostics, Atlanta, GA, USA) and rehydrated through a gradient of descending alcohols. Endogenous peroxidase activity was blocked by incubating the sections in 3% hydrogen peroxide in methanol for 20 min. Tissue sections were then exposed to 10 mM citrate buffer (pH 6.0) in a steamer for antigen retrieval. Sections were blocked for 1 h at 25˚C in 1% bovine serum albumin (BSA, Sigma) in 1X Tris-buffered saline with 0.05% Tween 20 (TBST). The primary antibody, rabbit anti-human CD31 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), was then applied at 1:50 for 1 h at 25˚C. After incubation with primary antibody, slides were washed in TBST and biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA) was applied at 1:200 for 20 min at 25˚C. The peroxidase-based Vectastain Elite ABC kit (Vector Laboratories) was used and color was developed using the chromogen Vector VIP (Vector Laboratories). All sections were counterstained with pre-heated Methyl Green (Vector Laboratories), dehydrated and cover slips were mounted using Permount mounting medium (Fisher Scientific). Negative control sections were treated in an identical manner, with the exclusion of the primary antibody. Representative images were captured using a Zeiss Axioplan 2 imaging microscope and a Zeiss Axiocam digital camera. To obtain mean vessel density measurements for quantitation of CD-31 staining, measurements were performed by two separate individuals by counting vessels stained with anti-CD-31 antibodies within 10 random x40 fields on 4 separate tissue sections isolated from both MCF-7/Cox-2 Clone 10 and MCF-7/empty vector control tumors.

Protein isolation and Western blot analysis of human Cox-2 in regional lymph nodes adjacent to MCF-7/Cox-2 clone 10 breast tumor xenografts. Frozen tumor samples were homogenized in a tissue homogenizer and protein was extracted using the mirVana PARIS kit per manufacturer's instructions (Ambion, Austin, TX, USA). Protein concentration was determined using the BIO-RAD Protein Assay (Biorad, Hercules, CA, USA) with BSA as a control, and 40 mg of protein was separated by SDS-PAGE (10% gel). Protein was then transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA), and membranes were blocked in Blotto (1xPBS, 0.1% Tween 20, 5% nonfat dried milk, and 1% BSA) for 1 h at 25˚C. During this time, the monoclonal anti-human Cox-2 antibody (1:250, Cayman Chemical, Ann Arbor, MI, USA) and anti-mouse IgG-horseradish peroxidase (HRP) (1:5000, Amersham) were pre-incubated at 37˚C for 1 h. Membranes were then probed with the primary and secondary antibodies together overnight at 4˚C. Blots were developed using the ECL+plus Western blotting detection system (Amersham). The monoclonal Cox-2 antibody does not cross react with Cox-1, nor does it cross-react with murine Cox-2.

Results

In vivo development of MCF-7/Cox-2 clone 10 human breast tumors. Ovariectomized female Crl:Nu-Foxn1^{nu} mice were implanted with 17-β estradiol pellets and 24 h later their mammary fat pads were injected with $5x10^6$ MCF-7/Cox-2 Clone 10 human breast tumor cells contained in 200 μl Matrigel. At 15 days after tumor cell injection, palpable human breast tumors developed. At 60 days after injection of tumor cells, tumor volumes of MCF-7/Cox-2 Clone 10 human breast tumors were 4-fold greater ($p<0.01$) compared with tumor volumes of MCF-7/empy vector control tumors (371.62 mm^3±77.7 mm^3 compared to 86.99 mm^3±37.7 mm^3) (Figure 1).

Histopathology of MCF-7/Cox-2 clone 10 human breast tumors. Human breast tumors were isolated and stained with H&E and viewed using a light microscope to define the histology of the tumors. At 60 days post injection of $5x10^6$ tumor cells into mammary fat pads of ovariectomized Crl:Nu-Foxn1^{nu} mice, MCF-7/empty vector control tumor cells formed smaller human breast tumors than those formed

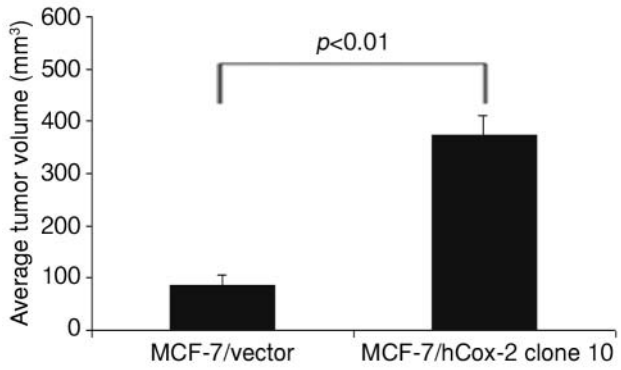


Figure 1. *Comparison of average tumor volumes of MCF-7/Cox-2 Clone 10 human breast tumors and MCF-7/empty vector breast tumors at 60 days after injection of 5x10^6 tumor cells into mammary fat pads of ovariectomized Crl:Nu-Foxn1 nu female mice.*

in the mice injected with MCF-7/MCF-7/Cox-2 Clone 10 human breast tumor cells, which was consistent with a 4-fold ($p<0.01$) significant difference in tumor volumes (Figure 1).

The human tumors formed at 60 days following injection of MCF-7/empty vector tumor cells were composed of organized nests of tumor cells within the Matrigel® matrix of the mammary fat pad into which the cells had been injected (Figure 2A). The white box shown in Figure 2A is the area in which the magnification of MCF-7/empty vector cells are shown at x40 magnification (Figure 2B). The small tumors formed at 60 days following injection of MCF-7/empty vector control cells contained cells that were apoptotic, with histological evidence of necrosis in the center of the tumors (Figure 2B, white arrows). At 60 days after injection of 5x10^6 human MCF-7/Cox-2 Clone 10 human breast tumor cells into mammary fat pads, histological examination revealed that the large MCF-7/Cox-2 Clone 10 human breast tumors were disorganized, with the presence of prominent nuclei and visible Indian filing (Figure 2C and D, white arrows). The MCF-7/Cox-2 Clone 10 human breast tumors formed at 60 days cells also had visible nuclear molding (Figure 2D white arrows) and a high nuclear:cytoplasmic ratio. The white box shown in Figure 2 C is the area in which the magnification of MCF-7/Cox-2 Clone 10 human breast tumor cells are shown at x40 magnification (Figure 2D). In contrast to the MCF-7/empty clone tumors, there was no visible Matrigel® matrix within the mammary fat pad of mice injected at 60 days with MCF-7/Cox-2 Clone 10 human breast tumors cells (Figure 2 A compared to Figure 2C).

Visible angiogenic blood vessels, CD-31 immunohistochemical staining and quantiation of mean vessel density in MCF-7/Cox-2 clone 10 breast tumors. Human breast tumor xenografts that developed at 60 days after injection of MCF-7/Cox-2 Clone 10 breast tumor cells into ovariectomized Crl:Nu-Foxn1^{nu} mice contained large and numerous blood vessels (Figure 3A) compared to the few and small blood vessels formed in ovariectomized Crl:Nu-Foxn1^{nu} mice injected with the MCF-7/vector control (data not shown).

Following immunohistochemical staining of tissue sections taken from both MCF-7/empty vector control tumors and MCF-7/Cox-2 Clone 10 human breast tumors with anti-CD31 antibodies (Figures 3B-E), quantification of staining was performed to provide a measure of microvessel density (MVD), which delineates the angiogenic blood vessels within the tumors. CD-31 staining has been reported to commonly be located at the periphery of tumors (29, 30), which is consistent with the findings of this present study (Figures 3B-E) for both MCF-7/empty vector tumors and MCF-7/Cox-2 Clone 10 human breast tumors. Based on mean vessel density, MCF-7/Cox-2 Clone 10 human breast tumors contained significantly greater numbers of blood vessels compared to tumors that developed at 60 days following injection of MCF-7/empty vector control tumor cells (Figure 3F; $p<0.01$).

Invasion of MCF-7/Cox-2 clone 10 human breast tumor into adjacent regional lymph nodes. At day 60, MCF-7/Cox-2 Clone 10 breast tumor cells were visible within adjacent regional lymph nodes, indicating that theMCF-7/Cox-2 Clone 10 breast tumor cells were invasive (Figure 4A and B). The white box shown in Figure 4A is the area in which the magnification of MCF-7Cox-2 Clone 10 human breast tumor cells are shown at 40 x magnification (Figure4 B).

The invasion of MCF-7/Cox-2 Clone 10 breast tumor cells into regional lymph nodes adjacent to mammary fat pads was confirmed not only by histological examination but also by Western blotting for Cox-2 protein in lymph node tissue (Figure 4C). In contrast, MCF-7/empty vector control cells were not visible in adjacent regional lymph nodes and there was no Cox-2 protein detected within lymph nodes adjacent to mammary fat pads where MCF-7/empty vector cells had been injected (Figure 4C).

Discussion

Our previous studies characterized the direct effects of the presence of human Cox-2 cDNA on *in vitro* biological characteristics of human breast tumor cells (25, 26). The MCF-7/Cox-2 clone 10 was generated by stable transfection of the MCF-7 human breast cancer cell line with human Cox-2 cDNA. The MCF-7/Cox-2 Clone 10 cell line proliferates at a 3-fold higher rate than Cox-2 null MCF-7/empty vector control tumor cells and expressed higher levels of mRNA of specific heparin binding proteins of the vascular endothelial growth factor (VEGF) family of pro-angiogenic growth factors (25)

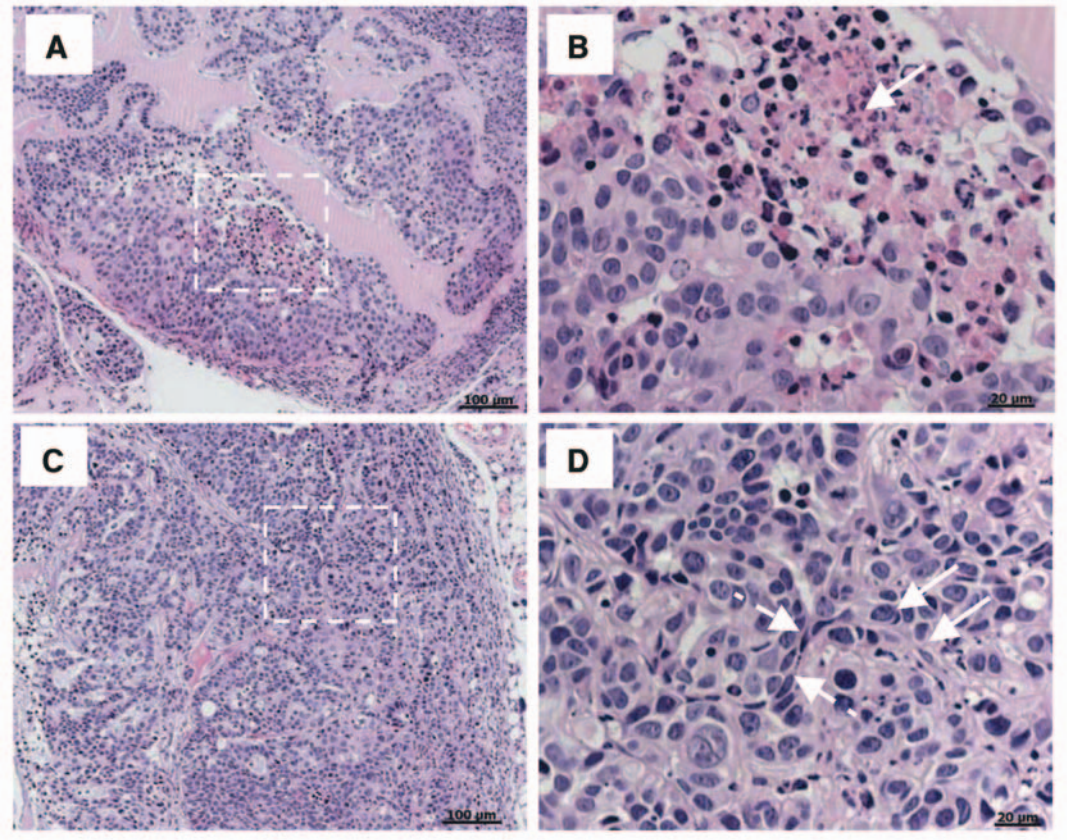
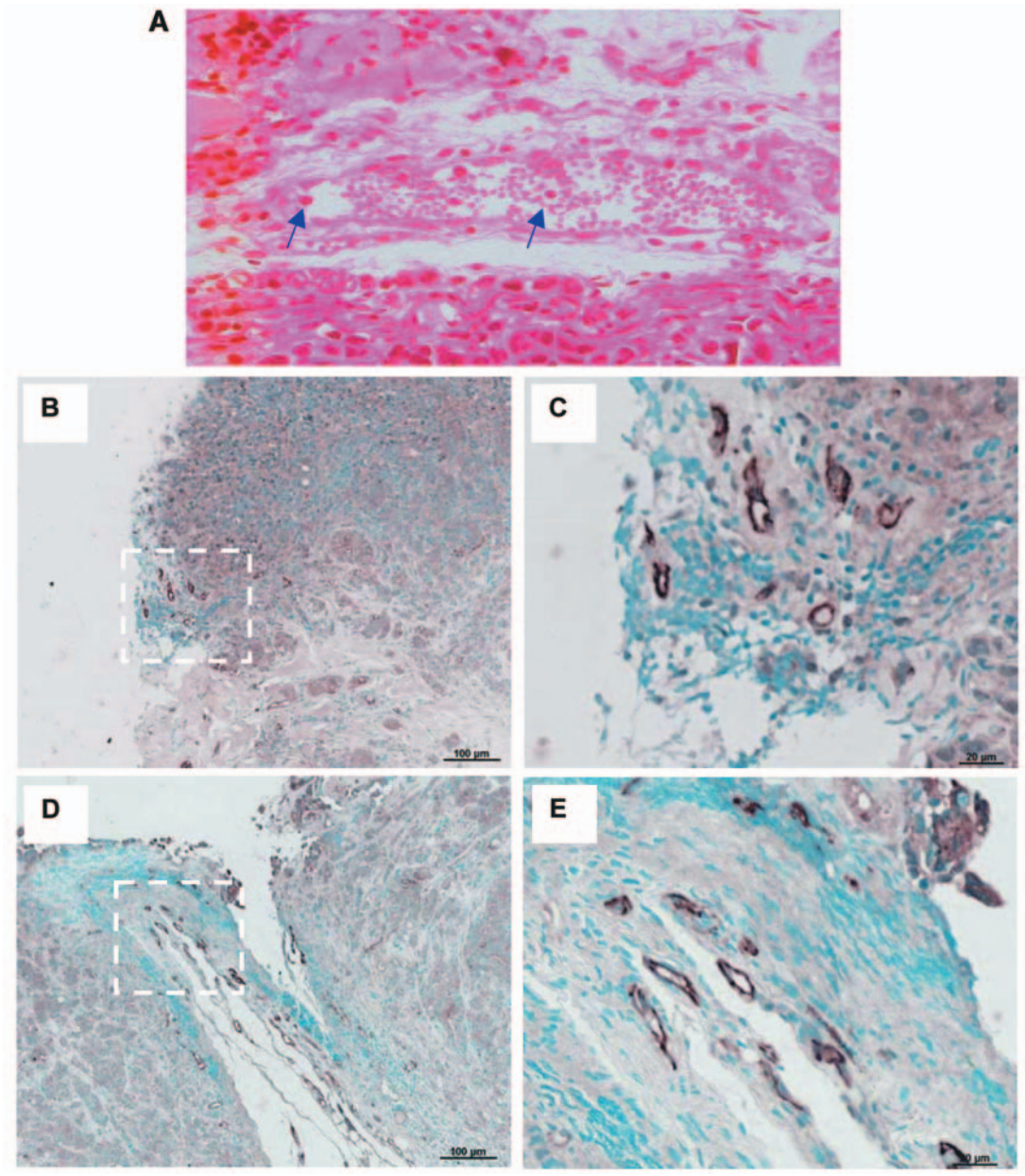


Figure 2. *Comparative histological analysis of human breast tumors formed within mammary fat pads of ovariectomized Crl:Nu-Foxn1^{nu} female mice at 60 days after injection of 5x10^{6} MCF-7/empty vector control cells (A and B) and 5x10^{6} MCF-7/Cox-2 Clone 10 human breast tumor cells (C and D).*

The present results describe the development of a unique *in vivo* model of human breast tumor xenografts which was developed to evaluate the direct *in vivo* biological activities of Cox-2 cDNA on the biological activities of breast tumor cells. Injection of MCF-7/Cox-2 Clone 10 human breast tumor cells into mammary fat pads of ovariectomized female Crl:Nu-Foxn1^{nu} mice that had been implanted into the subcapular area with slow release 17-β estradiol pellets rapidly formed angiogenic and invasive human breast tumors that histologically recapitulated ductal carcinoma breast tumors, and were invasive to adjacent regional lymph nodes. Formation of large, invasive breast tumors after injection of the MCF-7/Cox-2 Clone 10 human breast tumor cells demonstrates the critical role of Cox-2 in rapid development of human breast tumors, their very early local invasion into regional lymph nodes, and the formation of angiogenic blood vessels at very early times after MCF-7/Cox-2 Clone 10 human breast tumor cell injection into mammary fat pads. In addition, the presence of Cox-2 cDNA increased the volume of human breast tumors by 4-fold.

Histological evaluation of the breast tumors revealed that at 60 days after injection, MCF-7/Cox-2 Clone 10 human breast tumors were highly disorganized and had histological characteristics, such as Indian filing, that were indicative of a highly invasive phenotype. This is in comparison to the tumors formed after injection of the MCF-7/vector control cell line which remained organized and had necrotic cells wthin the central areas of the tumors with visible apoptotic cells. While the MCF-7/Cox-2 Clone 10 human tumors were visible outside the mammary fat pad, the MCF-7/empty vector cells remained within the Matrigel substrate in which they had been injected at all time periods examined (15-60 days). Although we evaluated proteins known to be involved in regulation of apoptosis, including bcl-2, bax and bad, we did not observe any differences between these two cell lines (data not shown). These results suggest that mechanisms other than those involving the bcl-2 family of proteins may be involved in providing the MCF-7/Cox-2 Clone 10 human breast tumors with the ability to resist undergoing apoptosis compared to the apoptosis observed in the MCF-7/empty vector tumors.



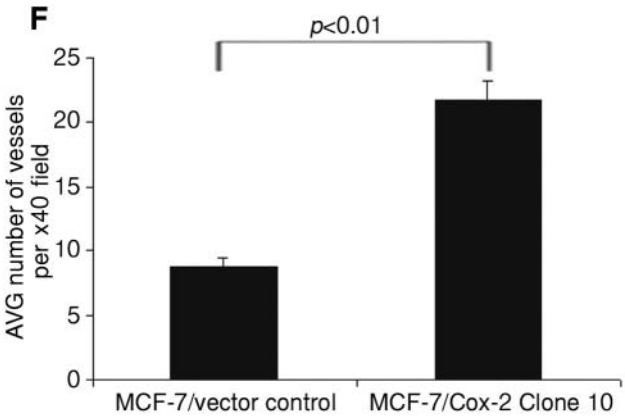


Figure 3. *A) Visible angiogenic blood vessel in MCF-7/Cox-2 Clone 10 breast tumor isolated at 60 days after injection of 5x106 tumor cells into mammary fat pads of ovariectomized Crl:Nu-Foxn1^{nu} female mice. B-E) CD31 staining of tissue sections of human breast tumors isolated from ovariectomized Crl:Nu-Foxn1^{nu} mice sacrificed at 60 days post-injection of either 5x10^{6} MCF-7/vector control cells (A and B) or MCF-7/Cox-2 Clone 10 cells (C and D). Magnification is x10 (A and C) and x40 (B and D). The white hatched boxes (A and C) denote the areas that are further magnified. F) Quantification of mean vessel density based on anti-CD31 stained blood vessels in MCF-7/Cox-2 Clone 10 human breast tumors compared to MCF-7/empty vector control tumors.*

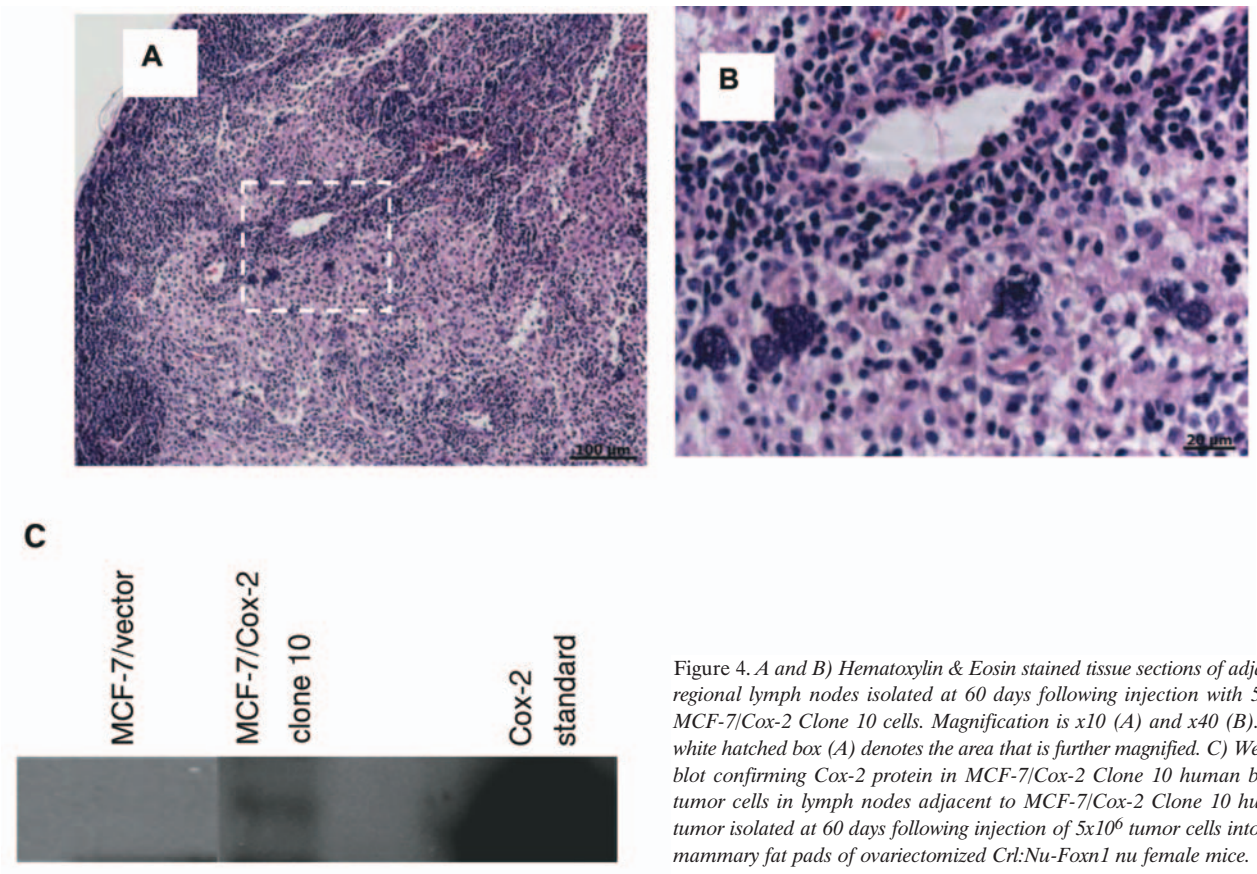


Figure 4. *A and B) Hematoxylin & Eosin stained tissue sections of adjacent regional lymph nodes isolated at 60 days following injection with 5x10^6 MCF-7/Cox-2 Clone 10 cells. Magnification is x10 (A) and x40 (B). The white hatched box (A) denotes the area that is further magnified. C) Western blot confirming Cox-2 protein in MCF-7/Cox-2 Clone 10 human breast tumor cells in lymph nodes adjacent to MCF-7/Cox-2 Clone 10 human tumor isolated at 60 days following injection of 5x10^6 tumor cells into into mammary fat pads of ovariectomized Crl:Nu-Foxn1 nu female mice.*

In addition to forming large human breast tumors, MCF-7/Cox-2 Clone 10 tumor cells also invaded the adjacent regional lymph nodes. The local invasion of MCF-7/Cox-2 Clone 10 tumor cells into adjacent lymph nodes observed in the present study is consistent with observations from our *in vitro* studies of the MCF-7/Cox-2 Clone 10 cell line which demonstrated that MCF-7/Cox-2 Clone 10 cells invaded across an artificial Matrigel® matrix basement membrane and formed large anchorage-independent colonies in soft agar (25).

The observations that the MCF-7/Cox-2 Clone 10 breast tumors *in vivo* are invasive are also consistent with reports from other laboratories which observed that the presence of Cox-2 in the human breast cancer cell line, Hs578T, resulted in production of elevated levels of proteolytic enzymes, which are known to degrade basement membrane components. These results suggest that Cox-2 stimulates enzymes that provide a pathway for tumor cell migration and invasion (28). Furthermore, using gene and tissue arrays combined with patient samples taken from non-small cell lung tumors and gastric tumors, Cox-2 expression has been associated with lymphovascular invasion of lymph nodes through activation of proteolytic enzymes such as the matrix metalloproteinases (MMP)-2 and MMP-9 (28, 30). Taken together, these observations suggest that the presence of Cox-2 appears to play a critical role in breast tumor cell migration and invasion, which was confirmed by the present *in vivo* studies.

The formation of new blood vessels is a requirement for the expansion of tumors beyond 1-2 mm in size. This process, defined as tumor-associated angiogenesis, is critical for a tumor to obtain adequate supplies of nutrients and oxygen as it develops and expands (31-33). One marker of new blood vessel formation is the protein CD31 or platelet-endothelial cell adhesion molecule-1 (PECAM-1), a member of the immunoglobulin superfamily. The present study used microvessel density as a representation of active tumor-associated angiogenesis based on quantification of the CD31 staining within human breast tumors formed by MCF-7/Cox-2 Clone 10 breast tumor cells and MCF-7/vector control cells. Previous studies have shown a positive association between Cox-2 and angiogenesis in invasive human breast tumors through the activation of VEGF pro-angiogenic growth factors or their associated VEGF receptors (22, 24), which is in agreement with the findings of our *in vitro* studies demonstrating that specific heparin binding proteins within

the family of VEGF A family of pro-angiogenic growth factors including $VEGF_{145}$, $VEGF_{165}$ and $VEGF_{189}$ are increased in the MCF-7/Cox-2 Clone 10 human breast tumor cells compared to the MCF-7/vector control cells (25).

Upon visual inspection of each tumor prior to its isolation, human breast tumors derived from injection of MCF-7/Cox-2 Clone 10 human breast tumor cells into ovariectomized Crl:Nu-Foxn1^{nu} mice had large vascular supplies surrounding each tumor. The significant number of angiogenic blood vessels that we observed in the MCF-7/Cox-2 human tumors is also consistent with our *in vitro* studies demonstrating the angiogenic phenotype of the MCF-7/Cox-2 Clone 10 human breast tumor cells (25).

In contrast to the rapid development of human breast tumors following injection of MCF-7/Cox-2 Clone 10 breast tumor cells, the MMTV-Cox-2 transgenic model system of mouse mammary tumor development requires successive rounds of pregnancy, lactation and weaning prior to the development of mammary tumors (20, 22). In this model, Cox-2 expression is under the control of the MMTV, which is hormonally regulated; these rounds of hormonal fluctuation are required for sufficient Cox-2 expression. Additionally, the mammary gland undergoes proliferation and apoptosis during pregnancy, lactation and the ductal involution that occurs during weaning, which may also contribute to the mammary tumor formation in MMTV-Cox-2 transgenic mice. Although the chemically induced rodent models of mammary carcinogenesis as well as the MMTV-Cox-2 transgenic mouse model of mammary tumorigenesis have been invaluable in identifying mechanisms and pathways involved in mammary carcinogenesis mediated by the presence of Cox-2, the present studies provide a unique human breast tumor model system with which to evaluate agents that may target development, rapid tumor proliferation and expansion, invasion, angiogenesis and estrogen production of human breast tumors. This model of human breast carcinoma, which rapidly recapitulates invasive and angiogenic human ductal carcinoma, may be useful for the evaluation of new agents such as prostaglandin (EP) antagonists which may replace selective Cox-2 inhibitors; it can also be used to evaluate aromatase inhibitors with a spectrum of activities which may function through specific aromatase promoter regions, as well as estrogen receptor antagonists. In addition, this unique model of human tumorigenesis may be uniquely suited to evaluating newly developing anti-angiogenic approaches for treatment of primary and metastatic human breast tumors as adjuvants to more conventional therapies. This human breast tumor model may also provide a means to evaluate very new approaches such as novel nanotechnology approaches that target both the breast tumor as well as the adjacent neovasculature (34). Since this model of human breast carcinoma is histologically closer to that observed in patients than the majority of mammary tumors, it may be useful for safety and efficacy preclinical studies of new agents under development for use as chemotherapeutic strategies, as adjuncts to conventional therapies, or as chemoprevention agents.

Acknowledgements

These studies were funded in part by NIH NCI U01-CO-12400 (MF).

References

1 Parrett ML, Harris RE, Joarder FS, Ross MS, Clausen KP and Robertson FM: Cyclooxygenase-2 gene expression in human breast cancer. Intl J Oncol *10*: 503-507, 1997.

2 Hla T, Bishop-Bailey D, Liu CH, Schaefers HJ and Trifan OC: Cyclooxygenase-1 and -2 isoenzymes. Int J Biochem Cell Biol *31(5)*: 551-557, 1999. Review.

3 Soslow RA, Dannenberg AJ, Rush D, Woerner BM, Khan KN, Masferrer J and Koki AT: COX-2 is expressed in human pulmonary, colonic, and mammary tumors. Cancer *89(12)*: 2637-2645, 2000.

4 Half E, Tang XM, Gwyn K, Sahin A, Wathen K and Sinicrope FA: Cyclooxygenase-2 expression in human breast cancers and adjacent ductal carcinoma *in situ*. Cancer Res *62(6)*: 1676-1681, 2002.

5 Ristimaki A, Sivula A, Lundin J, Lundin M, Salminen T, Haglund C, Joensuu H and Isola J: Prognostic significance of elevated cyclooxygenase-2 expression in breast cancer. Cancer Res *62(3)*: 632-635, 2002.

6 Parrett ML, Abou-Issa HM, Alshafie G, Ross MS, Harris RE and Robertson FM: Comparative ability of ibuprofen and *N*-(4-hydroxyphenyl)retinamide to inhibit development of rat mammary adenocarcinomas associated with differential inhibition of gene expression of cyclooxygenase isoforms. Anticancer Res *19(6B)*: 5079-5085, 1999.

7 Joarder FS, Abou-Issa H, Robertson FM, Parrett ML, Alshafie G and Harris RE: Growth arrest of DMBA-induced mammary carcinogenesis with ibuprofen treatment in female Sprague-Dawley rats. Oncol Reports *4*: 1271-1273, 1997.

8 Robertson FM, Parrett ML, Joarder FS, Ross M, Abou-Issa HM, Alshafie G and Harris RE: Ibuprofen-induced inhibition of cyclooxygenase isoform gene expression and regression of rat mammary carcinomas. Cancer Lett *122(1-2)*: 165-175, 1998.

9 Abbadessa G, Spaccamiglio A, Sartori ML, Nebbia C, Dacasto M, Di Carlo F and Racca S: The aspirin metabolite, salicylate, inhibits 7,12-dimethylbenz[a]anthracene-DNA adduct formation in breast cancer cells. Int J Oncol *28(5)*: 1131-1140, 2006.

10 Badawi AF, Eldeen MB, Liu Y, Ross EA and Badr MZ: Inhibition of rat mammary gland carcinogenesis by simultaneous targeting of cyclooxygenase-2 and peroxisome proliferator-activated receptor gamma. Cancer Res *64(3)*: 1181-1189, 2004. Retraction in: Cancer Res *65(17)*: 8057, 2005.

11 Kubatka P, Ahlers I, Ahlersova E, Adamekova E, Luk P, Bojkova B and Markova M: Chemoprevention of mammary carcinogenesis in female rats by rofecoxib. Cancer Lett *202(2)*: 131-136, 2003.

12 Jang TJ, Jung HG, Jung KH and MK O: Chemopreventive effect of celecoxib and expression of cyclooxygenase-1 and cyclooxygenase-2 on chemically-induced rat mammary tumours. Int J Exp Pathol *83(4)*: 173-182, 2002.
13 Singh-Ranger G and Mokbel K: The role of cyclooxygenase-2 (COX-2) in breast cancer, and implications of COX-2 inhibition. Eur J Surg Oncol *28(7)*: 729-737, 2002.
14 Kubatka P, Kalicka K, Chamilova M, Ahlersova E, Ahlers I, Bojkova B and Adamekova E: Nimesulide and melatonin in mammary carcinogenesis prevention in female Sprague-Dawley rats. Neoplasma *49(4)*: 255-259, 2002.
15 Abou-Issa HM, Alshafie GA, Seibert K, Koki AT, Masferrer JL and Harris RE: Dose-response effects of the COX-2 inhibitor, celecoxib, on the chemoprevention of mammary carcinogenesis. Anticancer Res *21(5)*: 3425-3432, 2001.
16 Nakatsugi S, Ohta T, Kawamori T, Mutoh M, Tanigawa T, Watanabe K, Sugie S, Sugimura T and Wakabayashi K: Chemoprevention by nimesulide, a selective cyclooxygenase-2 inhibitor, of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)-induced mammary gland carcinogenesis in rats. Jpn J Cancer Res *91(9)*: 886-892, 2000.
17 Badawi AF and Archer MC: Effect of hormonal status on the expression of the cyclooxygenase 1 and 2 genes and prostaglandin synthesis in rat mammary glands. Prostaglandins Other Lipid Mediat *56(2-3)*: 167-181, 1998.
18 Kundu N and Fulton AM: Selective cyclooxygenase (COX)-1 or COX-2 inhibitors control metastatic disease in a murine model of breast cancer. Cancer Res *62(8)*: 2343-2346, 2002.
19 Kundu N, Yang Q, Dorsey R and Fulton AM: Increased cyclooxygenase-2 (cox-2) expression and activity in a murine model of metastatic breast cancer. Int J Cancer *93(5)*: 681-686, 2001.
20 Liu CH, Chang SH, Narko K, Trifan OC, Wu MT, Smith E, Haudenschild C, Lane TF and Hla T: Overexpression of cyclooxygenase-2 is sufficient to induce tumorigenesis in transgenic mice. J Biol Chem *276(21)*: 18563-18569, 2001.
21 Narko K, Zweifel B, Trifan O, Ristimaki A, Lane TF and Hla T: COX-2 inhibitors and genetic background reduce mammary tumorigenesis in cyclooxygenase-2 transgenic mice. Prostaglandins Other Lipid Mediat *76(1-4)*: 86-94, 2005.
22 Chang SH, Liu CH, Conway R, Han DK, Nithipatikom K, Trifan OC, Lane TF and Hla T: Role of prostaglandin E2-dependent angiogenic switch in cyclooxygenase 2-induced breast cancer progression. Proc Natl Acad Sci USA *101(2)*: 591-596, 2004.
23 Subbaramaiah K, Howe LR, Port ER, Brogi E, Fishman J, Liu CH, Hla T, Hudis C and Dannenberg AJ: HER-2/neu status is a determinant of mammary aromatase activity *in vivo*: evidence for a cyclooxygenase-2-dependent mechanism. Cancer Res *66(10)*: 5504-5511, 2006.
24 Howe LR, Chang SH, Tolle KC, Dillon R, Young LJ, Cardiff RD, Newman RA, Yang P, Thaler HT, Muller WJ, Hudis C, Brown AM, Hla T, Subbaramaiah K and Dannenberg AJ: HER2/neu-induced mammary tumorigenesis and angiogenesis are reduced in cyclooxygenase-2 knockout mice. Cancer Res *65(21)*: 10113-10119, 2005.
25 Prosperi JR, Mallery SR, Kigerl KA, Erfurt AA and Robertson FM: Invasive and angiogenic phenotype of MCF-7 human breast tumor cells expressing human cyclooxygenase-2. Prostaglandins Other Lipid Mediat *73(3-4)*: 249-264, 2004.
26 Brueggemeier RW, Quinn AL, Parrett ML, Joarder FS, Harris RE and Robertson FM: Correlation of aromatase and cyclooxygenase gene expression in human breast cancer specimens. Cancer Lett *140(1-2)*: 27-35, 1999.
27 Prosperi JR and Robertson FM: Cyclooxygenase-2 directly regulates gene expression of P450 Cyp19 aromatase promoter regions pII, pI.3 and pI.7 and estradiol production in human breast tumor cells. Prostaglandins Other Lipid Mediat *81(1-2)*: 55-70, 2006.
28 Takahashi Y, Kawahara F, Noguchi M, Miwa K, Sato H, Seiki M, Inoue H, Tanabe T and Yoshimoto T: Activation of matrix metalloproteinase-2 in human breast cancer cells overexpressing cyclooxygenase-1 or -2. FEBS Lett *460(1)*: 145-148, 1999.
29 Dicken BJ, Graham K, Hamilton SM, Andrews S, Lai R, Listgarten J, Jhangri GS, Saunders LD, Damaraju S and Cass C: Lymphovascular invasion is associated with poor survival in gastric cancer: an application of gene-expression and tissue array techniques. Ann Surg *243(1)*: 64-73, 2006.
30 Byun JH, Lee MA, Roh SY, Shim BY, Hong SH, Ko YH, Ko SJ, Woo IS, Kang JH, Hong YS, Lee KS, Lee AW, Park GS and Lee KY: Association between cyclooxygenase-2 and matrix metalloproteinase-2 expression in non-small cell lung cancer. Jpn J Clin Oncol *36(5)*: 263-268, 2006.
31 Hanahan D, Christofori G, Naik P and Arbeit J: Transgenic mouse models of tumour angiogenesis: the angiogenic switch, its molecular controls, and prospects for preclinical therapeutic models. Eur J Cancer *32A(14)*: 2386-2393, 1996.
32 Parangi S, O'Reilly M, Christofori G, Holmgren L, Grosfeld J, Folkman J and Hanahan D: Antiangiogenic therapy of transgenic mice impairs *de novo* tumor growth. Proc Natl Acad Sci USA *93(5)*: 2002-2007, 1996.
33 Affara NI and Robertson FM: Vascular endothelial growth factor as a survival factor in tumor-associated angiogenesis. In Vivo *18(5)*: 525-542, 2004.
34 Robertson FM and Ferrari M: Rationale for nanotechnology in cancer therapy. *In*: Nanotechnology for Cancer Therapy. Amiji MA (ed.). CRC Press, Taylor & Francis, Boca Raton, FL, pp. 3-10, 2007.

Received January 17, 2007
Accepted February 5, 2007