Published in final edited form as:

Exp Toxicol Pathol. 2014 August ; 66(0): 235–242. doi:10.1016/j.etp.2014.02.003.

Characterization of azoxymethane-induced colon tumor metastasis to lung in a mouse model relevant to human sporadic colorectal cancer and evaluation of grape seed extract efficacy

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Abstract

The second leading cause of cancer-related deaths (both genders combined) in the United States is colorectal cancer (CRC). This emphasizes the need to develop both effective therapies for CRC patients and pre-clinical models mimicking human disease that carry translational potential in drug-development. Notably, at present there are no *in situ* models of CRC metastasis to lung. In our azoxymethane-induced colon tumorigenesis study in A/J mice assessing grape seed extract (GSE) efficacy, during necropsy we also found multiple lung nodules suggestive of colon tumor metastasis to lung that were significantly inhibited in GSE fed group. Both histopathological and molecular studies were performed to characterize and establish the origin of these lesions in lung. Histologically these nodules were determined as adenocarcinoma of mucin origin. Molecular analyses by immunohistochemistry (IHC) and RT-PCR revealed strong protein and transcript levels of colon specific markers CDX2 and CK20 in these lung nodules compared to uninvolved control lung tissue. Vis-à-vis, these nodules also showed minimally expressed lung specific biomarkers, specifically surfactant D and TTF-1, in IHC analysis. Additionally, 0.25% GSE supplementation in diet (w/w) decreased the incidence of these lung nodules by 53% and their total number by 66%. Together, the characterization of this unique *in situ* mouse model of CRC metastasis to lung provides translational opportunities in developing effective therapies to clinically manage and treat CRC at the advanced stage. Moreover, GSE efficacy in inhibiting CRC metastasis to lung in this model further supports its translational potential in controlling CRC growth, progression and metastasis in patients.

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Disclosure of potential conflicts of interest: No potential conflicts of interest were identified by any authors of this manuscript.

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Keywords

colorectal cancer; lung metastasis; biomarkers; chemoprevention; grape seed extract

1. Introduction

In most industrialized countries, cancer is the major cause of disease-related deaths; the majority of these deaths are a result of cancer metastatic growth (Taketo, 2011). Furthermore, colorectal cancer (CRC) is the second leading cause of cancer-related deaths (both genders combined) in the United States, and is expected to result in 50,830 deaths during 2013 alone (<http://seer.cancer.gov/csr>); most (~75%) of these CRC cases develop sporadically (Migliore et al., 2011). At the time of diagnosis, clinical and pathological staging of CRC is closely related to survival rate. For example, according to the United States Department of Health & Human Services, the overall 5-year relative survival rate of CRC confined to the bowel wall is 90% (<http://seer.cancer.gov/csr>), and a further progression of CRC to the lymph nodes decreases the 5-year survival rate to 70 % (<http://seer.cancer.gov/csr>). If distant metastasis occurs, the survival rate decreases to 12.5% (<http://seer.cancer.gov/csr>); common organ sites of clinical CRC metastasis, include liver, lung, peritoneum and ovaries (Van Loon and Venook, 2012). The tissue specific markers have been important tools utilized by diagnostic pathologists to determine metastatic tumor origin. For example, caudal type homeobox 2 (CDX2) is a highly sensitive marker of primary and metastatic colorectal adenocarcinomas, exhibiting 100% accuracy of intestinal tumors (Werling et al., 2003). Similarly, the biomarkers such as thyroid transcription factor-1 (TTF-1) are specifically expressed with in the lung, thyroid, and brain tissues (Boggaram, 2009).

Traditional treatment for CRC includes adjuvant chemotherapeutic agents and surgery, depending on the location of metastatic growth; however, the current chemotherapeutic agents induce toxicity, which also includes the risk of death (Ohe, 2002). Moreover, current chemotherapeutic agents are not capable of increasing long-term patient survival or possess the ability to cure metastatic disease. With such limitations in mind, it is evident that there is a clinical need to develop non-toxic, effective, long-term treatment regimens to control CRC growth, progression and metastasis to distal organs. In this regard, an additional approach to the current chemotherapeutic agents would include natural products that have limited or no toxicity and could be administered for long-term to prevent, suppress or reverse colon carcinogenesis (Rajamanickam and Agarwal, 2008). Correspondingly, there are multiple reports indicating that one-third of all cancer deaths in the US could be prevented through high consumption of fruits and vegetables or their bioactive components (Kaur et al., 2009, Kelloff et al., 1996, Rajamanickam and Agarwal, 2008). Natural agents such as grape seed extract (GSE) have been shown to be bioavailable, safe and efficacious during clinical trials against multiple cancer types; additionally GSE exhibits significant efficacy against CRC in pre-clinical mouse models (Eich et al., 2012, Kaur et al., 2009, Velmurugan et al., 2010a, Velmurugan et al., 2010b).

Scientific progression in the field of cancer molecular biology and genetics has fostered the development of effective patient therapies, which are attributed to the development of pre-

clinical animal models that mimic the human disease condition. However, the pre-clinical research field has not yet developed an efficient and effective model of carcinogen-induced colon tumorigenesis where colon tumors metastasize to lung; notably, this CRC metastatic tumor formation in lung accounts for 10–20% of the diagnosed patient population (Van Loon and Venook, 2012). Additionally, this lung metastasis patient population might not have the option of surgery, and thus, further decreasing the probability of patient survival. With this in mind, the aim of this study was to characterize lung nodules found in A/J mice following azoxymethane (AOM) exposure and establish their origin as colon tumors. Rodent experimental models of colon carcinogenesis have been utilized for over 80 years; the use of rodent models has a number of advantages to study the pathogenesis of CRC, including model reproducibility, access to different genetic backgrounds, and that these models recapitulate human cancer development (Rosenberg et al., 2009). To mimic human sporadic CRC development, there are a variety of carcinogens that induce colon tumors; however, AOM-induced colon carcinogenesis takes the advantage of the organotropism of the carcinogen where AOM specifically induces tumors in the colon and small intestine with highest incidence in the distal region of the colon (Rosenberg, et al., 2009). Notably, AOM-induced colon tumorigenesis model is the most commonly used pre-clinical animal model of human sporadic CRC (Rosenberg et al., 2009); however, previous studies have not reported the metastasis of colon tumors to the lung in this model. Additionally, investigating GSE efficacy in an *in situ* pre-clinical animal model of CRC metastasis is highly desirable, which combined with the vast pre-clinical GSE efficacy studies in various models of colon carcinogenesis, would help provide much needed evidence to initiate clinical trials investigating GSE efficacy against human CRC growth, progression and metastasis to distal organs including lung.

2. Material and Methods

2.1. Reagents

GSE-standardized preparation was a gift from Kikkoman Corp. (Nado City, Japan). The preparation composition is as follows: 89.3% procyanidins, 6.6% monomeric flavanols, 2.24% moisture content, 1.06% of protein, and 0.8% of ash, as reported recently (Derry et al., 2013, Velmurugan et al, 2010a, 2010b). Purchased antibodies include anti-CDX2, anti-CK20, anti-Surfactant D and anti-TTF-1 (all from Abcam). Anti-mouse and anti-rabbit horseradish peroxidase (HRP) secondary antibodies were from Invitrogen (Carlsbad, CA) and Cell Signaling Technology (Beverly, MA). RNA was isolated *via* the Qiagen RNeasy Kit, amplified *via* the Qiagen RT² RNA qPCR kit. Additionally, RNA transcript was quantified *via* specific RNA TaqMan primers for *Cdx2* from Life technologies (Grand Island, NY) and primers for mouse *Ck20* from Invitrogen.

2.2 Animals and treatments

Male A/J mice were purchased from Jackson Laboratory, and experiments were done with an approved protocol by IACUC (Derry et al., 2013). AOM was purchased from Sigma (St. Louis, MO) and dissolved in saline. GSE was mixed in AIN-76A powder diet at 0.25% (w/w). Animals, maintained under standard conditions with free access to water and food (AIN-76A powder diet), were divided in to 3 groups, and treated as: (1) untreated control

group (n=20), (2) AOM (positive) group (n=35), injected with 5 mg/kg dose of AOM i.p. once a week for 6 weeks, and (3) AOM+0.25% GSE (n=35), GSE containing diet feeding started 2 weeks post last AOM injection and continued till end of the study (28 weeks). Body weight and diet consumption were recorded weekly. At 43 weeks of age mice were sacrificed, entire lung was excised, gently flushed with ice-cold PBS, gross lung nodules counted and fixed flat in formalin, and were either embedded in paraffin for pathological and immunohistochemical (IHC) studies or frozen in liquid nitrogen for protein isolation, or stored in Qiagen RNeasy (Valencia, CA). Additionally, colon and small intestine tumors were also documented and stored as published recently (Derry et al., 2013).

2.3. Pathological and Immunohistochemical (IHC) Analyses

Fishers' Exact test was used to compare incidence/ number/ type of pathological lesions in different groups. The significance level was set at $P = 0.05$ for all tests. Paraffin-embedded sections (5 μ m) of lung tissue including nodules were subjected to standard H&E staining as well as IHC analysis as described previously (Derry et al., 2013, Velmurugan et al., 2010a). Primary anti-body dilution in each case was 1:100. Negative staining controls were used for each protein. Microscopic analyses were performed using Zeiss Axioscope 2 microscope; photomicrographs captured by Carl Zeiss AxioCam MrC5 camera with Axiovision Rel 4.5 software. All IHC data are shown as mean $\pm$ standard error mean (SEM) and are representative of 10 fields/section of 4 different mice per group. Statistically significant difference between untreated control, AOM and AOM+GSE groups was analyzed using one-way ANOVA (Sigma Stat 2.03, Jandel Scientific, San Rafael, CA). P values of 0.05 were considered significant.

2.4. RNA isolation and Real Time-RT-PCR (qRT-PCR)

Total RNA was isolated (from 20mg tissue in each case) employing Qiagen RNeasy Kit, as per vendor's protocol, and RNA concentration was determined with a NanoDrop 2000 (Thermo Scientific). Next, Qiagen RT² RNA qPCR kit was used following manufacturer's protocol and the First Strand cDNA Synthesis Reaction was stored at -20°C . In qRT-PCR, the commercially available and pre-validated TaqMan primer/probe set used was: *Cdx2* (*CDX2*; *Mm01212280_m1*). The primer set for *Ck20* was designed 5'-GCGTTTATGGGGGTGCTGGAG-3' (F) and 5'-AAGGCTTGGGCGGTGCGTCTC-3' (R). mRNA levels of triplicate samples from each group were measured by real-time quantitative reverse transcription-PCR using ABI PRISM 7700 at the Molecular Biology Shared Resources of the University of Colorado Cancer Center. Quantities of specific mRNA in each sample were normalized to the corresponding 18S rRNA levels to determine overall transcriptional modification.

3. Results

3.1. AOM-induced colon carcinogenesis also results in lung nodules formation, and dietary GSE feeding inhibits them

A previous group had shown 29% and 35% incidences of lung and liver tumors, respectively, in ICR mouse strain after 30 weeks of AOM injection (Fukutake et al., 1998). However, the authors neither characterized the origin of these tumors in lung and liver, nor

they evaluated whether these are colon tumors metastasized to lung and/or liver. During our AOM dose standardization study for colon tumorigenesis in A/J mice, we observed upon necropsy that, in addition to colon and small intestine tumors, 4 out of 6 mice also exhibited lung and liver nodules after 30 weeks post-last dose of AOM treatment (data not shown). Based on this unexpected finding, as detailed in Methods, we designed a long-term protocol to identify and characterize the origin of these lung nodules and to evaluate GSE efficacy towards them. At the end of the study, upon necropsy, compared to untreated controls, lung nodules were visible by the naked eyes in the AOM-treated group of mice (Figure 1A). In terms of an overall incidence, 11 out of 35 mice developed lung nodules in AOM alone group, accounting for a 31% incidence rate; however, only 5 out of 34 mice (15% incidence) displayed these lung nodules in AOM+GSE-fed group. Whereas these findings show that GSE feeding was able to inhibit lung nodule incidence by ~50%, the Fishers' Exact test used to compare lung nodule incidence did not show any statistically significant difference between AOM versus AOM+GSE-fed groups (Figure 1B). Regarding multiplicity, a total of 15 lung nodules were observed in AOM alone group of mice compared to only 5 lung nodules in AOM+GSE-fed group, accounting for a 67% decrease in total lung nodule number by GSE (Figure 1C). Also importantly, the lung nodule size in AOM+GSE group was <1mm in all cases compared to AOM alone where 9 out of 15 lung nodules were in 1–2 or 2–3 mm range (Figure 1D). Regarding origin and histology of lung nodules, upon AOM injections, lung nodule formation occurred most commonly on the right superior portion and the left inferior regions of the lung lobes. Pathological examination of control and AOM-treated lung tissue stained *via* H&E exhibited characteristics of adenocarcinoma formation, which originated from mucin cellular origin (Figure 1E). All the mice were also monitored throughout the study on a regular basis to investigate if dietary GSE had any negative impact; our primary measure of toxicity was body weight gain profile that was recorded weekly and did not differ significantly among the controls and GSE-fed group (data not shown).

3.2. Identification of colon tissue biomarkers within AOM-exposed lung nodules

Utilizing biomarkers that are specifically expressed in the colon tissue, we next aimed to identify and characterize the origin of the lung nodules that form upon AOM injection in A/J mice. Colon and lung tissues including colon tumors and lung nodules from untreated control and AOM-exposed groups were analyzed *via* IHC for the expression of a colon specific transcription factor, CDX2. As a positive control for CDX2 expression, as anticipated, there was a high level of CDX2 expression within the mucosal region of the untreated control colon tissue (data not shown). Conversely, normal lung tissue from untreated control mice showed a minimal CDX2 staining *via* IHC (Figures 2A and 2B); however, the lung tissue from AOM-treated mice exhibited an increased expression of CDX2 colon specific biomarker (Figure 2). To further quantify the levels of CDX2, we separated the lung tissue from AOM-treated mice into two groups, lung nodules and the tissue adjacent to lung nodules. Quantification of CDX2 levels revealed that lung nodules from AOM treated mice have ~7-fold higher CDX2 levels compared to normal lung tissue, and also importantly, the CDX2 level in the tissues adjacent to lung nodules from AOM-treated mice were more close to untreated normal lung tissue (Figures 2A and 2B); similar tissue samples from AOM+GSE group of mice showed comparable findings (Figure 2B).

These IHC results were further confirmed at the transcript levels, where RNA from colon and lung tissues from untreated control and AOM-treated mice was analyzed *via* qRT-PCR to detect *Cdx2* mRNA expression in these tissues. As anticipated, *Cdx2* mRNA expression was abundantly detectable in both normal colon mucosal tissue from untreated control as well as colon tumors from AOM-treated mice (Figure 2C); average *Cdx2* mRNA levels ranged from 320–395pg/ng 18s in these colon tissues (data not shown). However, normal lung tissue from untreated control mice and the lung tissue adjacent to lung nodules from AOM-treated mice exhibited not detectable to only minimal expression of *Cdx2* mRNA in (Figure 2D). Importantly, the lung nodules from AOM-treated mice showed ~14-fold higher *Cdx2* mRNA expression when compared to surrounding lung tissue (Figure 2D). This result, although statistically insignificant, is biologically significant as it suggests the presence of colon biomarker within these metastatic lung nodules, observed in A/J mice following AOM exposure.

To further confirm this AOM-induced lung metastatic CRC mouse model, we examined an additional colon biomarker, CK20; a cytokeratin that is expressed in epithelial cells and is present in virtually all cases of colorectal carcinomas (Chu et al., 2000). Similar to CDX2 data, IHC analysis revealed an increased CK20 protein expression in lung nodules from AOM exposed mice compared to normal lung tissue from untreated control mice (Figure 3A); both normal colon and colon tumors also showed high levels of CK20 (data not shown). Quantification of IHC staining clearly showed strong levels of CK20 in the lung nodules from AOM-treated mice compared to normal lung tissue from untreated control (~27-fold higher levels); the lung tissue adjacent to lung nodules from AOM-treated mice also showed relatively higher CK20 protein expression compared to normal lung, but it was much lower than that in the lung nodules (Figure 3B). Once again, the IHC data were further confirmed at the transcript levels, where RNA was extracted from control and AOM-treated colon and lung tissues and analyzed *via* qRT-PCR to detect *Ck20* mRNA expression in these tissues. *Ck20* mRNA expression was high in both normal colon mucosal tissues from control and colon tumors from AOM-treated animals (Figure 3C); average *Ck20* mRNA levels ranged from 469–569 pg/ng 18s in these colon tissues (data not shown). However, as expected, normal lung tissue from untreated control mice showed minimal expression of *Ck20* mRNA but there was a dramatically high *Ck20* mRNA expression in the lung nodules from AOM exposed mice (Figure 3D); importantly, a strong expression of *Ck20* mRNA, though less than that in lung nodules, was also observed in surrounding lung tissue (Figure 3D). Once again, although statistically insignificant, these findings were biologically significant as they further suggest the presence of colon biomarkers within these metastatic lung nodules/tumors originating from primary colon tumors in AOM exposed A/J mice.

3.3. Investigation of lung tissue biomarkers within AOM-exposed lung nodules

After confirming the presence of colon biomarkers in lung nodules/tumors in AOM exposed A/J mice, we also employed a reverse strategy to additionally investigate and confirm the presence (or lack) of lung tissue origin markers in the lung nodules from AOM-treated mice compared to normal lung tissue from untreated control mice. Our focus was to utilize the biomarkers that are expressed in the lung tissue and not in the colon tissue, thus we could further confirm the origin of the observed lung nodules in AOM-treated A/J mice. First we

selected Surfactant D (SP-D), which is expressed in type II cells within the lung tissue and is further up regulated in spontaneous adenocarcinoma (Zhang et al., 2003). Colon and lung tissues from untreated control and AOM-treated group were analyzed *via* IHC for the expression of the lung specific protein, SP-D. As a negative control for SP-D expression, we did not observe any SP-D levels in colon tissue samples from both control and AOM exposed mice (data not shown); however, normal lung tissue from untreated control mice showed a high expression of SP-D which was at a minimal level in the lung nodules from AOM-treated mice (Figure 4A). Interestingly, the lung tissue adjacent to lung nodules from AOM exposed mice showed 2-fold higher levels of SP-D compared to normal lung tissue (Figure 4A, right panel). Similar tissue samples from AOM+GSE group of mice showed comparable findings (Figure 4A, right panel).

With the above results in mind and further considering the fact that SP-D protein is important against host-defense, and therefore, could be up regulated in the lung tissues adjacent to lung nodules in AOM exposed mice due to increased inflammation, we aimed to investigate an additional lung origin biomarker, specifically TTF-1 (Zhang et al., 2003), to further confirm this AOM-induced lung metastatic CRC mouse model. TTF-1, also known as Nkx2.1, is a thyroid transcription factor that is essential to lung tissue formation and has been shown to be up regulated in lung adenocarcinoma (Kaufmann and Dietel, 2000, Kimura et al., 1996). IHC staining revealed high expression of TTF-1 in normal lung tissue from untreated control mice as well as in lung tissue adjacent to lung nodules from AOM-treated mice; however, TTF-1 expression was substantially reduced in the lung nodules from AOM-treated mice (Figure 4B). Furthermore, similar tissue samples from AOM+GSE group of mice showed comparable findings (Figure 4B, right panel).

4. Discussion

The present study aimed to identify and characterize lung metastasis of AOM-induced colon tumors in mouse model; this model is unique in that lung metastatic tumor formation occurs *in-situ* originating from primary colon tumors upon AOM-injection. Currently, there are no pre-clinical models of carcinogen-induced lung metastatic tumor growth originating from primary tumor growth in the colon; typical CRC metastasis animal models involve complicated surgical techniques utilizing immune compromised animals and tissue transplantation to study advanced CRC disease progression. For example, a model of CRC lymphatic metastasis involved nude mice that were injected subcutaneously with human HT-29 CRC cells on the side flanks of the animal; the resulting tumor was then removed and further surgically implanted into the cecum of the second generation of mice, which resulted in CRC adenocarcinoma formation and lymphatic metastasis (Sun et al., 2012). The hepatic metastatic CRC animal models utilize severe immune compromised mice and surgical splenic injection of human SW620 CRC cells; this initial surgery is then followed by a splenectomy (Xu et al., 2010). Another orthotopic hepatic model involves laparotomy to inject SW480 human CRC cells into the cecum of the animal that results in liver tumor formation after 8 weeks (Ding et al., 2011). Furthermore, additional orthotopic liver and lung metastasis models also include surgery and injection or implantation of human tissue into immune compromised animals at various tissue sites including colon and rectum, portal vein, intraperitoneal cavity, and the tail vein (Donigan et al., 2010, Ishihara et al., 2010,

Naomoto et al., 1987, Sirovich et al., 1999, Sturm et al., 2003). With the above information in mind, all the current pre-clinical models of metastatic CRC progression are costly, technically challenged, time-consuming, and do not represent an *in situ* model of human CRC metastasis. The model described in our study involves simple technical procedures and results in primary colon adenocarcinomas and their lung metastatic tumor formation; recapitulating the human disease progression in a fully immune competent A/J mice.

First evidence that supported this model as an AOM-induced model of lung metastatic CRC development was based on the histological properties of the lung tumors. The AOM-induced metastatic lung tumors formed on the exterior portion of the lung lobes; the location of these tumors suggested a potential lymphangitic spread, indicating that AOM-induced colon tumors had progression to lymphatic infiltration. While lymphatic infiltration can be identified by specific tumor marker proteins in lymphatic system, the non availability of such tissues limited our further investigation. However, we assume that if the metastatic spread had been arterial, we would have also observed tumor formation around the pulmonary arteries toward the center of the lung lobes. Moreover, we did not observe any spontaneous lung tumor formation in the untreated control animals; this observation suggests the observed lung tumors were a result of the AOM-induced colon tumorigenesis. We do realize the possibility that due to AOM exposure, we potentially could have induced metaplasia within the pulmonary tissue; metaplasia is the transformation of one tissue type to an alternative cell phenotype. Clinically, it is defined as the formation of intestinal tissue from the esophageal epithelium, known as Barrett's esophagus (Slack, 2004). More specifically, metaplasia is the process that involves the transformation of epithelia cells to goblet cells; however, histological examination of lung tumors/nodules in our study revealed intestinal gland-like structure instead of granule goblet cell type. Furthermore, pathologically, metaplasia would result in heterogeneous abnormal lung structure, which is composed of large air spaces lined with highly proliferative cuboidal epithelium (Okubo and Hogan, 2004); however, such metaplastic phenotype was not observed in our histological examination of the tissues.

To further characterize our pre-clinical AOM-induced lung metastasis CRC mouse model, we utilized specific cellular origin markers; these biomarkers are currently utilized in the clinic to identify the primary site of adenocarcinoma presenting at metastatic sites. The CDX2 protein plays an important role in proliferation and differentiation of intestinal tissue; it is normally expressed in the nucleus of epithelia cells within the colon, from the proximal to the distal regions; however, cytosolic expression of CDX2 is indicative of intestinal differentiation and adenocarcinoma formation (Wani et al., 2009, Werling et al., 2003). Furthermore, clinically, the expression of CDX2 has been shown to identify colonic origin in resected primary and metastatic adenocarcinomas from the lung; CDX2 is considered the gold standard biomarker due to its 100% percent specificity and 100% sensitivity (Werling et al., 2003). High levels of this protein has been found in clinical CRC adenocarcinoma specimens (Werling et al., 2003). To further confirm the origin of lung tumors in our pre-clinical AOM-induced lung metastasis CRC model, we examined an additional colonic origin marker namely CK20, which is a cytokeratin typically expressed in epithelial cells; positive expression of CK20 has been seen in all cases of CRC carcinomas; however, the

levels did not coordinate with clinical stage (Chu et al., 2000, Samija et al., 2013). Taken together the above information and the fact that we observed high expression of both CDX2 and CK20, known colonic biomarkers, at mRNA and protein levels in lung tumors/nodules suggests that these lung tumors possibly originated from primary colon tumors, in this AOM-induced CRC lung metastasis mouse model. Our IHC results for SP-D and TTF-1 further supported this conclusion.

Furthermore, investigating GSE efficacy in this pre-clinical AOM-induced lung metastasis CRC mouse model revealed significant inhibition of colon tumor metastasis to lung tumor formation. Additionally, examination of individual animal colon tumor multiplicity data revealed that the animals with the highest tumor multiplicity did not exhibit secondary metastatic lung tumor formation upon GSE supplementation; this indicates GSE's ability to decrease the angiogenic process and thus further inhibiting the metastatic spread. In this regard, our group and others have shown that GSE modulates a number of factors involved in the metastatic process; specifically, GSE treatment decreases VEGF, TNF- α , MMP-1,-3,-7,-8,-9 and MMP-13 protein expression (Chao et al., 2011, Kaur et al., 2009, La et al., 2009). In conclusion, the development of this preclinical AOM-induced lung metastasis CRC mouse model is a significant scientific contribution; this *in-situ* sporadic CRC metastatic lung model will aid in the development of effective clinical agents that inhibit CRC disease progression. Furthermore, agents such as GSE, that target multiple pathways which are involved in angiogenesis and distant metastatic tumor growth, could be ideal candidates for their clinical potential in controlling CRC growth, progression as well as metastasis to distal organs such as lung.

Acknowledgments

We thank Dr. David Orlicky, Department of Pathology, UCD and Dr. Carlyne Cool, National Jewish Health, Denver for their histological characterization of the lung nodules. We also acknowledge Umarani Pugazhenth, Ritambhara Wadhwa and Anil Jain, UCD for their technical assistance in these studies.

Grant support: This work was supported by NIH R01 AT003623, NCI R01 CA112304 and NCI Cancer Center P30 CA046934.

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Fig. 1. AOM-induced colon carcinogenesis in A/J mice results in lung nodule formation

The details of the experimental protocol for AOM-induced colon tumorigenesis resulting in lung nodule formation in A/J mice are outlined in the Methods section. (A) Gross examination of isolated lung lobes showing lung nodules after AOM exposure, (B) percent incidence of lung nodules, (C) total number of lung nodules, and (D) different sizes of lung nodules in mice of different groups exposed to AOM and AOM +GSE. (E) Representative pictographs (x100 and x400) depicting H&E staining of lung tissue and nodules. AOM, azoxymethane; GSE, grape seed extract; NS, not statistically significant.

Fig. 2. Identification of lung nodules to be of colon origin using CDX2 as a colon specific biomarker

Lung nodules formed in A/J mice after AOM exposure were analyzed for CDX2 protein and *Cdx2* mRNA expression. For comparative analysis lung tissue from untreated control mice, lung nodule, and tissue adjacent to lung nodules from AOM and AOM+GSE groups were analyzed. (A–B) Immunohistochemical staining for CDX2 was based on DAB staining as detailed in Methods section. Representative pictographs are depicted at x100 and x400. Immunoreactivity (represented by cytosolic intensity of brown staining) was scored as 0 (no staining), +1 (weak), +2 (moderate) and +3 (strong), and +4 (very strong). Quantification was done in tissues from four separate mice per group, examining 10 randomly selected areas in each animal. Columns represent mean $\pm$ SEM (error bars) in each group. Transcriptional expression of *Cdx2* assessed by qRT-PCR in (C) colon tissue from untreated

control and AOM-treated mice, and **(D)** lung tissue from untreated controls, lung nodules, and tissue adjacent to lung nodules in AOM-treated mice using qRT-PCR. Columns represent the mean transcript level (normalized to ribosomal RNA) of four mice/group $\pm$ SEM (error bars). AOM, azoxymethane; GSE, grape seed extract; NS, not statistically significant.

Fig. 3. Identification of lung nodules to be of colon origin using CK20 as a colon specific biomarker

Lung nodules formed in A/J mice after AOM exposures were analyzed for CK20 protein and *Ck20* mRNA expression. For comparative analysis lung tissue from untreated control mice, lung nodule, and tissue adjacent to lung nodules from AOM group was analyzed. (A–B) Immunohistochemical staining for CK20 was based on DAB staining as detailed in Methods section. Representative pictographs are depicted at x100 and x400. Immunoreactivity (represented by cytosolic intensity of brown staining) was scored as 0 (no staining), +1 (weak), +2 (moderate) and +3 (strong), and +4 (very strong). Quantification was done in tissues from four separate mice per group, examining 10 randomly selected

areas in each animal. Columns represent mean $\pm$ SEM (error bars) in each group. Transcriptional expression of *Ck20* assessed by qRT-PCR in **(C)** colon tissue from untreated control and AOM-treated mice, and **(D)** lung tissue from untreated controls, lung nodules, and tissue adjacent to lung nodules in AOM-treated mice. Columns represent the mean transcript level (normalized to ribosomal RNA) of four mice/group $\pm$ SEM (error bars). AOM, azoxymethane; NS, not statistically significant.

Fig. 4. Identification of lung nodules to be of non-lung origin using Surfactant D and TTF-1 as biomarkers

Lung nodules formed in A/J mice after AOM exposures were analyzed for Surfactant D and TTF-1 protein expression. For comparative analysis lung tissue from untreated control mice, lung nodule, and tissue adjacent to lung nodules from AOM and AOM+GSE groups were analyzed. Immunohistochemical staining for (A–B Surfactant D and (C–D) TTF-1 was based on DAB staining as detailed in Methods section. Representative pictographs are depicted at x100 and x400. Immunoreactivity (represented by cytosolic intensity of brown staining) was scored as 0 (no staining), +1 (weak), +2 (moderate) and +3 (strong), and +4 (very strong). Quantification was done in tissues from four separate mice per group,

examining 10 randomly selected areas in each animal. Columns represent mean $\pm$ SEM (error bars) in each group. AOM, azoxymethane; GSE, grape seed extract.