Published in final edited form as:

Clin Exp Metastasis. 2009 ; 26(8): 1033–1047. doi:10.1007/s10585-009-9293-4.

Evaluation of metastatic and angiogenic potentials of human colon carcinoma cells in chick embryo model systems

M. Cecilia Subauste, Tatyana A. Kupriyanova, Erin M. Conn, Veronica C. Ardi, James P. Quigley, and Elena I. Deryugina

Department of Cell Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA

Elena I. Deryugina: deryugin@scripps.edu

Abstract

Increased metastatic and angiogenic potentials of aggressive human colon carcinoma cells were verified in independent chick embryo models by comparing in vivo highly metastatic SW620 colon carcinoma cell line with its isogenic, non-metastatic SW480 cell variant. In the experimental metastasis model, both cell types rapidly arrested in the chorioallantoic membrane (CAM) vasculature as demonstrated by quantitative PCR and immunohistochemistry. Live cell imaging also indicated that both SW620 and SW480 cells efficiently extravasated from the CAM capillary system. However, only few SW480 cells were present in the CAM tissue after 24–48 h. In contrast, the numbers of SW620 cells increased exponentially, indicating proliferative and survival advantages of metastatic colon carcinoma cells in vivo. Multicellular SW620 foci were identified in close proximity to CAM blood vessels. A positive correlation between increased metastatic ability and VEGF-expression of colon carcinoma SW620 cells was demonstrated by the substantial inhibitory effects of anti-VEGF treatment on the levels of metastatic colonization and density of blood vessels adjacent to tumor cell foci. Furthermore, the chick embryo angiogenesis model confirmed high levels of VEGF-dependent angiogenesis induced by SW620 cells, but not SW480 cells. Thus, chick embryo experimental metastasis and CAM angiogenesis models appear to coordinately reflect critical features of advanced colon carcinomas, i.e., the acquisition of enhanced survival and increased angiogenic potentials, both constituting critical determinants of colon cancer progression. The use of rapid and quantitative chick embryo models might provide alternative approaches to conventional mammalian model systems for screening anti-cancer agents.

Keywords

Metastasis; Angiogenesis; Colon carcinoma; Cancer progression; Chick embryo models

Introduction

Colon cancer is one of the most prevalent malignancies worldwide. Many patients with colon cancer are diagnosed with advanced disease and 40–50% of patients will develop local or distant tumor recurrences [1]. Capacity to colonize distant organs is critical for the development of overt metastases by highly aggressive colon carcinoma cells [2]. The development of novel approaches and application of alternative metastasis models is required to study of the molecular events involved in colonization of target organs by tumor cells, the last step in the metastatic cascade.

The growth of primary colon adenocarcinomas as well as formation of distant metastases in advanced disease depends on tumor-induced angiogenesis [3–8]. Individual molecules

associated with colon cancer progression and angiogenesis, such as vascular endothelial growth factor (VEGF), have become both prognostic indicators and therapeutic targets for colon carcinoma patients [9, 10]. Serum concentrations of VEGF are 6.5-fold higher in colon carcinoma patients as compared to healthy individuals, and high serum level of VEGF serves as a factor strongly associated with reduced overall survival and disease-free survival [11, 12]. Correspondingly, when placed on regimens that include bevacizumab, a humanized monoclonal antibody against VEGF, patients with advanced colon carcinoma exhibit an increased median survival as compared to those undergoing conventional chemotherapeutic treatment [1, 13–16].

The majority of animal model systems to study colon cancer metastasis involve the use of immunocompromised mice and rats for i.v. inoculations and hetero- or orthotopic implantations of human tumor cells [2, 17–28]. Being naturally immunodeficient, the chick embryo represents an alternative model system to analyze several aspects of tumor biology. The spontaneous metastasis chick embryo model, based on the grafting of human tumor cells on the chorioallantoic membrane (CAM), has provided valuable information regarding key processes involved in human tumor progression, such as tumorigenesis and tumor cell intravasation [29–37]. The chick embryo has been used also in experimental metastasis studies, providing comprehensive information about metastatic events occurring after intravasation of tumor cells, i.e., survival of tumor cells in the circulation, their arrest in the vasculature, extravasation, and proliferation in the stroma [38–42]. In addition, a modification of J. Folkman's CAM angiogenesis assay [43], allowing for a quantitative analysis of angiogenic potential of human tumor cells in the chick embryo [44–46], has also provided an in vivo model to study the role of neovascularization in malignant progression.

Taking advantage of the multiple strengths of the chicken embryo model systems, we employed spontaneous metastasis, experimental metastasis, and angiogenesis models to evaluate progression of colon cancer by comparing colon carcinoma cell lines with different metastatic potentials. We analyzed the fate of the non-metastatic cell line, SW480, derived from a primary adenocarcinoma, versus that of the highly metastatic cell line, SW620, derived from a lymph node metastasis of the same patient after widespread dissemination of the recurrent cancer [47]. These isogenic colon carcinoma cells have been extensively employed in vitro [48–58] and also in rodent models in vivo [19, 21, 24, 27, 52, 59, 60]. High tumorigenic and metastatic potentials of SW620 cells as compared to non-metastatic SW480 cells demonstrated in xenotransplantation experiments have indicated that this pair of cell lines represents an appropriate model for studying colon cancer progression. The relevance of the SW480/SW620 comparative analysis to study late events in colon carcinogenesis is also highlighted by the fact that metastatic SW620 carcinoma cells over-express VEGF [48], one of the factors strongly associated with poor clinical prognosis [9, 10].

In this study, we demonstrated that during experimental metastasis in chick embryos, the two carcinoma cell variants arrested equally well in the CAM vasculature soon after i.v., injection. However, only SW620 cells survived and increased in numbers as determined by quantitative real-time *Alu* PCR. These initial observations were confirmed by detailed immunohistochemical and live cell imaging studies, which indicated that individual SW620 cells generated multicellular foci associated closely with small blood vessels and capillaries, thereby suggesting tumor-vascular interactions. The high angiogenic potential of SW620 cells was demonstrated in a quantitative angiogenic CAM assay. The importance of interactions between metastatic colon carcinoma cells and blood vessels during metastatic colonization was further indicated by the inhibition of SW620 colonization with a function-blocking anti-human VEGF antibody. Thus, in two quantitative chicken embryo models, the metastatic SW620 variant, but not its non-metastatic SW480 counterpart, exhibited

enhanced survival, proliferation and VEGF-mediated angiogenesis, all of which are critical features characteristic of advanced colon carcinoma. Therefore, the chick embryo metastasis and angiogenesis model systems could provide valuable tools for rapid and quantitative screening of novel therapeutics that target colon carcinoma metastatic outgrowth.

Materials and methods

Cell lines and culture conditions

SW480 and SW620 colon carcinoma cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). High disseminating HT-1080 human fibrosarcoma cells (HT-hi/diss) were generated as described [32]. Cells were cultured in Dulbecco's MEM (DMEM; Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal calf serum (HyClone, Logan, UT). Cell cultures were maintained at 37°C in a mixture of air and 5% CO₂ and passaged at confluence.

Chick embryo spontaneous metastasis model

All experiments involving the use of animals were performed in accordance with the protocols approved by the Scripps Research Institute's Animal Care and Use Committee. Chick embryo spontaneous metastasis assays were performed essentially as described [32]. Fertilized White Leghorn eggs (SPAFAS, North Franklin, CT) were incubated for in a rotary incubator at 38°C and 60–70% humidity. On day 10 of incubation, the top portion of the CAM was lowered, and $0.25\text{--}5 \times 10^6$ tumor cells were inoculated in 25 μl of serum-free DMEM onto the CAM through the small window in the eggshell. The windows were sealed and the eggs were returned to a stationary incubator. At day 7, primary tumors were excised and weighed and portions of distal CAM, liver, lungs and spleen were harvested and analyzed by quantitative *Alu* PCR to determine the number of human cells intravasated to the CAM and metastasized to the internal organs.

Chick embryo experimental metastasis model

Cultured cells were detached by brief trypsinization, washed, and resuspended in PBS. A total of 1 or 1.5×10^5 cells in 0.1 ml were injected into the allantoic vein of 12-day-old embryos. Where indicated, goat anti-human VEGF antibody or control goat IgG (both from R&D Systems, Minneapolis, MN) were injected i.v., at 50 μg per embryo 24 and 72 h after injection of SW620 cells. Following injections, the eggs were returned to a stationary incubator. At different time points indicated in the text, the liver, lungs and spleen, and portions of the CAM distant to the site of injection, were harvested and analyzed by quantitative *Alu* PCR for the relative numbers of human cells in the chick embryo tissues.

Real-time PCR for quantitative detection of human tumor cells

The number of human cells within chick embryo tissues was determined by quantitative PCR amplifying *Alu* repeats (*Alu* qPCR), which are uniquely present in a primate genome and lacked in the chicken DNA. The *Alu* qPCR was performed essentially as described [32]. Briefly, genomic DNA was extracted from harvested tissues using the Puregene kit (Genta Systems, Minneapolis, MN). Human *Alu* sequences were amplified by qPCR using 60 ng of genomic DNA as template in a 10 μl reaction, containing 2 mM MgCl₂, 200 μM dNTP, 0.4 units of Platinum Taq polymerase (Invitrogen Corp., Carlsbad, CA), 1:10⁵ dilution of SYBR[®]Green dye (Molecular Probes, Eugene, OR) and 0.4 μM of each *Alu*-sense (5'-ACGCCTGTAATCCCAGCACTT-3') and *Alu*-antisense (5'-TCGCCAGGCTGGAGTGCA-3') primers. *Alu* qPCR was carried out at 95°C for 4 min, followed by 30 cycles at 95°C for 30 s, 63°C for 30 s, and 72°C for 30 s. Each assay included negative controls (samples of tissues from embryos that have not received any

human cells), a positive control (human genomic DNA) and the experimental samples in duplicate. Relative numbers of tumor cells in each tissue sample were determined using a standard curve generated by a serial (1:10) dilution of human tumor cells within a constant number (1×10^6) of chick embryo fibroblasts. The standard curve is linear within a range of 10 – 10^5 human cells per 1×10^6 chicken cells and the detection sensitivity of the assay constitutes ~ 20 – 25 human cells per 10^6 chicken cells.

Immunohistochemistry

Samples of the CAM were fixed in Zn-formalin and paraffin embedded. Deparaffinated sections were stained with H&E. To visualize human tumor cells, sections were stained immunohistochemically with a mixture of anti-human pan-cytokeratin monoclonal antibodies (mAbs) (Sigma, Saint Louis, MO). Sections were treated with 0.3% hydrogen peroxide and blocked with PBS supplemented with 2% BSA and 5% normal goat serum. For antigen retrieval, sections were incubated for 10 min at 37°C with pepsin (Dako, Carpinteria, CA). Incubation with $10 \mu\text{g/ml}$ primary antibodies was performed overnight at 4°C , followed by washing and incubation with secondary anti-mouse biotinylated antibodies for 1 h at room temperature. Avidin D-horseradish peroxidase conjugate (Vector) was added for 30 min, followed by addition of a DAB chromogenic substrate. Sections were counterstained with Mayer's hematoxylin. Digital images were captured using the Olympus BX60 microscope equipped with a digital DVC video camera and processed with Adobe Photoshop 6.0 software (Adobe Systems, Inc.).

Live cell imaging

SW480 and SW620 cells were labeled with $2 \mu\text{M}$ green CellTracker CMFDA (Molecular Probes) and injected i.v., at 1×10^5 cells per embryo. To highlight vasculature, the embryos were injected with $50 \mu\text{g}$ of rhodamine-conjugated Lens *culinaris* agglutinin (LCA, Vector). At indicated time points, the embryos were sacrificed and the CAM was stretched on glass slides, and examined in a Carl Zeiss Axio Imager.M1m microscope. Digital images were taken with Axiovision Rel. 4.6 software (Carl Zeiss MicroImaging GmbH) and processed with CS3 Adobe Photoshop software.

CAM angiogenesis assay

CAM angiogenesis assays were performed using shell-free 10-day-old chick embryos essentially as described [46]. At day 3 of incubation, the contents of the eggs were transferred to sterile plastic weigh boats, covered with square Petri dishes, and placed into a stationary incubator at 38°C with 85% humidity for an additional 7 days. To prepare collagen for the angiogenesis assay, eight volumes of non-pepsin-treated, type I rat tail collagen (BD Biosciences, Franklin Lakes, NJ) were neutralized with 1 volume of $10\times$ MEM and 1 volume of 0.15 M NaOH. Human tumor cells were added in serum-free DMEM at 3×10^4 cells per $30 \mu\text{l}$ of collagen mixture with a final concentration of collagen at 2.1 mg/ml and 0.25 mM HEPES buffer (pH 7.4). Where indicated, the anti-VEGF antibody was added to the tumor cell-collagen mixture at a final concentration of $25 \mu\text{g/ml}$. To assemble collagen grafts, $30 \mu\text{l}$ of the final collagen mixture was placed atop two layers of nylon mesh (Tetko, Kansas City, MO) and allowed to polymerize at 37°C for 20–30 min, making so called “onplants”. Solidified collagen onplants were grafted on top of the CAM of day-10 chick embryos incubated ex ovo. The angiogenic, newly-formed blood vessels were scored within the grids of the top mesh at 65–84 h using a stereomicroscope. Data are presented as an angiogenic index, i.e., a ratio of the number of grids containing angiogenic blood vessels over the total number of grids scored.

Data analysis and presentation

Data processing and statistical analysis were performed with GraphPad Prism software (GraphPad Software, Inc., San Diego, CA). The total number of samples is indicated in the text or figure legends. The data are presented as bar graphs or scattergrams depicting means $\pm$ SEM or medians from a representative experiment or from normalized data of pooled experiments. Two-tailed Student's *t* test for $P < 0.05$ was used to compare significance of differences between the experimental groups.

Results

SW480 and SW620 carcinoma cells in the spontaneous metastasis chick embryo model

The chick embryo has been successfully used as a host to study tumor cell intravasation and dissemination of different types of human carcinomas, including breast [36], prostate [61], cervical [62] and epidermoid [29, 35, 37] carcinomas. Therefore, we verified whether SW480 and SW620 colon carcinoma cells would form primary tumors and spontaneously metastasize in the chick embryo model by grafting the cells on the CAM of day 10 embryos. A highly disseminating variant of human fibrosarcoma HT-1080, HT-hi/diss, previously shown to generate primary tumors and efficiently intravasate into the CAM vasculature [32], was used as a positive control. Within 7 days, 2.5×10^5 HT-hi/diss cells formed sizable primary tumors with an average weight of ~500 mg. However, both SW480 and SW620 carcinomas grafted even at 5×10^6 cells per embryo generated fivefold smaller tumors (Fig. 1a). On day 7, HT-hi/diss cells were readily detectable in the distal CAM by *Alu* qPCR, confirming their high levels of spontaneous dissemination, whereas negligible, background numbers of colon carcinoma cells were identified in the distal CAM (Fig. 1b). Similarly, almost no colon carcinoma cells were detected in the internal organs, including liver, lungs, and spleen (data not shown). Therefore, despite overall ability to generate primary tumors on the CAM, both SW480 and SW620 colon carcinomas are apparently inefficient in spontaneous metastasis in the chick embryo model. However, this is not surprising, because of the short term of the spontaneous dissemination assay in the chick embryo (5–9 days) in contrast to much longer time (several weeks) required for colon carcinoma cells to spontaneously metastasize in the rodent models.

Experimental metastasis of SW480 and SW620 carcinoma cells in the chick embryo

We next analyzed the capacity of SW620 and SW480 cells to colonize various organs of chick embryos in the experimental metastasis model. Equal numbers of SW480 and SW620 cells (1×10^5 cells per embryo) were injected into the allantoic vein of 12-day-old embryos. Following additional 7 day incubation, portions of the CAM most distant from the site of inoculation, and the internal organs, including liver, spleen, and lungs, were harvested and analyzed by *Alu* qPCR to determine numbers of human cells within the host tissues. In six independent experiments, SW620 cells demonstrated significantly higher efficiency of CAM colonization compared to SW480 cells (Fig. 1c). The mean number of SW620 cells detected per 10^6 cells of the CAM tissue was approximately 17-fold higher ($P < 0.0001$) than that for SW480 cells and comparable to that for HT-hi/diss cells. These findings demonstrate that only metastatic SW620 colon carcinoma cells efficiently colonize the CAM tissue of the chick embryo, while its non-metastatic SW480 counterpart is essentially deficient in CAM colonization.

Consistent with the very low levels of CAM colonization by SW480 cells, very few human cells were detected in the internal organs of the embryos injected with SW480 cells (Table 1). Colonization of internal embryo organs was very low (liver) or at background levels (lungs, spleen) also for SW620 cells (Table 1). This colonization pattern is quite different from relatively high levels exhibited by human HT-1080 fibrosarcoma in experimental

metastasis. These results suggested that CAM tissue might provide a microenvironment uniquely suitable for initial arrest and subsequent proliferation of aggressive colon carcinoma cells, which likely reflects that the CAM in the embryo is a functional analogue of the lung in the adult animal serving as a major repository of i.v. inoculated tumor cells.

Kinetic analysis of SW480 and SW620 experimental metastasis

To gain insight into the fate of SW480 and SW620 cells in the CAM and internal organs, quantitative kinetic studies of CAM and liver colonization were performed following their inoculation into allantoic vein (Fig. 2). Analysis by *Alu* qPCR indicated that 2 h after i.v. injection both cell types were arrested in the CAM vasculature with a comparable efficiency. However, by 24 h following inoculation, the number of SW480 cells dropped almost 90%; the cells became practically undetectable at day 3 and did not increase significantly in numbers by the end of experiments. In contrast, SW620 cells appeared to begin proliferating after a lag-period and exponentially increased in numbers between day 3 and day 9 (Fig. 2a).

To determine the time-frame of SW480 disappearance from the CAM, we performed a more detailed analysis. To increase detection levels within the 48 h length of the experiment, chick embryos were injected i.v. with 1.5×10^5 SW480 cells instead of 1.0×10^5 cells. As seen in Fig. 2b, the numbers of SW480 cells arrested at 2 h steadily decreased and less than 10% of these cells were identified at 48 h. These results indicate that immediately following i.v. inoculation, both SW620 and SW480 colon carcinomas arrest with equal efficiency in the CAM vasculature. However, only the highly metastatic SW620 carcinoma variant is capable of surviving and subsequently proliferating in the CAM tissue after initial vascular arrest.

To analyze whether colon carcinoma cells efficiently arrested in the capillary system of internal organs, we performed a kinetic analysis of liver colonization by SW480 and SW620 cells (Fig. 2c). The data clearly indicate that initial, 2 h colonization of the liver by both cell types was comparable to the levels of demonstrated for the CAM (Fig. 2a). However, by 24 h less than 10% of initially arrested cells were identified in the liver, indicating rapid and dramatic tumor cell disappearance. Neither cell type regained their numbers by day 5 after cell inoculations. This kinetic analysis strengthened the notion that in the chick embryo, the CAM capillary system, which functions as the analog of adult lung vasculature, is the only tissue that allows for not only the initial arrest of circulating SW480 and SW620 colon carcinoma cells, but also for sustained proliferation of aggressive SW620 cells.

Immunohistochemical analysis of CAM colonization by SW620 and SW480 colon carcinoma cells

Immunohistochemical staining with anti-human pan-cytokeratin antibodies was undertaken in order to localize SW480 and SW620 carcinoma cells in the CAM tissue. Two hours after inoculation, individual SW480 and SW620 cells were identified consistently in the capillary plexus beneath the CAM ectoderm (Fig. 3). It appears that initially both cell types adhere equally well to the CAM endothelium and arrest in the CAM vasculature with the same efficiency. No substantial aggregate formation was observed in either of the colon carcinoma variants at that time point. Within the next 3 days, small multicellular foci, comprised of up to 8–16 cells, were formed in the capillary plexus of embryos injected with SW620 cells. In contrast, no SW480 cells were identified in the CAM tissue by immunohistochemistry, which is consistent with the very low levels of detection by *Alu* qPCR (Fig. 2a). While SW480 cells were not visualized even at day 7, SW620 metastatic foci appeared to significantly increase in size after 3 days, suggesting extensive cell proliferation. Mitotic figures were frequently identified within SW620 cell congregates, which remained associated with the plexus capillaries and underlying mesodermal blood

vessels. At days 7 and 9 after injection, SW620 foci appeared to be expanding into the CAM mesoderm, with individual carcinoma cells associated more loosely with each other, but still in close proximity to blood vessels. At this time point, very rare SW480 cells were identified in the CAM tissue, but in only one out of three injected embryos (Fig. 3).

Therefore, our immunohistological studies reinforced the *Alu* qPCR kinetics and confirmed the high experimental metastasis levels of the aggressive SW620 carcinoma variant. In addition, the histology suggested the close and continuous relationships between the arrested SW620 cells and the CAM vasculature.

Live image analysis of CAM colonization by SW480 and SW620 cells

To gain information on physiological processes which are involved in the early events following tumor cell inoculation into the circulation and to highlight the differential in behavioral characteristics between SW480 and SW620 cells, we performed live image analysis of CAM colonization. The cells were pre-labeled with green fluorescence dye, while the CAM vasculature was illuminated by red fluorescence lectin. Kinetic analysis of non-fixed CAM tissue was performed at different time points after SW480 and SW620 cells were injected into the allantoic vein of the chick embryo (Fig. 4).

The CAM vasculature system consists of venous and arterial vessels inter-connected through the network of tiny capillaries called ectoderm plexus. Within 2 h, both SW480 and SW620 cells reached the tips of terminal arterioles of the CAM (Fig. 4, top panels), although some cells were still found intravascularly (Fig. 4a, left bottom panel). Most of the arrested cells appear round, but some of them were observed as actively extravasating into honeycomb-like extravascular spaces in the ectoderm capillary plexus (Fig. 4a, right panels). No major differences in the overall appearance of SW480 and SW620 cells arrested in the CAM tissue were detected at this time point. However, by 24 h, the majority of SW480 cells have disappeared and only a very few cells could be found in the CAM (Fig. 4b, top panels). While these cells were found at some distance from the terminal capillaries, they were still round, not displaying signs of spreading or active movement in the CAM mesoderm (Fig. 4b, top panels). In contrast, SW620 cells were abundant and found both distant from and nearby the terminal capillaries (Fig. 4b, bottom panels). Some of the SW620 cells displayed migratory appearance and cells were frequently found in doublets, indicative of initiation of cell proliferation. Importantly, fragmented cells resembling apoptotic bodies were identified among SW480 cells, which suggested that these cells have undergone apoptosis (Fig. 4b, top right panel, arrow). Unfortunately, the extremely limited numbers of SW480 cells identifiable at this time period did not allow for their detection with the available *in vivo* probes for apoptosis (such as FLIVO) and for statistical analysis of apoptotic events. Very rarely, some fragmented cells were found also among SW620 cells, the majority of which appear as just extravasated from the terminal capillaries (Fig. 4b, right bottom panel, arrow). Although these fragmented cells might suggest some level of apoptosis, their very low numbers would indicate rather low levels of apoptosis for SW620 cells.

The overall appearance of rare SW480 cells identified by fluorescence did not change by 48 h following their inoculation (Fig. 4c, top panels). The cells were still rounded and almost never found in doublets, indicating the lack of proliferation. In contrast, SW620 cells appeared actively proliferating since more cell doublets and also clusters of four cells were easily identified (Fig. 4c, bottom panels). In two more days, at the 96 h time point, SW620 cells could be found in clusters of 16 or more cells, indicating approximately a 20–24 h doubling time for some of the SW620 cells *in vivo*. Interestingly, these clusters of SW620 cells appear to wrap around blood vessels suggestive of vasculotropism and a dependence of growth of micrometastases on close interactions with the vasculature.

This kinetic analysis performed by live cell imaging allowed us to demonstrate that SW480 cells were as efficient as their metastatic counterpart in early survival in the circulation and initial arrest in the CAM vascular system. In addition, SW480 cells appeared to be similar to SW620 cells in their ability to extravasate into extravascular spaces of the capillary plexus, but their dramatic disappearance from the CAM tissue and an apparent lack of proliferation strongly suggested that SW480 cells have greatly diminished *in vivo* survival ability.

Angiogenic potential of colon carcinoma cell variants

The immunohistological findings and live cell image analysis suggested that survival and proliferation of SW620 colon carcinoma depended on interactions with the capillary endothelium and blood vessels of the CAM. To verify that the metastatic SW620 variant is more angiogenic than its non-metastatic SW480 counterpart, we compared the two cell types in the quantitative CAM angiogenesis model (Fig. 5a). Approximately a twofold increase in angiogenesis was observed when SW620 cells were incorporated into collagen rafts as compared with control onplants containing no tumor cells ($P < 0.005$). In parallel, the highly-disseminating HT-hi/diss cells also exhibited high angiogenic potential and induced angiogenesis at levels more than twofold over those observed in control ($P < 0.001$). In contrast, SW480 cells failed to induce angiogenesis over background, consistent with their very low metastatic potential. Thus, the ability of metastatic SW620 colon carcinoma cells to induce angiogenesis in the CAM model (Fig. 5a) positively correlated with their capacity to colonize the CAM tissue in experimental metastasis model (Figs. 1c, 2a).

Since SW620 cells have previously been demonstrated to secrete more VEGF than SW480 cells [48], we investigated whether higher levels of VEGF might be responsible for the differential in angiogenesis manifested by the two colon carcinoma cell lines. To this end, collagen onplants containing SW620 cells were additionally supplemented with function-blocking anti-VEGF antibody. As shown in Fig. 5a, anti-VEGF treatment completely abrogated SW620-induced angiogenesis, bringing the angiogenesis index to the level observed in SW480-containing onplants and control onplants containing no tumor cells.

Experimental metastasis of SW620 colon carcinoma cells is VEGF-dependent

SW620-secreted VEGF may facilitate the cross-talk between tumor cells and endothelial cells, thereby enabling enhanced survival and ensuing proliferation of SW620 cells upon their arrest in the circulation during early phases of experimental metastasis. In order to analyze this hypothesis, chick embryos, which had been injected with SW620 cells, were treated with the function-blocking anti-human VEGF antibody (Fig. 5b). Previously, this antibody, highly specific to human VEGF, has been shown not to affect normal CAM angiogenesis in the chick embryo [63], thereby allowing us to address the specific role of tumor-derived VEGF in SW620 colonization. A total of 50 μ g of anti-human VEGF antibody or normal IgG was administered *i.v.* on day 1 and 3 following *i.v.* inoculations of 1×10^5 SW620 cells. On day 6, SW620 colonization was analyzed by *Alu* qPCR. As shown in Fig. 5b, the level of SW620 colonization in the embryos treated with an anti-VEGF antibody was significantly reduced to ~30% of control levels ($P < 0.05$). Complementing our quantification of experimental metastasis by *Alu* qPCR, immunohistochemical analysis (illustrated in Fig. 5c) indicated that anti-VEGF treatment caused a 2.5-fold ($P < 0.005$) decrease in the relative number of SW620 metastatic foci (Fig. 5d, top). In addition, anti-VEGF treatment significantly reduced the density of CAM blood vessels, adjacent to metastatic foci (Fig. 5d, bottom). Thus, SW620 colonization of the CAM appears to be VEGF-dependent, supporting the notion that survival and initial proliferation of circulating carcinoma cells after their arrest in the capillary network involve specific tumor cell-endothelial cell interactions.

Discussion

In order to complement current rodent models employed for screening of therapeutic compounds and target molecules, we have evaluated whether chick embryo model systems would suitably reflect high metastatic and angiogenic potentials of advanced colon carcinoma cells by comparing non-metastatic SW480 and metastatic SW620 isogenic variants in three independent model systems. Despite the capacity to form primary tumors, neither cell line exhibited the ability to spontaneously disseminate following grafting on the CAM of the chick embryo. While this finding may seem disappointing at first glance, it can actually reflect the general inability of colon carcinomas to spontaneously disseminate in the mouse subcutaneous metastasis model [17, 18]. It is generally accepted that human colon carcinomas do not metastasize unless they are implanted orthotopically into the cecum, which apparently provides colon cancer cells with a specific microenvironment required for their successful spontaneous metastasis to liver [2, 18, 19, 28, 64]. In addition, even after orthotopic implantation the establishment of spontaneous metastases require relatively long time periods (weeks or months), a requirement which cannot be met in the 5–9 day-long chick embryo assay.

Experimental metastasis models such as intrasplenicportal injections, which bypass early steps of the metastatic cascade, are often employed to study metastasis of aggressive human colon carcinoma cells [17, 18, 20, 27, 28, 65]. In this regard, SW480 and SW620 cells exhibited appropriate phenotypic and behavioral differentials in the chick embryo experimental metastasis model, consistent with their hierarchy in colon cancer progression demonstrated in different mammalian model systems [19, 21, 24, 59, 60].

The capacity of highly aggressive SW620 colon carcinoma cells to survive days after their direct inoculation into circulation of the chick embryo is in agreement with a previously shown correlation between the enhanced resistance of SW620 cells to apoptosis and their metastatic aggressiveness [24]. Interestingly, the initial arrest of human colon carcinoma cells in the liver and lungs of immunodeficient rats was independent of their ability to colonize these target organs [26]. Accordingly, within 2–4 h after i.v. inoculation, SW480 and SW620 cells arrested in the CAM of the chick embryo with similar efficiency, indicating comparable abilities of both cell types to adhere to the CAM endothelium or underlying basal membrane. However, the majority of non-metastatic SW480 cells disappeared from the CAM within 48 h following injection, which correlates well with the low colonization efficiency of these colon carcinoma cells demonstrated in rodent models [19, 21, 59, 60]. Importantly, SW620 and SW480 cells have been shown to display similar adherence to major ECM proteins, including laminin, type I collagen and fibronectin [54], thus making adhesion deficiency an unlikely cause of SW480 cell disappearance. More likely, the decline in numbers of SW480 cells could be attributed to their higher susceptibility to pro-apoptotic stimuli during or soon after extravasation. In this regard, SW480 cells exhibit higher levels of apoptosis mediated by CD95 (Fas/Apo-1), a receptor, the function of which is impaired in SW620 cells, making them resistant to the inducers of CD95-dependent apoptotic cell death [24, 49, 58].

In the chick embryo, rare SW480 cells were capable of surviving in the CAM tissue and at later time points were identifiable by immunohistology and live cell imaging as sparse foci consisting of few tumor cells. These findings could reflect an intrinsic heterogeneity of the primary premalignant colon neoplasia, having potential to give rise to some aggressive, metastatic colon carcinomas [17]. While the great majority of SW480 colon carcinoma cells disappeared from the CAM, SW620 cells exponentially increased in numbers and generated large, multi-cellular metastatic foci extending into the underlying CAM mesoderm. Overall, the differential capacity of the two colon carcinoma variants to colonize the CAM of the

chick embryo correlates well with their metastatic behavior observed in rodent model systems. These findings indicate that the chick embryo experimental metastasis model can accurately reflect both the survival ability and metastatic potential of human colon carcinomas and might be useful for probing the molecular/mechanistic aspects of colon carcinoma progression.

The survival and ensuing proliferation of tumor cells after their initial arrest in a subset of secondary organs are critical for the development of metastases [66]. These steps of metastatic progression depend on close interactions of tumor cells with the organ-specific microenvironment [67]. In contrast to the CAM, the liver, lungs or spleen of the embryo did not provide a supportive environment for proliferation of colon carcinoma SW620 cells despite their efficient original arrest at these intra-embryonal sites. The dense capillary network and/or increased blood circulation in the CAM as compared to internal organs [68], could contribute to the preferential colonization of the CAM tissue by SW620 colon carcinoma cells. Interestingly, human tumor cell lines derived from other cancer types, e.g., fibrosarcoma [32], cervical carcinoma [62], and prostate carcinoma [61] efficiently colonize the chick embryo liver, lungs or brain following i.v. inoculation. It appears that the CAM capillary plexus, which functions as an analogue of the capillary system of adult lungs, provides an ideal environment for survival and eventual metastatic outgrowth of aggressive colon carcinoma cells arrested after their injection into the allantoic vein. Continuous proliferation of SW620 colon carcinoma cells was sustained by the CAM microenvironment for at least 7–9 days, resulting in ~4–5 doublings of the initially arrested cell population. This is an important aspect of the chick embryo experimental metastasis model since colonization of the CAM might allow for studying the microenvironment-dependent events involved in colon carcinoma outgrowth.

Tumor dissemination and outgrowth, late steps in the metastatic progression, are dependent not only on angiogenic onset in the primary tumor, but also on close interactions with the vasculature and tumor-induced angiogenesis in secondary sites [66]. Accordingly, colon cancer progression has been strongly associated with the increased production of pro-angiogenic growth factors and cytokines such as VEGF [11, 12] and IL-8 [69]. In this regard, SW620 colon carcinoma cells have been shown to produce VEGF at levels much higher than those observed in SW480 cells [48]. Correspondingly, a differential in the angiogenic stimulation by conditioned media from SW480 and SW620 carcinoma cells was indicated in a cornea vascularization mouse model [70]. Reciprocal modulation of low levels of VEGF in SW480 cells by transfection with VEGF cDNA and treatment of SW620 cells with anti-sense VEGF, respectively, increased and decreased proliferation of endothelial cells induced in vitro by tumor cell conditioned medium [48].

Consistent with higher levels of VEGF expression, SW620 colon carcinoma cells in our study induced high levels of VEGF-dependent angiogenesis in a quantitative chick embryo angiogenesis model [46]. The levels of angiogenesis stimulated by SW620 cells were comparable with the levels induced by highly angiogenic human HT-1080 fibrosarcoma [45]. As opposed to the majority of other CAM angiogenesis models, the assay employed in this study allows to discriminate between the true angiogenic blood vessels sprouting upward in collagen grafts versus pre-existing blood vessels converging to the source of pro-angiogenic stimuli [46]. It is important that in this model system SW480 cells did not induce angiogenesis over control levels, indicating the low angiogenic potential of this non-metastatic colon carcinoma variant. Therefore, yet another aspect of colon carcinoma progression, i.e., increased angiogenic potential of metastatic cells, was demonstrated using the chick embryo angiogenesis model.

Within the CAM tissue, exponentially growing SW620 cells appeared to retain close associations with blood vessels. This finding suggested the existence of mutually beneficial interactions between the aggressive colon carcinoma cells, producing pro-angiogenic factors, and the CAM vasculature, supplying the growing metastatic foci with oxygen, growth factors and metabolites. Chemotherapeutic regimens involving anti-VEGF antibodies have demonstrated efficacy in treatment of advanced colon carcinomas, mainly through the inhibition of angiogenesis at the primary tumor and metastatic sites [1, 13–16]. In addition to specific anti-angiogenic effects, anti-VEGF antibodies also provide anti-tumor effects on colon carcinomas in xenograft models [20, 64]. In this study, we demonstrated that treatment with anti-VEGF antibody significantly suppressed colonization of SW620 cells in the chick embryo experimental metastasis model. Levels of experimental metastasis, analyzed by *Alu* qPCR and immunohistochemistry in the CAM tissue within 5 days after inoculation of SW620 cells, were substantially reduced by anti-VEGF treatment. These data are consistent also with the inhibitory effects of stable siRNA silencing of VEGF expression on experimental metastasis of SW620 cells in immunodeficient mice [65].

In the chick embryo experimental metastasis model, the growth of metastatic foci per se might not critically depend on VEGF-induced angiogenesis since the CAM itself is a rich network of developing angiogenic vessels. The establishment of an individual metastatic focus may rather depend on initial survival of single cells after their arrest in the capillary system and their extravasation, requiring adequate VEGF-mediated signaling and interaction between tumor and endothelial cells. Interestingly, immunohistochemical analysis did not demonstrate that anti-VEGF antibody affected the relative number of cells per individual focus, suggesting that anti-VEGF treatment likely affected single tumor cells. In mouse model systems, anti-VEGF antibodies have been shown to reduce tumor growth, consistent with inhibition of colon carcinoma cell proliferation [20]. However, in vitro data indicate that both additions of exogenous VEGF or anti-VEGF function-blocking antibodies might not affect proliferation of colon carcinoma cells [20], but rather increase their susceptibility to apoptosis [71]. These findings might explain why anti-VEGF treatment of embryos inoculated with SW620 cells reduced the number of metastatic foci rather than their size. In addition to these effects, our immunohistochemical analysis in anti-VEGF-treated embryos indicated a reduced number of blood vessels in close vicinity to SW620 metastatic foci. Although speculative, this observation could be also explained by reduced convergence of pre-existing blood vessel to the SW620 foci, in which VEGF has been blocked with function-blocking antibody. Therefore, reduction in both the density of blood vessels surrounding metastatic colonies and the density of metastatic foci observed in the chick embryo could be attributed to some of the mechanisms underlying anti-VEGF therapy in mammalian settings.

In summary, dramatic differences in survival, growth and angiogenic potentials between metastatic and primary colon carcinoma cells were validated in two independent chicken embryo models: The chick embryo experimental metastasis model accurately reflected high metastatic potential of aggressive colon carcinoma cells, while the chick embryo angiogenesis model demonstrated that metastatic colon carcinoma cells induced high levels of VEGF-mediated angiogenesis. Thus, these quantitative chick embryo models might complement current rodent model systems and facilitate rapid, preliminary testing of potential new agents to aid molecular-based cancer therapy.

Abbreviations

CAM	Chorioallantoic membrane
DMEM	Dulbecco's MEM

qPCR	Quantitative PCR
VEGF	Vascular endothelial growth factor

Acknowledgments

The authors thank Chen-Xing Li and Lauren Hyden for excellent technical assistance.

References

1. Saif MW. Targeted agents for adjuvant therapy of colon cancer. *Clin Colorectal Cancer*. 2006; 6(1): 46–51. [PubMed: 16796791]
2. Kuo TH, Kubota T, Watanabe M, et al. Liver colonization competence governs colon cancer metastasis. *Proc Natl Acad Sci USA*. 1995; 92(26):12085–12089. [PubMed: 8618849]
3. Takahashi Y, Kitadai Y, Bucana CD, et al. Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer. *Cancer Res*. 1995; 55(18):3964–3968. [PubMed: 7664263]
4. Frank RE, Saclarides TJ, Leurgans S, et al. Tumor angiogenesis as a predictor of recurrence and survival in patients with node-negative colon cancer. *Ann Surg*. 1995; 222(6):695–699. [PubMed: 8526575]
5. Engel CJ, Bennett ST, Chambers AF, et al. Tumor angiogenesis predicts recurrence in invasive colorectal cancer when controlled for Dukes staging. *Am J Surg Pathol*. 1996; 20(10):1260–1265. [PubMed: 8827033]
6. Choi HJ, Hyun MS, Jung GJ, et al. Tumor angiogenesis as a prognostic predictor in colorectal carcinoma with special reference to mode of metastasis and recurrence. *Oncology*. 1998; 55(6):575–581. [PubMed: 9778626]
7. Takahashi Y, Bucana CD, Cleary KR, et al. p53, vessel count, and vascular endothelial growth factor expression in human colon cancer. *Int J Cancer*. 1998; 79(1):34–38. [PubMed: 9495355]
8. Staton CA, Chetwood AS, Cameron IC, et al. The angiogenic switch occurs at the adenoma stage of the adenoma carcinoma sequence in colorectal cancer. *Gut*. 2007; 56(10):1426–1432. [PubMed: 17566019]
9. Ellis LM, Takahashi Y, Liu W, et al. Vascular endothelial growth factor in human colon cancer: biology and therapeutic implications. *Oncologist*. 2000; 5 Suppl 1:11–15. [PubMed: 10804085]
10. Ellis LM, Hicklin DJ. VEGF-targeted therapy: mechanisms of anti-tumour activity. *Nat Rev Cancer*. 2008; 8(8):579–591. [PubMed: 18596824]
11. Werther K, Christensen IJ, Brunner N, et al. Soluble vascular endothelial growth factor levels in patients with primary colorectal carcinoma. The Danish RANX05 colorectal cancer study group. *Eur J Surg Oncol*. 2000; 26(7):657–662. [PubMed: 11078612]
12. De Vita F, Orditura M, Lieto E, et al. Elevated perioperative serum vascular endothelial growth factor levels in patients with colon carcinoma. *Cancer*. 2004; 100(2):270–278. [PubMed: 14716760]
13. Hurwitz H, Fehrenbacher L, Novotny W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med*. 2004; 350(23):2335–2342. [PubMed: 15175435]
14. Culy C. Bevacizumab: antiangiogenic cancer therapy. *Drugs Today (Barc)*. 2005; 41(1):23–36. [PubMed: 15753967]
15. Vanhoefer U. Molecular mechanisms and targeting of colorectal cancer. *Semin Oncol*. 2005; 32(6 Suppl 8):7–10. [PubMed: 16360005]
16. Duff SE, Jeziorska M, Rosa DD, et al. Vascular endothelial growth factors and receptors in colorectal cancer: implications for anti-angiogenic therapy. *Eur J Cancer*. 2006; 42(1):112–117. [PubMed: 16321517]

17. Morikawa K, Walker SM, Nakajima M, et al. Influence of organ environment on the growth, selection, and metastasis of human colon carcinoma cells in nude mice. *Cancer Res.* 1988; 48(23): 6863–6871. [PubMed: 2846163]
18. Fidler IJ. Orthotopic implantation of human colon carcinomas into nude mice provides a valuable model for the biology and therapy of metastasis. *Cancer Metastasis Rev.* 1991; 10(3):229–243. [PubMed: 1764766]
19. Witty JP, McDonnell S, Newell KJ, et al. Modulation of matrilysin levels in colon carcinoma cell lines affects tumorigenicity in vivo. *Cancer Res.* 1994; 54(17):4805–4812. [PubMed: 8062282]
20. Warren RS, Yuan H, Matli MR, et al. Regulation by vascular endothelial growth factor of human colon cancer tumorigenesis in a mouse model of experimental liver metastasis. *J Clin Invest.* 1995; 95(4):1789–1797. [PubMed: 7535799]
21. Sampson-Johannes A, Wang W, Shtivelman E. Colonization of human lung grafts in SCID-hu mice by human colon carcinoma cells. *Int J Cancer.* 1996; 65(6):864–869. [PubMed: 8631605]
22. Killion JJ, Radinsky R, Fidler IJ. Orthotopic models are necessary to predict therapy of transplantable tumors in mice. *Cancer Metastasis Rev.* 1998; 17(3):279–284. [PubMed: 10352881]
23. Kuniyasu H, Yasui W, Shinohara H, et al. Induction of angiogenesis by hyperplastic colonic mucosa adjacent to colon cancer. *Am J Pathol.* 2000; 157(5):1523–1535. [PubMed: 11073812]
24. Hewitt RE, McMarlin A, Kleiner D, et al. Validation of a model of colon cancer progression. *J Pathol.* 2000; 192(4):446–454. [PubMed: 11113861]
25. Ramachandran C, Nair PK, Alamo A, et al. Anticancer effects of amooranin in human colon carcinoma cell line in vitro and in nude mice xenografts. *Int J Cancer.* 2006; 119(10):2443–2454. [PubMed: 16894569]
26. Schluter K, Gassmann P, Enns A, et al. Organ-specific metastatic tumor cell adhesion and extravasation of colon carcinoma cells with different metastatic potential. *Am J Pathol.* 2006; 169(3):1064–1073. [PubMed: 16936278]
27. Thalheimer A, Otto C, Bueter M, et al. The intraportal injection model: a practical animal model for hepatic metastases and tumor cell dissemination in human colon cancer. *BMC Cancer.* 2009; 9:29. [PubMed: 19166621]
28. Thalheimer A, Otto C, Bueter M, et al. Tumor cell dissemination in a human colon cancer animal model: orthotopic implantation or intraportal injection? *Eur Surg Res.* 2009; 42(3):195–200. [PubMed: 19270457]
29. Kim J, Yu W, Kovalski K, et al. Requirement for specific proteases in cancer cell intravasation as revealed by a novel semiquantitative PCR-based assay. *Cell.* 1998; 94(3):353–362. [PubMed: 9708737]
30. Zijlstra A, Mellor R, Panzarella G, et al. A quantitative analysis of rate-limiting steps in the metastatic cascade using human-specific real-time polymerase chain reaction. *Cancer Res.* 2002; 62(23):7083–7092. [PubMed: 12460930]
31. Mira E, Lacalle RA, Gomez-Mouton C, et al. Quantitative determination of tumor cell intravasation in a real-time polymerase chain reaction-based assay. *Clin Exp Metastasis.* 2002; 19(4):313–318. [PubMed: 12090471]
32. Deryugina EI, Zijlstra A, Partridge JJ, et al. Unexpected effect of matrix metalloproteinase down-regulation on vascular intravasation and metastasis of human fibrosarcoma cells selected in vivo for high rates of dissemination. *Cancer Res.* 2005; 65(23):10959–10969. [PubMed: 16322244]
33. Hagedorn M, Javerzat S, Gilges D, et al. Accessing key steps of human tumor progression in vivo by using an avian embryo model. *Proc Natl Acad Sci USA.* 2005; 102(5):1643–1648. [PubMed: 15665100]
34. Stupack DG, Teitz T, Potter MD, et al. Potentiation of neuroblastoma metastasis by loss of caspase-8. *Nature.* 2006; 439(7072):95–99. [PubMed: 16397500]
35. Lewis JD, Destito G, Zijlstra A, et al. Viral nanoparticles as tools for intravital vascular imaging. *Nat Med.* 2006; 12(3):354–360. [PubMed: 16501571]
36. Lester RD, Jo M, Montel V, et al. uPAR induces epithelial-mesenchymal transition in hypoxic breast cancer cells. *J Cell Biol.* 2007; 178(3):425–436. [PubMed: 17664334]

37. Zijlstra A, Lewis J, Degryse B, et al. The inhibition of tumor cell intravasation and subsequent metastasis via regulation of in vivo tumor cell motility by the tetraspanin CD151. *Cancer Cell*. 2008; 13(3):221–234. [PubMed: 18328426]
38. Chambers AF, Schmidt EE, MacDonald IC, et al. Early steps in hematogenous metastasis of B16F1 melanoma cells in chick embryos studied by high-resolution intravital videomicroscopy. *J Natl Cancer Inst*. 1992; 84(10):797–803. [PubMed: 1573668]
39. Koop S, MacDonald IC, Luzzi K, et al. Fate of melanoma cells entering the microcirculation: over 80% survive and extravasate. *Cancer Res*. 1995; 55(12):2520–2523. [PubMed: 7780961]
40. Shioda T, Munn LL, Fenner MH, et al. Early events of metastasis in the microcirculation involve changes in gene expression of cancer cells. Tracking mRNA levels of metastasizing cancer cells in the chick embryo chorioallantoic membrane. *Am J Pathol*. 1997; 150(6):2099–2112. [PubMed: 9176401]
41. Kobayashi T, Koshida K, Endo Y, et al. A chick embryo model for metastatic human prostate cancer. *Eur Urol*. 1998; 34(2):154–160. [PubMed: 9693252]
42. Bobek V, Plachy J, Pinterova D, et al. Development of a green fluorescent protein metastatic-cancer chick-embryo drug-screen model. *Clin Exp Metastasis*. 2004; 21(4):347–352. [PubMed: 15554391]
43. Nguyen M, Shing Y, Folkman J. Quantitation of angiogenesis and antiangiogenesis in the chick embryo chorioallantoic membrane. *Microvasc Res*. 1994; 47(1):31–40. [PubMed: 7517489]
44. Seandel M, Noack-Kunmann K, Zhu D, et al. Growth factor-induced angiogenesis in vivo requires specific cleavage of fibrillar type I collagen. *Blood*. 2001; 97(8):2323–2332. [PubMed: 11290594]
45. Zijlstra A, Seandel M, Kupriyanova TA, et al. Proangiogenic role of neutrophil-like inflammatory heterophils during neovascularization induced by growth factors and human tumor cells. *Blood*. 2006; 107(1):317–327. [PubMed: 16174763]
46. Deryugina EI, Quigley JP. Chapter 2. Chick embryo chorioallantoic membrane models to quantify angiogenesis induced by inflammatory and tumor cells or purified effector molecules. *Methods Enzymol*. 2008; 444:21–41. [PubMed: 19007659]
47. Leibovitz A, Stinson JC, McCombs WB 3rd, et al. Classification of human colorectal adenocarcinoma cell lines. *Cancer Res*. 1976; 36(12):4562–4569. [PubMed: 1000501]
48. Ellis LM, Liu W, Wilson M. Down-regulation of vascular endothelial growth factor in human colon carcinoma cell lines by antisense transfection decreases endothelial cell proliferation. *Surgery*. 1996; 120(5):871–878. [PubMed: 8909524]
49. von Reyher U, Strater J, Kittstein W, et al. Colon carcinoma cells use different mechanisms to escape CD95-mediated apoptosis. *Cancer Res*. 1998; 58(3):526–534. [PubMed: 9458101]
50. Hegde P, Qi R, Gaspard R, et al. Identification of tumor markers in models of human colorectal cancer using a 19,200-element complementary DNA microarray. *Cancer Res*. 2001; 61(21):7792–7797. [PubMed: 11691794]
51. Palmer HG, Gonzalez-Sancho JM, Espada J, et al. Vitamin D(3) promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of beta-catenin signaling. *J Cell Biol*. 2001; 154(2):369–387. [PubMed: 11470825]
52. Tan B, Wang JH, Wu QD, et al. Sodium hyaluronate enhances colorectal tumour cell metastatic potential in vitro and in vivo. *Br J Surg*. 2001; 88(2):246–250. [PubMed: 11167875]
53. Sengupta PK, Smith EM, Kim K, et al. DNA hypermethylation near the transcription start site of collagen alpha2(I) gene occurs in both cancer cell lines and primary colorectal cancers. *Cancer Res*. 2003; 63(8):1789–1797. [PubMed: 12702564]
54. Wang JH, Manning BJ, Wu QD, et al. Endotoxin/lipopolysaccharide activates NF-kappa B and enhances tumor cell adhesion and invasion through a beta 1 integrin-dependent mechanism. *J Immunol*. 2003; 170(2):795–804. [PubMed: 12517943]
55. Ottaiano A, di Palma A, Napolitano M, et al. Inhibitory effects of anti-CXCR4 antibodies on human colon cancer cells. *Cancer Immunol Immunother*. 2005; 54(8):781–791. [PubMed: 15592929]

56. Schimanski CC, Schwald S, Simiantonaki N, et al. Effect of chemokine receptors CXCR4 and CCR7 on the metastatic behavior of human colorectal cancer. *Clin Cancer Res*. 2005; 11(5):1743–1750. [PubMed: 15755995]
57. Sonvilla G, Allerstorfer S, Stattner S, et al. FGF18 in colorectal tumour cells: autocrine and paracrine effects. *Carcinogenesis*. 2008; 29(1):15–24. [PubMed: 17890768]
58. Walker T, Mitchell C, Park MA, et al. Sorafenib and vorinostat kill colon cancer cells by CD95-dependent and -independent mechanisms. *Mol Pharmacol*. 2009; 76(2):342–355. [PubMed: 19483104]
59. Liu K, McDuffie E, Abrams SI. Exposure of human primary colon carcinoma cells to anti-Fas interactions influences the emergence of pre-existing Fas-resistant metastatic subpopulations. *J Immunol*. 2003; 171(8):4164–4174. [PubMed: 14530339]
60. Katayama M, Nakano H, Ishiuchi A, et al. Protein pattern difference in the colon cancer cell lines examined by two-dimensional differential in-gel electrophoresis and mass spectrometry. *Surg Today*. 2006; 36(12):1085–1093. [PubMed: 17123137]
61. Conn EM, Botkjaer KA, Kupriyanova TA, et al. Comparative analysis of metastasis variants derived from human prostate carcinoma cells. Roles in intravasation of VEGF-mediated angiogenesis and uPA-mediated invasion. *Am J Pathol*. 2009; 175(4):1638–1652. [PubMed: 19729488]
62. Deryugina EI, Conn EM, Wortmann A, et al. Functional role of cell surface CUB domain-containing protein 1 in tumor cell dissemination. *Mol Cancer Res*. 2009; 7(8):1197–1211. [PubMed: 19671673]
63. de Paulis A, Prevete N, Fiorentino I, et al. Expression and functions of the vascular endothelial growth factors and their receptors in human basophils. *J Immunol*. 2006; 177(10):7322–7331. [PubMed: 17082651]
64. Kanai T, Konno H, Tanaka T, et al. Anti-tumor and anti-metastatic effects of human-vascular-endothelial-growth-factor-neutralizing antibody on human colon and gastric carcinoma xenotransplanted orthotopically into nude mice. *Int J Cancer*. 1998; 77(6):933–936. [PubMed: 9714067]
65. Wang S, Liu H, Ren L, et al. Inhibiting colorectal carcinoma growth and metastasis by blocking the expression of VEGF using RNA interference. *Neoplasia*. 2008; 10(4):399–407. [PubMed: 18392139]
66. Steeg PS. Tumor metastasis: mechanistic insights and clinical challenges. *Nat Med*. 2006; 12(8):895–904. [PubMed: 16892035]
67. Horak CE, Steeg PS. Metastasis gets site specific. *Cancer Cell*. 2005; 8(2):93–95. [PubMed: 16098461]
68. Romanoff, AL. The avian embryo. New York: The Macmillan; 1960.
69. Li A, Varney ML, Singh RK. Expression of interleukin 8 and its receptors in human colon carcinoma cells with different metastatic potentials. *Clin Cancer Res*. 2001; 7(10):3298–3304. [PubMed: 11595728]
70. Fernandez-Garcia NI, Palmer HG, Garcia M, et al. 1alpha, 25-Dihydroxyvitamin D3 regulates the expression of Id1 and Id2 genes and the angiogenic phenotype of human colon carcinoma cells. *Oncogene*. 2005; 24(43):6533–6544. [PubMed: 16007183]
71. Shaheen RM, Ahmad SA, Liu W, et al. Inhibited growth of colon cancer carcinomatosis by antibodies to vascular endothelial and epidermal growth factor receptors. *Br J Cancer*. 2001; 85(4):584–589. [PubMed: 11506500]

Fig. 1.

a and **b** Metastatic potential of SW480 and SW620 colon carcinomas in the chick embryo spontaneous metastasis models. Colon carcinoma SW480 and SW620 cells (5×10^6) and fibrosarcoma HT-hi/diss cells (2.5×10^5) were grafted on the CAM of day 10 chick embryos. Seven days later, the primary CAM tumors were excised and weighed (**a**) and the distal portions of the CAM were analyzed by *Alu* qPCR to determine relative numbers of human cells within chicken background (**b**). The average weight of HT-hi/diss tumors was 479 ± 81 mg ($n = 9$), while SW480 and S620 cells generated tumors of 106 ± 9 mg ($n = 12$) and 167 ± 17 mg ($n = 13$), respectively. The average number of HT-1080 cells disseminated to the CAM was $1,197 \pm 300$ cells per 10^6 chicken cells, while SW480 cells and SW620

cells were detected at 7 ± 3 and 12 ± 3 per 10^6 chicken cells, respectively. Presented are cumulative data from three independent experiments employing from 9 to 13 embryos per cell variant. Statistical significance was determined in comparison with SW480 group. * $P < 0.005$, ** $P < 0.001$, and *** $P < 0.0001$. **c** Metastatic potential of SW480 and SW620 colon carcinomas in the chick embryo experimental metastasis model. A total of 1×10^5 SW480, SW620 and HT-hi/diss cells were injected into the allantoic vein of day 12 chick embryos. Following 7 day incubation, the embryos were sacrificed and portions of the CAM were analyzed by *Alu* qPCR to determine the numbers of human cells that colonized the tissue. Data are presented as a cumulative scattergram from six independent experiments. The mean number of SW480 and SW620 cells colonized the CAM was 284 ± 117 ($n = 49$) and $4,791 \pm 543$ ($n = 46$), respectively. The mean number of HT-1080 cells constituted $4,505 \pm 1,164$ ($n = 14$). Lines represent mean number of human cells per 1×10^6 chicken cells. Statistical significance was determined in comparison with SW480 group. *** $P < 0.0001$

Fig. 2.

a Kinetic analysis of CAM colonization by SW480 and SW620 colon carcinoma cells. A total of 1×10^5 SW480 or SW620 cells were injected into the allantoic vein of day 12 chick embryos. Presented is one of two independent experiments employing from 5 to 7 embryos per each time-point. **b** and **c** Time-point analysis of the disappearance of SW480 cells from the CAM (**b**) or liver (**c**). A total of 1.5×10^5 SW480 cells were injected into the allantoic vein of day 12 chick embryos. The data are from a representative experiment employing from 5 to 7 embryos per each time-point. At the time points indicated in the graphs, the embryos were sacrificed and the numbers of human cells that colonized the tissue were determined by *Alu* qPCR

Fig. 3.

Immunohistological analysis of CAM colonization by SW480 and SW620 colon carcinoma cells. A total of 1×10^5 SW480 and SW620 cells were injected into allantoic vein of day 12 chick embryos. At the time points indicated in the figure panels, the embryos were sacrificed and portions of the CAM were processed for immunohistochemical analysis of human cells. Human cells were stained with anti-human pan-cytokeratin antibodies (*brown staining*) and the CAM tissue was counterstained with hematoxylin. While both SW480 cells (*left panels*) and SW620 cells (*central and right panels*) can be visualized 2 h after i.v. inoculation as individual cells arrested in the capillary plexus (arrowheads point to some of the capillaries in the plexus), only SW620 cells are readily detected after day 1 following injection. Rare

SW480 cells were found at day 9 after cell inoculation, at which time large clusters of SW620 cells protrude into the CAM mesoderm. Note CAM blood vessels and capillaries in close vicinity of SW620 clusters (*arrows*)

Fig. 4.

Live image analysis of CAM colonization by SW480 and SW620 cells. Colon carcinoma SW480 and SW620 cells were pre-labeled with green fluorescence CellTracker and injected i.v. into chick embryos. The CAM vasculature was highlighted with red fluorescence LCA 30–45 min before imaging. At indicated time points, the samples of unfixed CAM were visualized in a fluorescent microscope equipped with Nomarski differential interference contrast (DIC) prism. The boxed images on the left depict areas from the same field taken at different magnifications (original objective magnifications of $\times 10$, $\times 20$, and $\times 40$ and ocular magnification of $\times 10$). The images in the *boxes* on the far right represent separate fields acquired at the original magnification of $\times 400$. Arrows point to fragmented cells

representing apoptotic bodies. Honeycomb-like background is the ectoderm capillary plexus, where *dark holes* represent extravascular spaces. *Bars* are 100 μm for $\times 100$, 50 μm for $\times 20$, and 20 μm for $\times 400$ magnifications

Fig. 5.

a Angiogenic potential of SW480 and SW620 cells. Colon carcinoma SW480 and SW620 cells and HT-hi/diss fibrosarcoma cells were analyzed in the quantitative angiogenesis assay employing collagen onplants grafted on the CAM of shell-less chick embryos. Control onplants contained no tumor cells. Where indicated, anti-human VEGF antibody was incorporated into collagen mixture along with SW620 cells. Angiogenic index was determined on day 3 as a ratio of grids in the onplants containing newly-formed blood vessels over total number of grids scored. Data are presented as a scattergram from three independent experiments employing from 4 to 6 embryos per variable. Lines are medians. ** $P < 0.05$; *** $P < 0.001$. **b** Effects of anti-human VEGF antibody on experimental

metastasis of SW620 colon carcinoma cells. SW620 cells (1×10^5) were injected i.v. into day-12 chick embryos. Control IgG or anti-human VEGF antibody was inoculated twice, on day 1 and 3 after cell injections. CAM tissue was harvested on day 6 and analyzed by *Alu* qPCR for the relative numbers of human cells. Presented is a cumulative graph from three independent experiments employing 20 (control IgG) and 15 (anti-VEGF) embryos. * $P < 0.05$. **c** Immunohistological analysis of SW620 colonization. Sections of the CAM were harvested from the embryos injected with SW620 cells and treated with control IgG or anti-VEGF antibody and stained with anti-cytokeratin antibody to visualize human colon carcinoma cells (*brown staining*). *Upper panels* allow depict metastatic foci (clusters of brown-colored cells). *Bar*, 100 μm ; original magnification, $\times 40$. *Lower panels* depict *boxed* areas in the corresponding upper panels at higher magnification ($\times 200$). *Arrows* point to small blood vessels in the close vicinity of the tumor cell clusters. **d** The bar graph on the top illustrates the relative density of metastatic foci determined during immunohistochemical analysis as a number of tumor clusters per arbitrary square filled with CAM tissue. The *bar graph* on the bottom depicts the relative density of CAM vessels adjacent to metastatic foci. ** $P < 0.005$; *** $P < 0.0001$

Table 1

Colonization of internal organs by SW480 and SW620 colon carcinoma cells in comparison to HT-1080 fibrosarcoma

Internal organ	SW480 colon carcinoma	SW620 colon carcinoma	HT-1080 fibrosarcoma	<i>P</i> value
Liver	19 ± 2 (45)	54 ± 14 (69)	2,886 ± 462 (56)	<i>P</i> < 0.0001
Lung	7 ± 1 (7)	8 ± 3 (9)	353 ± 48 (37)	<i>P</i> < 0.005
Spleen	1 ± 1 (32)	8 ± 4 (34)	619 ± 382 (4)	<i>P</i> < 0.0001

The numbers of human cells per 1×10^6 chicken cells in the indicated organs were determined by *Alu* qPCR 5–7 days after i.v. inoculation of the cells. The data are means ± SEM. The number of analyzed embryos is indicated in parentheses. Significance (*P* value) of difference was calculated for HT-1080 cell data set versus sets for SW480 and SW620 cells