Investigating the Cellular Mechanisms Promoting Tumorigenesis and an Inflammatory Microenvironment in *Tpl2* Knockout Mice

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Abstract

Chronic production or abnormal use of inflammatory factors is related to tumor initiation, development, and progression. One of the important molecular pathways involved in the regulation of inflammation is the MAPK cascade, which helps control mitosis, cell differentiation, and apoptosis. Early studies assumed *Tpl2,* a member of this pathway, to be an oncogene due to its role in inflammation. However, a 2010 study conducted by Dr. K. DeCicco-Skinner revealed the opposite: mice without the gene had a higher incidence of skin tumors and inflammation, suggesting that the gene plays a tumor suppressor role in the skin. This capstone hypothesized that in the absence of the MAPK signaling cascade, compensatory molecular pathways are activated which are at least partially responsible for heightened levels of tumorigenesis and inflammation observed in knockout mice. Western blotting and immunohistochemistry were used to identify differences in gene expression between wild-type mice and *Tpl2* knockout mice. Analysis revealed significant differences in cyclooxygenase-2 (COX-2) and COX-2 dependent prostanoids and prostanoid receptors. COXs are inflammatory enzymes which play an essential role in inflammation-related cancers; COX-2 in particular is often overexpressed in skin cancer. Further research into COX-2's exact interactions with *Tpl2* could reveal new methods to downregulate it, which may have implications for treatment options.

Introduction

The Hallmarks of Cancer

Cancer is a title given to an extremely diverse and complex group of diseases, with varied types, causes, behaviors, symptoms, and treatments. Cancer can be found in the brain, the blood, or the breast, arise due to genetics, chemical exposure, or simple bad luck; it can be highly debilitating or hardly noticeable, lethal or treatable. There are, however, a handful of characteristics shared between most, if not all, types which help scientists better understand and study the immense entity of cancer. In 2000, Hanahan and Weinberg of the University of California at San Francisco identified the six "hallmarks of cancer," as displayed in Figure 1. Opponents have argued that the list is a drastic oversimplification. Hanahan and Weinberg hold that it is a necessary starting point which promotes cohesion and the use of a common vocabulary in the world of oncology research. Indeed, it is a useful tool as long as it is noted that each and every cancer achieves malignancy via different cellular mechanisms, in different sequences, and to varying degrees (Hanahan & Weinberg, 2000).

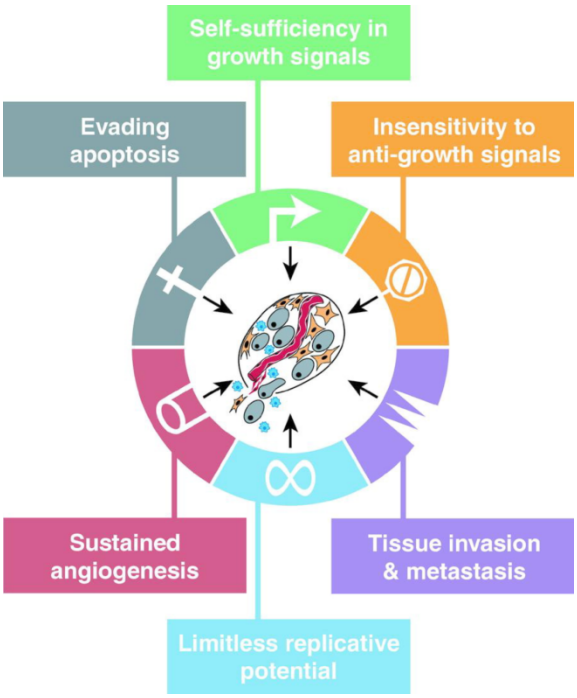

Figure 1: A representation of the six hallmarks of cancer, as proposed by Hanahan & Weinberg in 2000 in *Cell.* The six hallmarks, characteristics shared by most cancers, are: evasion of apoptosis, self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, limitless replicative potential, and sustained angiogenesis (order insignificant). The role of each in successful tumor development and progression has been heavily supported by research.

First, metastasis is the process of cancerous cells separating from the original tumor site and travelling throughout the body, often settling, congregating, and proliferating in new locations. Once a cancer enters the lymphatic system and invades other tissues it logically becomes more difficult to control and treat, as geographic isolation is no longer relevant. In fact, these distant settlements of cancerous cells, called metastases, are responsible for 90% of cancer deaths, and are one of the major indicators of malignancy (Sporn, 1996).

Second, angiogenesis is the process by which tumors establish their own network of blood vessels. Vascularization is normally a highly regulated and coordinated process in the body. Initially, tumor cells are avascular, lacking the ability to form new vessels and therefore remaining localized and relatively benign. The later vascular stage is when angiogenesis occurs, and it is at this point that malignancy usually becomes relevant. This phase allows for tumors to grow unhindered by the supply of blood, nutrients, and oxygen afforded solely by the body's capillary system (Ribatti & Crivelatto, 2011).

Third, cancerous cells exhibit an insensitivity to antigrowth signals. These include those signals which normally stimulate cells to enter a temporary G0 state, as well as those which force cells permanently out of the cell cycle so that they may achieve differentiation. Tumors work in complex ways at the molecular level to disrupt the pathways which respond to growth inhibitors, thereby allowing for the continuation of proliferative behavior even in the presence of such signals (Hanahan & Weinberg, 2000).

The forth hallmark is related: tumors have the ability to avoid apoptosis, or cell death, and thus can continue to proliferate despite receiving cell signals calling for self-destruction. The body's initial response to damaged DNA is to initiate DNA repair mechanisms. If these are unsuccessful, growth arrest is pursued. Then, apoptosis is utilized as a last resort to prevent the

expansion of potentially malignant cells (Evan & Littlewood, 1998). However, if mutated cells do not respond to cell death signals, they retain the ability to proliferate, and may eventually form tumors. The most widespread mechanism of apoptosis avoidance in cancerous cells is p53 inactivation via a mutation or deletion; this is the gene which normally initiates cell death in appropriate situations (Evan & Littlewood, 1998).

Instead, tumors achieve self-sufficiency in growth signals, which is the fifth hallmark. Normal tissue exhibits a dependence on external mitogenic signals, derived from either paracrine or endocrine means, to initiate growth. Conversely, cancerous cells are able to proliferate without them. Some simply alter incoming growth factor (GF) signals or the mechanisms by which those signals are transduced in the cell. Others produce GFs of their own, establishing a continuous positive feedback loop termed autocrine stimulation. Both methods limit the amount of external control exerted on the tumor and establish a dangerous degree of independence (Hanahan & Weinberg, 2000).

The sixth and final hallmark is a limitless replicative potential. In some ways, this can be considered the cumulative effect of the five previous characteristics. However, it also involves a unique mechanism not formerly discussed. Normal cells are only able to undergo a finite number of divisions due to the shortening of the sequences at the end of chromosomes, called telomeres, which occurs with each mitotic cycle. In contrast, cancerous cells have been shown to have the ability to overcome replicative senescence, and thus, to become immortal. Such behavior in tumor cells can be accounted for by the expression of telomerase, an enzyme which restores telomere length, as well as telomere stabilization via briefer original telomere sequences (Counter *et al*, 1992).

Though not included in the list of hallmarks, Hanahan and Weinberg also identified genomic instability as an "enabling characteristic" of cancer. Cancer is experienced as the result of a mutation, which can be inherited, caused by faulty DNA polymerase functioning during DNA replication, or triggered by any number of environmental factors (Hanahan & Weinberg, 2000; Hanahan & Weinberg, 2011). Normally, such mutations are detected and amended by the appropriate cellular machinery. The comparably few circumstances when they are not corrected allow for the six hallmarks to begin to manifest themselves, and for the multi-step process of tumorigenesis to get underway (Lengauer, Kinzler, and Vogelsteon, 1998).

Inflammation and Cancer Progression

As recently as 2009, a seventh hallmark of cancer was proposed and recognized by many oncology experts: inflammation (Colotta *et al*, 2009). In fact, in a 2011 review of their original paper, Hanahan and Weinberg themselves recognized it as an additional vital "enabling characteristic" of cancer (Hanahan & Weinberg, 2011). Regardless of the official label, an inflammatory microenvironment has inarguably been linked to successful initiation, promotion, and progression of tumorigenesis (Mueller, 2006). The connection between the two was actually first suggested as early as the nineteenth century, when Virchow observed tumors arising in chronically inflamed tissue and noted the presence of inflammatory cells in tumor samples, predicting for the first time that there was a functional relationship between inflammation and tumorigenesis (Mantovani *et al*, 2008; Mueller, 2006). However, the idea did not resurface until very recently, when a number of studies have produced a range of evidence confirming the link. Now, it is estimated that 15-20% of cancer deaths are directly related to infections and their inflammatory responses, and it is a generally accepted fact that inflammation does indeed

promote initial tumor growth as well as subsequent tumor development, by encouraging multiple aspects of malignancy (Mantovani *et al,* 2008).

Like many other processes involved in cancerous growth, inflammation occurs normally in the body and is necessary for healthy, everyday functioning (Cordon-Cardo & Prives, 1999). Only when it starts becoming unresponsive to the body's control mechanisms, or falls into the category of "chronic," does it become problematic and potentially dangerous. A healthy person experiences inflammation as a result of infection and mechanical injury. In both cases, the presence of inflammatory cells and inflammatory mediators, such as cytokines, prostaglandins, and chemokines, serve to initiate the appropriate immune system by recruiting macrophages and lymphocytes to the site of tissue damage (Cordon-Cardo & Prives, 1999).

Epithelial tissue, specifically, experiences constant mechanical stress and undergoes this process often. Physical or chemical injury immediately initiates the formation of a fibrin blood clot composed of extracellular matrix proteins and platelets to avert infection (Mueller, 2006; Coussens *et al*, 1999). Then, growth factors are released. They recruit inflammatory cells such as mast cells and neutrophils to the site, which in turn attract macrophages and lymphocytes. A complex set of events – the "inflammatory tissue response" – is orchestrated between these different cells and molecules, in order to promote angiogenesis and complete the healing and closure of the wound. Once this is achieved, the entire response is down-regulated; the numbers of inflammatory mediators and immune cells returns to their low, basal levels (Mueller, 2006; Coussens *et al*, 1999).

When these proinflammatory factors are produced excessively or chronically, tumor initiation and progression becomes a possibility (Lin & Karin, 2007). In such cases, the levels of inflammatory cytokines remain constitutively high, which in turn causes the continuous

recruitment of inflammatory and immune cell types to the area. The development of such a microenvironment is conducive to the maintenance of angiogenesis, metastasis, tissue invasion, and other malignant behaviors (Mueller, 2006; Coussens *et al*, 1999). The differences between the two responses – the normal inflammatory tissue response and the chronic inflammatory response leading to invasive carcinoma – are depicted in Figure 2 (Mueller, 2006).

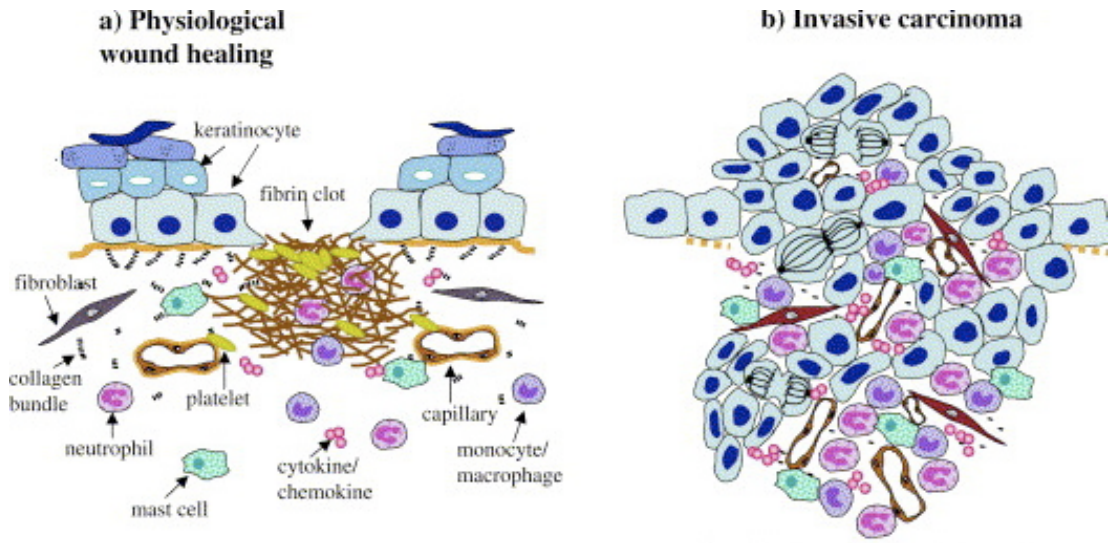

Figure 2: A depiction of the difference between (a) the highly-regulated normal cellular inflammatory response to mechanical injury or stress and (b) the disorganized chronic inflammatory state which may lead to invasive tumorigenesis. Graphic originally published by Mueller in 1999 in the *European Journal of Cancer.*

The evidence linking inflammation and cancer is plentiful and exists at both the whole organism and the molecular levels. Perhaps the simplest stream of evidence is the detection of inflammatory cells in the microenvironments of all tumors, in all experimental animals and human patients, throughout all stages of cancer development (Mantovani *et al*, 2008). In addition, it is known that patients suffering from chronic inflammatory diseases such as inflammatory bowel disease or prostatitis have a much higher risk of developing a related form of cancer in their lifetime. Such observations challenged oncologists to investigate further and

establish cause and effect relationships through controlled experimental research. They were certainly successful in doing so. Molecular pathways involved in inflammation have been shown to operate downstream of mutations linked to tumorigenesis, such as those in the genes coding for the proteins RAS, MYC, and RET. In addition, the selective targeting of molecules involved in inflammatory processes (including cytokines, chemokines, and relevant transcription factors like NF-κB) has been proven to decrease the incidence and limit the spread of cancer. It has also been demonstrated that transplanting inflammatory cells into specimens, or promoting the overexpression of inflammatory mediators, increases the rate of tumor development (Mantovani *et al*, 2008). Finally, the use of nonsteroidal anti-inflammatory drugs (NSAIDs) has been shown to reduce the incidence of multiple types of malignant growth, including colorectal cancer (Mantovani *et al*, 2008; Ricchi *et al*, 2003).

Squamous Cell Carcinoma

Skin cancer is the most common form of cancer in the United States, with over 3.5 million new cases diagnosed annually (Jemal & Siegel, 2011). One in five Americans will develop a form of skin cancer at some point in their lifetime, and there are more new cases of skin cancer every year than breast, prostate, colon, and lung cancers combined (Polsky & Wang, 2011). Non-melanoma skin cancer (NMSC) includes basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) and is the most common type of skin cancer in the United States, accounting for over 95% of skin cancer diagnoses (Jemal & Siegel, 2011). Nine out of ten cases of NMSC are directly linked to ultraviolet (UV) radiation exposure, which forms thymine dimers. These are pre-mutagenic lesions which will manifest as mutations if they are not repaired

by the appropriate cellular machinery in a timely and effective manner (Hawrot. Alam, & Ratner, 2003).

Of the more than 3 million new NMSC cases diagnosed every year, 80% are classified as BCC. As their name implies, these tumors grow in the basal layer, the innermost section of the epidermis, which in turn constitutes the outermost layer of the skin. They are rarely fatal, typically growing slowly and showing minimal signs of metastasis. SCC tumors, on the other hand, while accounting for just 20% of NMSC diagnoses, are often highly invasive and metastatic and, therefore, tend to be more fatal. They grow in the epidermis and result in approximately 2,500 deaths in the U.S. every year (Hawrot. Alam, & Ratner, 2003).

A SCC tumor results from the uncontrolled growth of keratinocytes, the cells making up the bulk of the epidermis,. Under normal conditions, keratinocytes regulate skin function through controlled proliferation and differentiation (Polsky & Wang, 2011). The interruption of these vital control mechanisms (via the six hallmarks of cancer) is what allows tumorigenesis to be initiated. The majority of SCC tumors arise in body areas experiencing sustained sunlight exposure, with the most common sites being the ears, head, and neck. The extremities are the next most affected site. Approximately 20% of tumors develop elsewhere, in less-exposed areas, such as in the trunk region, within mucus membranes, or on the genitals (Polsky & Wang, 2011).

Recent data linking skin type and squamous cell carcinoma development has further supported the marking of radiation exposure as the greatest risk factor for SCC tumorigenesis. A number of Scandinavian studies showed lighter-skinned individuals to have a significantly higher incidence rate than darker-skinned individuals (Hawrot. Alam, & Ratner, 2003). For example, non-Hispanic whites had an SCC tumor development rate of 11 times greater than Hispanics. Furthermore, persons living in geographic areas with high levels of UV radiation

were found to be at least three times more likely to develop SCC than persons residing farther from the equator (Hawrot. Alam, & Ratner, 2003). However, radiation is not the sole cause of squamous cell carcinoma. These tumors may also result from exposure to chemical agents, immunosupression, human papillomavirus, or chronically injured or inflamed areas of skin (Hawrot. Alam, & Ratner, 2003).

Squamous cell carcinomas often look similar to elevated warts, appearing scaly, red, and rough with a raised, irregular border (Polsky & Wang). They may also form an outer crust layer or turn into open, bleeding sores if irritated. When identified early by a physician, most SCC tumors can simply be excised; they tend to remain confined to the epidermis for some time. Topical medications and radiation, laser, and photodynamic therapies are other potential, though less popular, treatment options. The larger a tumor becomes, the more likely it is to metastasize, spreading through the lymph system to other tissues and organs. In such cases, treatment is not as straight-forward or successful. Metastasis occurs in about 2-10% of diagnoses, most of which are life-threatening (Polsky & Wang).

As is the case with many types of cancers, the incidence and mortality of squamous cell carcinoma in the United States is continually rising (Sporn, 1996). This is despite the few logical and relatively simple preventative measures recommended by health professionals: early detection, regular professional and self-examinations, a reduction of radiation exposure, and effective sun protection. Because of the proven success of these steps, many physicians have begun to promote dependence on proactive preventive measures such as these rather than on reactive cures once the disease has reached an advanced stage. The emphasis on the “cure for cancer” is complicated by the comparable complexity of the disease once it reaches these later stages. Not only is metastasis and heterogeneity of tumor cells more likely, but multiple drug

resistance and other survival mechanisms are often been developed by this point as well (Sporn, 1996). Nevertheless, finding a cure to squamous cell carcinoma and other cancers is central to modern oncology research. Gaining a complete understanding of the disease's mechanisms at the cellular level is the first step in achieving this lofty goal; the hope is that therapies can be aimed at earlier stages of the disease, when tumors tend to be more localized and homogeneous and less resistant.

The MAPK Transduction Cascade

One of the most important cellular pathways that takes place in inflammatory cells is the mitogen-activated protein kinase (MAPK) transduction cascade. The pathway can be initiated by extracellular signals, stress (including injury and inflammation), or cytokine presence, and works through a series of phosphorylation events to bring about a physiological response (Kumar, Boehm & Lee, 2003). Eventually, a product of the pathway reaches the nucleus and effects transcription. It is via this method that the MAPK pathway activates or inhibits target genes, controlling such vital functions as cell growth, mitosis, cell survival, cell differentiation, and apoptosis (Pearson *et al*, 2001).

The pathway, outlined in Figure 3 (Kumar, Boehm & Lee, 2003), consists of a family of Proline-directed Serine/Threonine kinases and includes three classes of protein kinases: a MAPK and two upstream components responsible for its phosphorylation, a MAPK kinase (MAPKK) and a MAPKK kinase (MAPKKK). To date, at least five separate MAPK pathways have been identified in mammalian cells. Extracellular regulating kinases (ERK1, 2, and 5) are MAPKs which tend to be activated by signals dealing with mitosis and proliferation. Environmental stressors (ultraviolet radiation, heat, osmotic pressure, and inflammatory cytokines), on the other

hand, are more likely to activate c-Jun N-terminal kinase 1 (JNK1) or p38α MAPK (Kumar, Boehm & Lee, 2003; Pearson *et al*, 2001). Different stimuli will activate slightly different pathways and elicit slightly different sets of cellular responses.

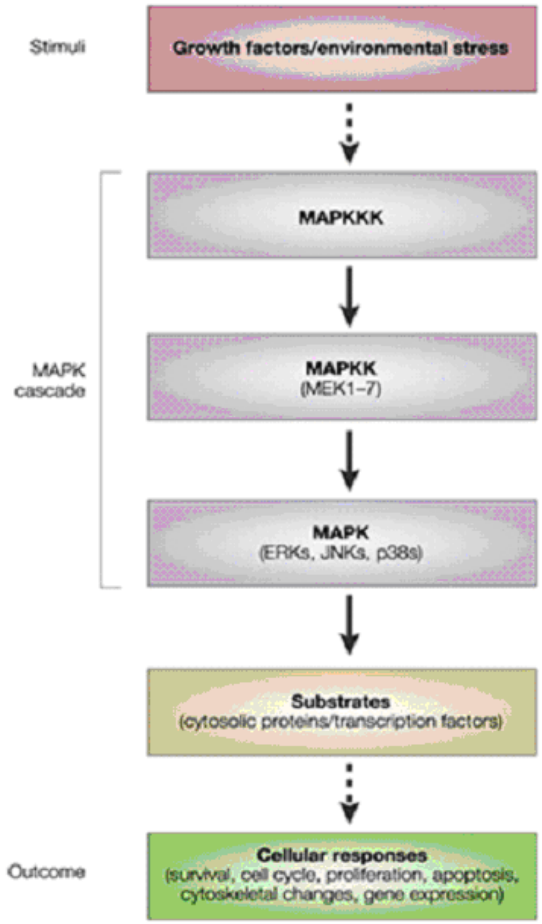

Figure 3: A graphic outlining the MAPK signal transduction cascade, from Kumar, Boehm & Lee, *Nature Reviews Drug Discovery,* 2003. The pathway is initiated by an environmental stressor, the presence of certain inflammatory molecules, or an extracellular signal. A series of phosphorylation events occur and the amplified signal reaches the nucleus, where it effects gene transcription and initiates a specific physiological response in the cell.

MAP3K8 is a member of the MAPK signal transduction cascade; its analog in mice is called *Tpl2* and in humans, *Cot*. MAP3K8 is, more specifically, a stress-activated MAPKK, or a MEKK (Kyriakis & Avruch, 2001). Because of the role of the entire MAPK pathway in tumor-promoting processes such as inflammation, cell survival, and cell proliferation, MAP3K8 was initially predicted to be an protooncogene (a protein promoting tumorigenesis). Early

experimentation seemed to support this claim (Kumar, Boehm & Lee, 2003). The inhibition of other members of the MAPK family was shown to down-regulate inflammatory factors such as interleukins (IL-1 and IL-6) and tumor-necrosis factors (TNFs), and induce other inflammatory molecules, including cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). MAPKs were found to promote the development of an inflammatory microenvironment; thus, it was predicted that they would also promote cancerous growth (Kumar, Boehm & Lee, 2003; Kyriakis & Avruch, 2001). In addition, MAP3K8 mutations specifically have been linked directly to a number of cancers, including lung cancer (Clark *et al*, 2004). However, activating mutations are rarely found and overexpression of MAP3K8 *in vivo* often doesn't result in tumor formation.

A 2010 study by Trovato & DeCicco-Skinner at American University and the National Institutes of Health discovered virtually the opposite in the context of squamous cell carcinoma. *Tpl2* knockout mice had a significantly greater rate of tumorigenesis than wild-type mice. Knockouts displayed a greater number of tumors per mouse, higher overall tumor incidence, decreased tumor latency, and higher levels of inflammation than wild-type mice. These results, some of which are summarized in Figures 4 and 5, suggested a tumor suppressor role for *Tpl2* in the skin (Trovato & DeCicco-Skinner, 2010).

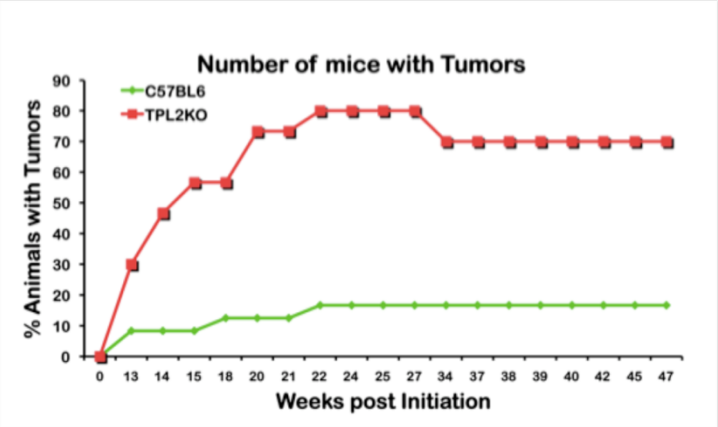

Figure 4: A sampling of the data from Trovato & DeCicco-Skinner, 2010. Tumors were initiated using 9,10-Dimethylbenz-A-Anthracene (DMBA), followed by biweekly applications of 12-O-tetradecanoyl-phorbol-13-acetate (TPA). Enhanced tumorigenesis was observed in the *Tpl2* knockouts.

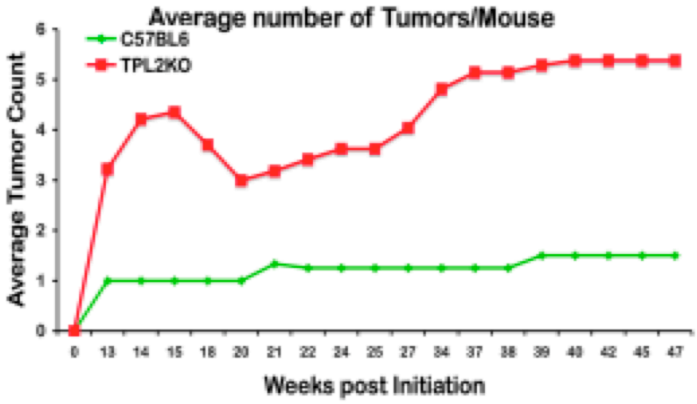

Figure 5: A sampling of data from Trovato & DeCicco-Skinner, 2010. Tumors were counted twice weekly. A greater number of tumors per mouse was observed in the *Tpl2* knockouts, compared to the wild-type mice.

It has been hypothesized that, in the absence of the *Tpl2* pathway, compensatory inflammatory pathways are activated in the cell, accounting for the heightened levels of inflammation and tumorigenesis observed in *Tpl2* knockout specimens (Trovato & DeCicco-Skinner, 2010). This is logical because, however important, the MAPK pathway does not operate in isolation. Crosstalk takes place between the MAPK transduction cascade and a broad array of other cellular pathways, including many involved in inflammatory processes in the cell. Understanding the complex interactions between these different pathways is necessary in order to comprehend exactly how and why these skin tumors arise at a cellular level in *Tpl2* knockout mice. Linkages established between pathways and a detailed understanding of the molecular mechanisms involved could also potentially be harnessed in the development of future treatment options.

Objectives

The purpose of this experiment was to investigate molecular pathways that could account for the heightened levels of tumorigenesis and inflammation observed in *Tpl2* knockout mice, compared to wild-type specimens. It was assumed that in the absence of the MAPK signal

transduction cascade, a number of other pathways are upregulated in the cell, and that these could help explain the observed effects. Immunohistochemistry (IHC) and western blotting analyses were used to compare the expression of various inflammatory and tumorigenic genes in wild-type and *Tpl2* knockout mice treated with the cancer-promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA) over various time points.

Materials & Methods

Immunohistochemistry Analysis

Immunohistochemistry was used to investigate *in vivo* expression of COX-2, EP2, and EP4 in the skin. Skin tissue sections from *Tpl2* knockout ($^{-/-}$) and C57BL/6 wildtype (WT) mice, which were bred and maintained at the NIH Animal Facility in Bathesda, MD in accordance NIH animal protocol guidelines, were fixed onto slides using paraffin. The slides were placed in HistoChoice twice for 3 minutes to dissolve the paraffin and the tissue section was rehydrated using 3 minute exposures to solutions of decreasing ethanol concentrations (100%, 90%, 70%, and 50%). The slides were washed in PBS for 5 minutes. Antigen retrieval was then performed. The solution was made using 20 mL of unmasking solution and 180 mL of deionized water. This solution was heated in a microwave for 2 minutes 45 seconds at maximum power. The slides were then placed inside and heated at power level 3 for 4 minutes. After cooling for 6 minutes, the slides were washed in PBS for 5 minutes. They were placed in a solution consisting of 196 mL of methanol and 4 mL of hydrogen peroxide (H_2O_2) for 20 minutes, then washed twice in PBS for 2 minutes. The H_2O_2 quenches endogenous peroxidase activity in the cells. The tissue sections were isolated from one another with a Liquid Blocker Super PAP pen. 150 μL of serum-free Protein Block was incubated for 20 minutes on each section in a humidity chamber at room

temperature. This binds non-specific antigen with "non-tagged" complement. Slides were then washed in PBS for 5 minutes. Primary antibodies for COX-2, EP2, or EP4 were appropriately diluted with 3% BSA-PBS and 150 μL was applied to each section. 150 μL of 3% BSA-PBS was used as a negative control. Incubation was completed overnight in a humidity chamber at 4 degrees.

The following day, the slides were washed with PBS. Donkey anti-Goat Biotinylated secondary antibody was diluted in 3% BSA-PBS, applied to the sections, and left to incubate for 40 minutes in a humidity chamber at room temperature. The slides were washed with PBS and ABC reagent from VectaStain (consisting of Avidin and Biotin) was placed on the sections for 30 minutes. This tightly binds to the secondary antibody, which is bound to the primary antibody, and thereby enhances staining. The slides were then washed with PBS and stained for 5-20 minutes with a staining solution from a peroxidase Substrate kit. Slides were placed in water to block the stain and then in Mayer's Hematocylin for 90 seconds to stain the nucleic acids. The slides were dipped in water multiple times to remove excess staining solution. They were washed with PBS and then placed in increasing concentrations of ethanol to dehydrate the stained sections. They were left to air dry for 30 minutes. A drop of permanent mounting medium was placed on each section, followed by a cover slip. Representative areas were photographed using a Nikon Eclipse E800 digital camera at 10x magnification.

Western Blotting Analysis

Western blotting was used to investigate if signal transduction cascade differences exist between *Tpl2*$^{-/-}$ and C57BL/6 WT mice. Primary keratinocytes were extracted from 1-3 day old newborn pups. This included sacrificing the pups, removing the skin, isolating the keratinocytes,

and plating them in 6-well tissue culture plates. When near 100% keratinocyte confluency was achieved, DMSO vehicle control or 12-O-tetradecanoyl-phorbol-13-acetate (TPA) treatment was given. TPA treatment was applied for varying time points (24 hour, 18 hour, 12 hour, 6 hour, 4 hour, 3 hour, 1 hour, or 30 minutes) at a concentration of 10 ng/mL. The cells were scraped off the plates, placed in a microtube, and rocked for 15 minutes to encourage cell lysis. The microtubes were then incubated on wet ice for 35 minutes and centrifuged at 13200 rpm at 4 degrees Celsius for 15 minutes. The supernatant contained the intracellular protein; it was transferred to a new microtube and stored at -80 degrees Celsius.

Extracted protein concentrations were measured using BCA Protein Assay Kits. The resulting concentrations were used to make protein samples for western blotting so that concentrations of each genotype and time point were equal. 20 μg of each sample was used for electrophoresis. Appropriate amounts of MPER buffer and loading dye were added to each sample. Complete samples were then heated at 70 degrees Celsius for 10 minutes. 4-12% Bis-Tris gels, SeeBlue Plus2 Prestained Standard marker, and NuPAGE MES SDS Running buffer were used for electrophoresis. The gels were run at 200 volts for 35 minutes. They were then transferred onto membranes using Invitrolon PVDF Filter Paper Sandwich by applying 30 volts for 80 minutes. The membranes were placed in 5% milk solution overnight at 4 degrees Celsius to ensure proper blocking.

For blotting, the membranes were first washed once with TBST for 10 minutes on a shaker. 10 mL of 5% milk solution and appropriate primary rabbit antibody concentration (1:400-1:1000) for COX-2, EP1, EP2, EP3, EP4, or Beta-Actin was applied to the membranes and allowed to incubate for 1 hour at room temperature on a rocker. The membranes were washed three times with TBST for 10 minutes on a shaker. 10 mL of 5% milk solution and anti-

rabbit HRP secondary antibody (1:2000) was added and incubated for 45 minutes. The membranes were again washed three times with TBST for 10 minutes on a shaker. The chemi-luminescent substrate SuperSignal West Dura Extended Duration Substrate was applied to the membranes for 5 minutes. The membranes were then visualized and photographed using a ChemiDok-It Imaging System, and subsequently stripped by incubation with Restore Western Blot Stripping Buffer for 30 minutes on a shaker. Membranes were placed in 5% milk solution after they were washed twice with TBST for 7-10 minutes on a shaker. The membranes were probed up to 6 times using different antibodies, including a Beta-Actin control the final time. Antibodies were probed on different membranes at least 3 times to ensure consistency of results.

Results

Tpl2 knockout ($^{-/-}$) mice have heightened expression of the inflammatory enzyme cyclooxygenase-2 (COX-2), compared to wild-type (WT) mice.

COX-2 is an important inflammatory enzyme activated by NF-κB activity. Western analysis revealed elevated levels of the enzyme in *Tpl2*$^{-/-}$ mice (Figure 6). Extracted keratinocytes were left untreated, treated with the tumor inducer TPA, or treated with 5, 10, or 20 μM solutions of the COX-2 inhibitor SN50. The controls (no SN50 treatment) revealed virtually undetectable COX-2 levels in WT mice, and heightened basal COX-2 levels in *Tpl2*$^{-/-}$ mice. Treatment with the tumor inducer TPA increased the expression of the enzyme in both genotypes. As the COX-2 inhibitor SN50 was applied in increasing concentrations, COX-2 expression in WT mice fell, revealing an effective inhibition process. In *Tpl2*$^{-/-}$ mice, on the other hand, COX-2 expression remained heightened despite SN50 exposure. This suggests that COX-2

expression in *Tpl2*$^{-/-}$ keratinocytes is elevated, rendering the inhibition process largely ineffective.

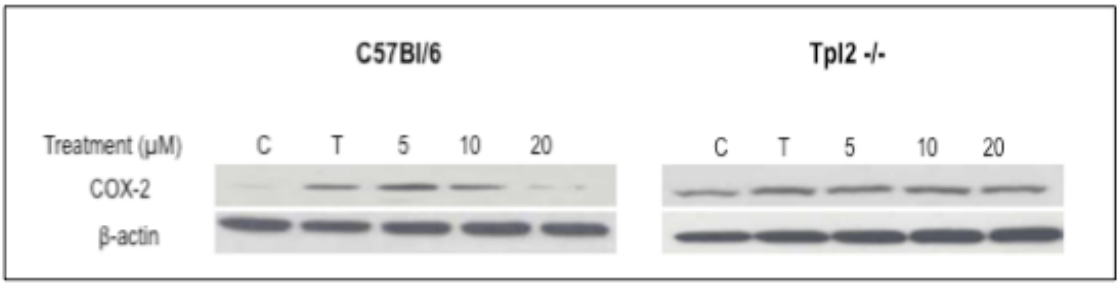

Figure 6: Western analysis of COX-2 expression between WT and *Tpl2*$^{-/-}$ keratinocytes, following treatment with the COX-2 inhibitor SN50. Beta-actin was used as a control.

Immunohistochemistry analysis further supported the observation of heightened COX-2 expression in the *Tpl2*$^{-/-}$ genotype (Figure 7). Skin sections were treated with TPA for 24 or 48 hours and then immunostained with COX-2 at a 1:200 dilution. The results revelaed elevated levels of the inflammatory enzyme in knockouts, compared to controls, which displayed very little staining.

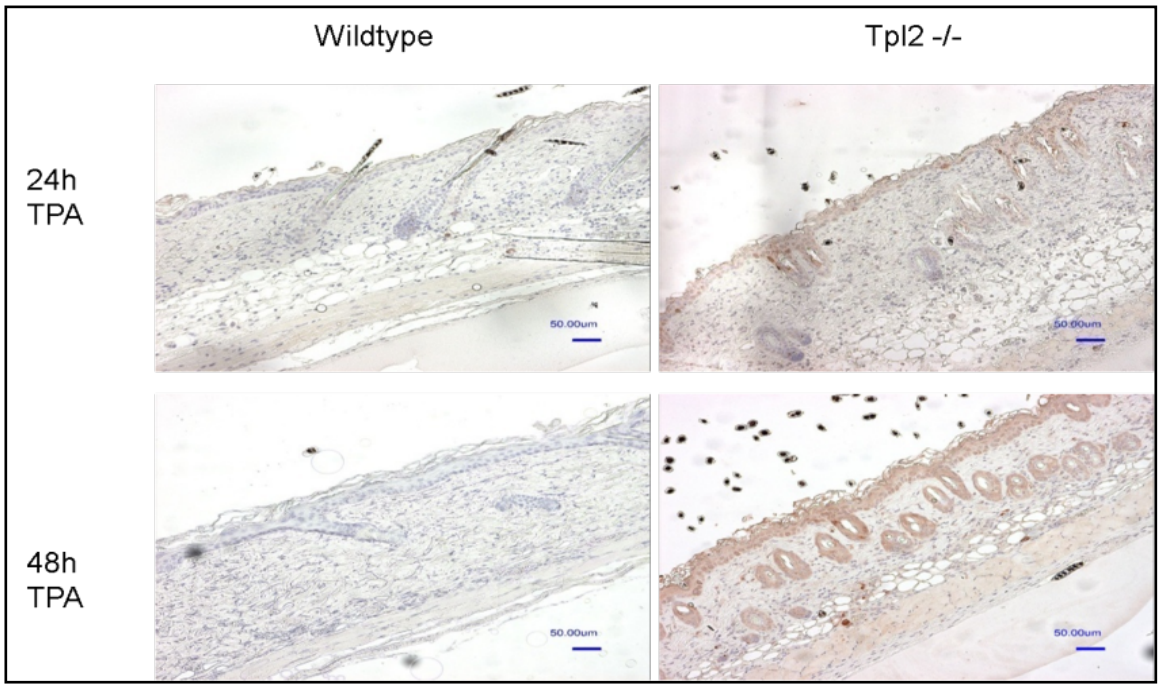

Figure 7: Immunohistochemistry analysis of COX-2 expression between WT and *Tpl2*$^{-/-}$ skin sections, following either 24 or 48 hour treatment with TPA. Magnification: 10x.

The PGE_2 nuclear EP receptors 1, 2, 3, and 4 are differentially expressed between *Tpl2*$^{-/-}$ and WT mice.

PGE_2 is an important product of the COX-2 pathway, and is the dominant molecule through which the pathway exerts its effects at the cellular level. This prostanoid binds to one of four nuclear receptors: EP1, EP2, EP3, or EP4.

Extracted keratinocytes were treated with TPA for 0, 1, 3, 6, 12, or 24 hours and analyzed via western blotting (Figure 8). Differential expression of the EP2, EP3, and EP4 receptors between the *Tpl2*$^{-/-}$ and WT genotypes was observed. No significant differences in EP1 levels were seen between the genotypes, which is agreeable with previous findings suggesting the irrelevance of EP1 in tumorigenic processes.

Basal expression of EP2 was four times greater and peaked earlier in TPA treatment in *Tpl2*$^{-/-}$ mice. Additionally, TPA treatment decreased EP4 expression in WT mice and increased it in *Tpl2*$^{-/-}$ mice. EP3 showed an expression pattern opposite that of EP4: as TPA treatment increased, EP3 expression increased in WT cells and decreased in *Tpl2*$^{-/-}$ cells.

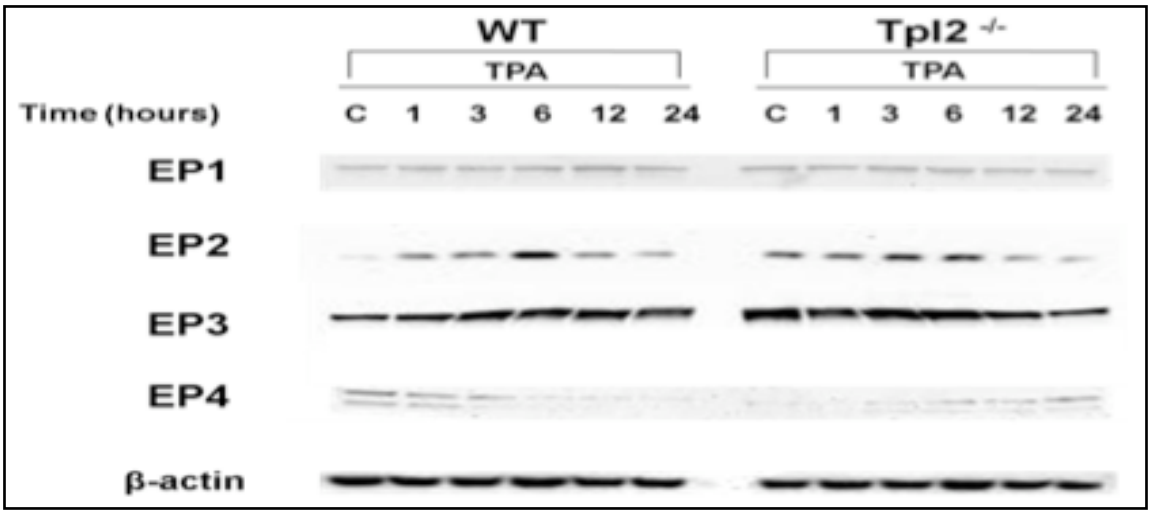

Figure 8: Western analysis of EP1, EP2, EP3, and EP4 expression between WT and *Tpl2*$^{-/-}$ keratinocytes, following treatment with TPA for 0, 1, 3, 6, 12, or 24 hours. Beta-actin was used as a control.

Tpl2$^{-/-}$ mice have heightened expression of the PGE_2 nuclear receptor EP2, compared to WT mice.

Western analysis results suggesting heightened EP2 expression in the *Tpl2*$^{-/-}$ genotype were further supported by immunohistochemistry analysis (Figure 9). Skin sections from *Tpl2*$^{-/-}$ or WT mice were treated with TPA for 0, 4, 8, 12, 24, or 48 hour periods and then immunostained with EP2 at a 1:200 dilution. EP2 expression was consistently higher in *Tpl2*$^{-/-}$ skin sections compared to WT skin sections at the basal level, as well as for all TPA treatment times. In WT mice, EP2 levels show a mild increase over 24 hours, falling off by 48 hours. Conversely, in *Tpl2*$^{-/-}$ mice, EP2 levels are noticably heightened within 4 hours of treatment and maintain this elevated state through 48 hours.

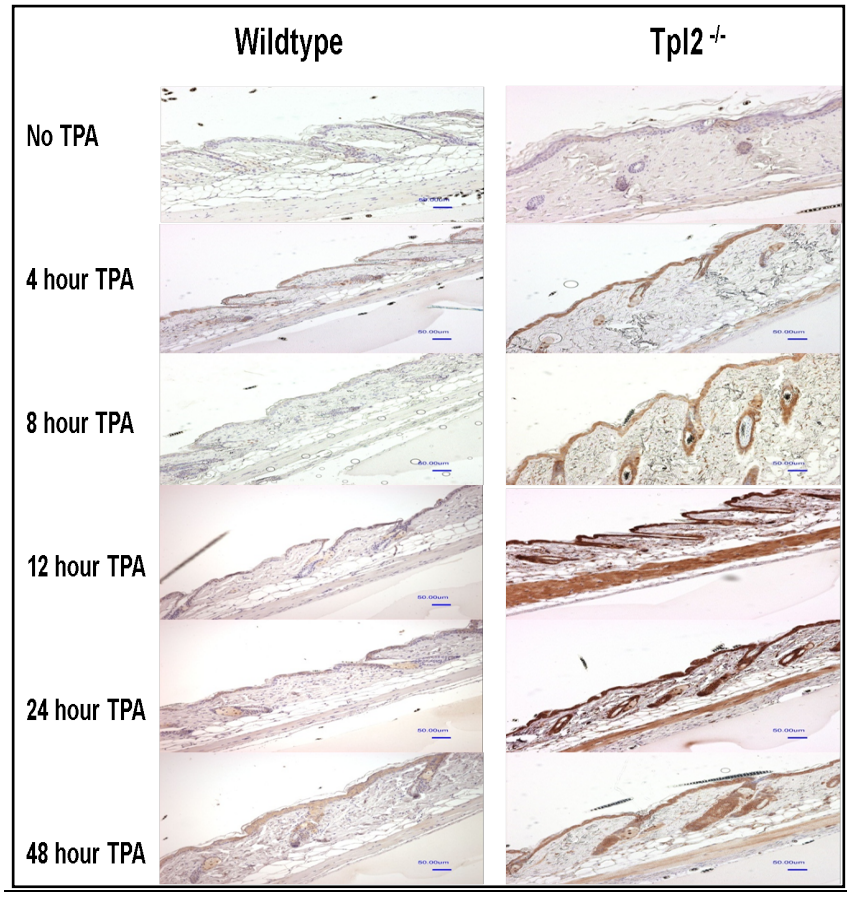

Figure 9: Immunohistochemistry analysis of EP2 expression between WT and *Tpl2*$^{-/-}$ skin sections, following treatment with TPA for 0, 4, 8, 12, 24, or 48 hours. Magnification: 10x.

Tpl2$^{-/-}$ mice have heightened expression of the PGE_2 nuclear receptor EP4, compared to WT mice.

Western analysis results suggesting differential EP4 expression in the *Tpl2*$^{-/-}$ genotype were further supported by immunohistochemistry analysis (Figure 10). Skin sections from *Tpl2*$^{-/-}$ or WT mice were treated with TPA for 0, 4, 8, 12, 24, or 48 hour periods and then immunostained with EP4 at a 1:200 dilution. In WT mice, EP4 expression is highest in controls and declines as TPA treatment is increased, reaching minimal levels by 8 hours. In *Tpl2*$^{-/-}$ mice, EP4 expression increases from its low basal levels in controls, up through 24 hours.

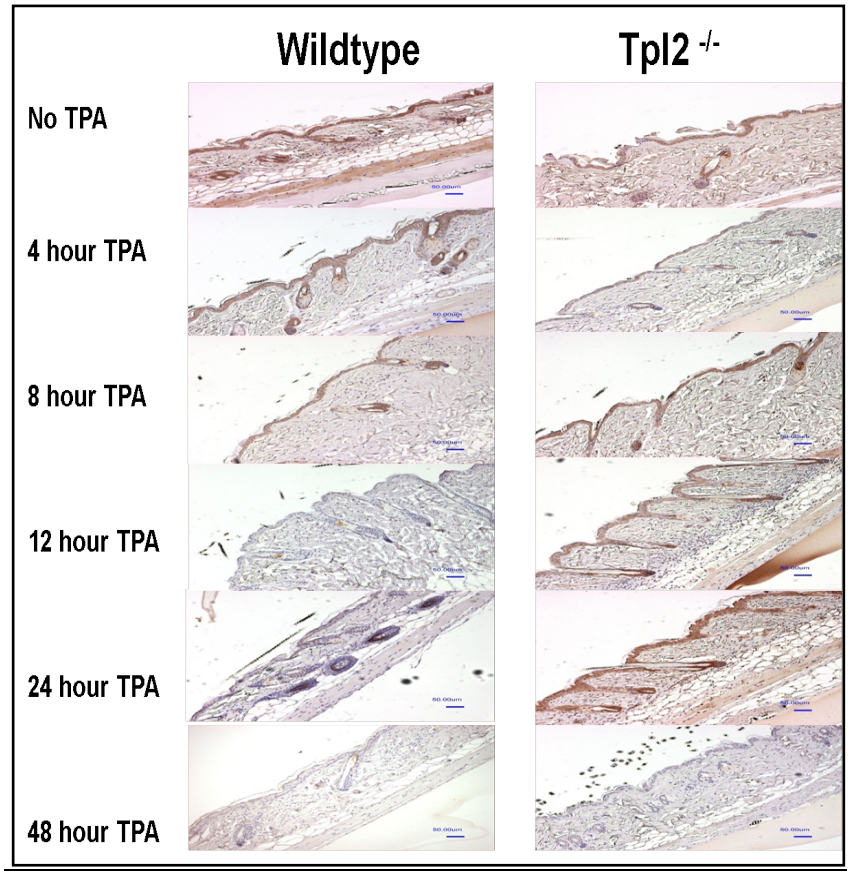

Figure 10: Immunohistochemistry analysis of EP4 expression between WT and *Tpl2*$^{-/-}$ skin sections, following treatment with TPA for 0, 4, 8, 12, 24, or 48 hours. Magnification: 10x.

Discussion

Cyclooxygenases (COXs) are important inflammatory enzymes. COX-2 has been of particular interest in oncology and is activated by NF-kB, a transcription factor which is normally rendered inactive in the cell by its association with *Tpl2* (Smith *et al*, 1996). Thus, the removal of *Tpl2* would cause the release of active NF-kB, which could then activate COX-2. COX-2 levels are virtually undetectable in healthy tissue but their overexpression has been observed in a variety of cancer types, including prostate, colon, lung, breast, cervix, pancreas, skin, intestine, and stomach. The enzyme has been shown to be activated by injury and aids in inflammation while also inhibiting apoptosis, stimulating angiogenesis and tissue invasion, and participating in peroxidase reactions which convert pro-carcinogens to carcinogens (Tsatsanis *et al,* 2006; Futugami *et al*, 2002). Previous research has established a direct causal relationship between COX-2 expression and tumorigenesis in the skin. Tumor development has been recorded in COX-2 transgenic mice even without treatment with a tumor-promoter such as TPA. Conversely, deleting, inhibiting, or otherwise down-regulating COX-2 in mice has been correlated with a 75% drop in the development and progression of chemically or UV-induced skin tumors (Sung *et al*, 2006; Kagoura, 2001).

The molecular pathway through which COXs have effects at the cellular level is outlined in Figure 11. COXs catalyze the conversion of arachadonic acid (AA) to the intermediate product prostaglandin H_2 (PGH_2). PGH_2 can then be converted to a number of biologically active prostanoids, one of which is PGE_2. PGE_2 manifests its biological activity by binding to one of four different transmembrane G-protein coupled receptors known as EP1, EP2, EP3, and EP4. EP2 and EP4 are both coupled to andenylate cyclase (AC), the enzyme which catalyzes the conversion of ATP to cyclic AMP (cAMP), a molecule which in turn binds to protein kinase A

(PKA), NF-kB, or the transcription factor CREB. The activation of these cAMP-controlled genes has been linked to the growth of carcinomas as well as angionesis and the avoidance of apoptosis (Smith *et al*, 1996; Abramovitch, R. *et al*, 2004).

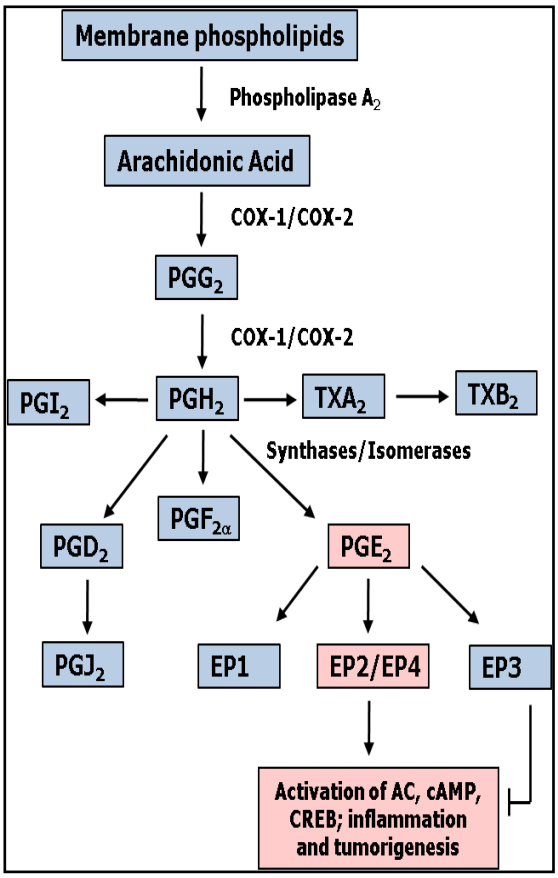

Figure 11: The Prostaglandin Pathway.

This study found that TPA-treated skin and TPA-treated keratinocytes from *Tpl2*$^{-/-}$ mice have increased expression of COX-2 and its downstream signalling pathway members, including the EP2 and EP4 receptors. E. Trovato and K. DeCicco-Skinner's 2010 study found heightened levels of inflammation and tumorigenesis in *Tpl2*$^{-/-}$ mice, and these results offer a possible explanation of their observations, at the molecular level. As expected due to its tumorigenic and inflammation-promoting capacity, COX-2 expression was shown to be elevated in *Tpl2*$^{-/-}$ mice. Western analysis showed greater basal expression levels in knockouts, as well as a largely

inefficient inhibition of expression by SN50, whose inhibitory effects were readily observable in WT controls. Additionally, immunohistochemistry of TPA-treated skin sections confirmed the observation of elevated COX-2 in the *Tpl2*$^{-/-}$ genotype.

A number of previous studies have established an association between EP2 expression and cancer promotion in the skin. EP2-transgenic mice have enhanced skin tumor development, survival, proliferation, angiogenesis, and inflammation, and ablating EP2 causes a drop in inflammation and skin tumorigenesis levels (Sung, He & Fischer, 2005; Sung *et al*, 2006). EP2 knockout mice develop fewer tumors, have reduced cellular proliferation in skin keratinocytes, and have a thinner epidermis, fewer blood vessels, and a large reduction in the number of inflammatory cells compared to wildtype controls (Sung, He & Fischer, 2005; Chun *et al*, 2009). Lastly, EP2 has been shown to induce expression of the cell survival protein survivin, and thus to avoid UV-induced apoptosis (Chun & Langenbach, 2011).

In this experiment, higher levels of EP2 expression in *Tpl2*$^{-/-}$ mice was observed. Western analysis revealed basal expression of EP2 that was four times greater and peaked earlier in TPA treatment in *Tpl2*$^{-/-}$ mice. Immunohistochemistry confirmed this differential expression. Basal EP2 levels were higher in *Tpl2*$^{-/-}$ skin sections, and this difference was maintained for all TPA treatment times. EP2 levels showed a slight increase in WT mice over 24 hours, and fell off to nearly indetectable levels by 48 hours. Knockout EP2 expression rose more dramatically; it was noticably heightened within 4 hours and reamained elevated through 48 hours.

EP4 has also been linked to tumorigenesis, with one study finding moderate EP4 expression in papillomas and squamous cell carcinomas, compared to virtually undetectable EP4 levels in both untreated and UV-irradiated skin sections (Lee *et al*, 2005). Here, western analysis showed that expression was heightened in *Tpl2* knockout mice, though the patterns of this

expression differed from that of EP2. Western analysis showed that TPA treatment decreased EP4 expression in WT mice but increased it in *Tpl2*$^{-/-}$ mice. These opposing trends between the genotypes were also observed in immunohistochemistry analysis. In WT specimens, EP4 expression was highest in controls, declined as TPA treatment increased, and was virtually undetectable by 8 hours. In contrast, in *Tpl2*$^{-/-}$ mice, EP4 expression consistently increased from low basal levels in controls, up through 24 hours.

EP3 showed an expression pattern almost exactly opposite that of EP4: as TPA treatment increased, EP3 expression increased in WT cells and decreased in *Tpl2*$^{-/-}$ cells. This agrees with previous investigations into general EP3 expression and actions in the cell, both of which have been shown to be opposite of EP2 and EP4. EP3 receptors are linked to G_i receptors and therefore typically work to inhibit, rather than encourage, cAMP production (Abramovitch, R *et al,* 2004; Klein *et al*, 2007; Sung, He & Fischer, 2005).

The links between tumorigenesis and many of the molecules investigated here (including *Tpl2*$^{-/-}$, COX-2, EP2, and EP4) were previously established and have been heavily supported with varied data. However, this study offers the first evidence of an explicit relationship between the *Tpl2*$^{-/-}$ and COX-2 signalling pathways. We propose that activation of the COX-2 pathway, inducing elevated COX-2, EP2, and EP4 and reduced EP3, contributes to the heightened levels of inflammation and tumorigenesis in the absence of the *Tpl2*$^{-/-}$ pathway. This may be due to the rise in cAMP production expected to accompany the upregulation of the EP2 and EP4 receptors.

A number of COX-2 selective inhibitors are currently offered as chemopreventative agents. However, there are possible health concerns associated with these pharmaceuticals, such as long-term use causing a predisposition to adverse cardiovascular symptoms, that have yet to be corrected (Danjani & Islam, 2008). Future research should aim to further expand the

understanding of the molecular mechanisms underlying the interaction between the *Tpl2* and COX-2 pathways. This information could prove to be useful in the development of future skin cancer treatment options. For example, the COX-2 pathway may be able to be more efficiently and safely down-regulated by modulating its interactions with the *Tpl2*$^{-/-}$ pathway.

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