

Research proposal: Identification of a Key Metastasis-Associated Fibroblast- Specific Mediator in Colorectal Cancer Dissemination to Lung

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1. Introduction and state of the art

Metastasis, the process that results from cancer cells which spread through the body and generate new tumors at distant sites, is the cause of most of cancer-associated deaths. Indeed, most of the patients who suffer from metastatic disease cannot be cured by current therapeutic strategies(1). However, in contrast to the extensive knowledge acquired about the pathogenic mechanisms of primary tumor formation and development, the biological principles of metastasis remain obscure.

Metastatic disease generation is a complex multi-step process named “metastatic cascade”. It consists of the invasion of cancer cells into the adjacent parenchyma, the intravasation of these cells into the bloodstream and their survival in circulation, the arrest and extravasation at distant organ sites and the colonization of these new tissue environments(2,3). Metastatic colonization involves the transformation of micrometastasis into clinically detectable lesions, named macrometastasis. It seems to be the most limiting step of the cascade, with an efficiency as low as 0.01%(4). After traveling through the circulation, tumor cells find themselves in a new environment, distinct from the one present at the primary tumor site, to which they have to adapt to(5). The metastatic microenvironment, such as the primary tumor microenvironment, is composed of non-malignant cells, blood vessels, extracellular matrix and signaling factors which facilitate tumor progression(6,7).

The first contribution to the elucidation of the role of the tumor microenvironment in the metastatic context was made by Paget more than a hundred years ago, when he postulated the “seed and soil hypothesis”. He observed that breast cancer dissemination was not random, but it obeyed a specific pattern. Thanks to that he came up with the idea that cancer cells (the “seed”) colonize specific organs which microenvironment (the “soil”) favors their settlement and proliferation, which means that not all foreign tissue microenvironments are suitable for metastatic progression(8). Subsequent studies showed that many cancer subtypes follow characteristic spreading patterns, fact that support the existence of organ-specific metastatic niches or “fertile soils”(9).

Metastatic organotropism requires bidirectional interplay between the disseminated tumor cells and their host microenvironment. These cancer cells must undergo some phenotypic adaptation in order to form stable macrometastasis at distant sites(5). There is evidence that suggest that such adaptations are organ-specific, and they may thus partially depend on distinct signals emitted by the cells of the metastatic microenvironment. At the same time, cancer cells release factors which alter the metastatic microenvironment, as they do in primary tumor sites.

One of the cell types which more actively enhances the abilities of cancer cells in primary tumors are cancer associated fibroblasts (CAFs), which are now considered as a central character in tumor progression, as they enhance tumorigenesis, tumor invasion, angiogenesis and metastasis processes(10,11). They are similar to myofibroblasts, normal cells involved in physiological programs of wound healing and fibrosis(12). Some examples of mediators secreted by CAFs are the hepatocyte growth factor (HGF), members of the epidermal growth factor (EGF) family, matrix-metalloproteinases (MMPs) and various cytokines and chemokines(13). At the same time, tumor cells enhance the transformation of normal fibroblasts into CAFs through secretion of other factors, such as TGF- β (14). As it happens in primary tumors, fibroblasts of distant organ sites may also be important for metastasis development, but there are fewer studies which describe their functions in this context. These metastasis-associated fibroblasts (MAFs) may establish a fine-tuned crosstalk with cancer cells and potentiate their adaptation to the new microenvironment in an organ-specific manner. Indeed, O’Connell et al. et al. reported that S100A4+ stromal cells, which are mainly fibroblasts, enhance

metastatic colonization through the secretion of several growth factors and ECM components, specially Tenascin-C and VEGF-A(15). However, the existence of specific molecules secreted by MAFs which may drive cancer specific metastatic colonization remains uncertain.

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths worldwide(16). In addition, ~20% of patients show metastasis at the time of diagnosis(17). CRC displays marked metastatic organotropism, being the liver the most common metastatic site, followed by the lung(18,19). The high frequency of liver metastasis can be easily explained by the design of the circulatory system, as the liver is the first organ encountered by CRC cells through the portal vein(3). By contrast, their predilection for the lung cannot be solely explained by a circulatory relationship. Therefore, there may be specific components in the lung microenvironment which mediate colonization of colorectal cancer cells and convert the lung tissue into a permissive “soil” for them. As fibroblasts comprise a great part of lung interstitial compartment(20), lung MAFs may contribute to “fertile soil” generation by secretion of specific molecules, which allow CRC cells metastatic lung colonization and enhance their malignant capabilities (Fig.1).

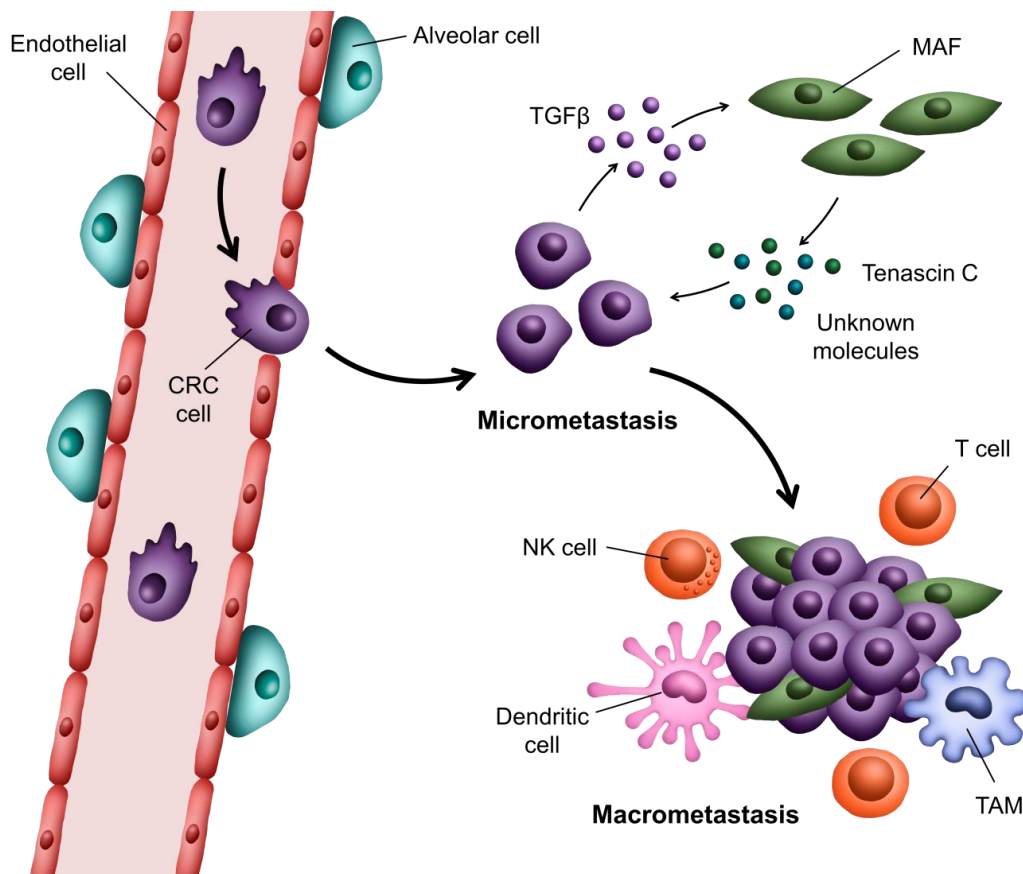


Figure 1. Metastatic colonization of the lung. CRC cells travel through the bloodstream and extravasate by crossing the lung capillaries. In the lung microenvironment, they interact with various cell types, such as metastasis-associated fibroblasts (MAFs), NK cells, T cells, tumor-associated macrophages (TAMs) and dendritic cells. Tumor cells establish a fine-tuned crosstalk with MAFs, by releasing mediators such as TGFβ, enhancing MAFs tumor-promoting capabilities. In addition, MAFs secrete other mediators, such as Tenascin C, which stimulate CRC cells malignant skills. *Self-created figure.*

2. Hypothesis

Our aim is to test the following hypothesis: pulmonary MAFs show a differential gene expression profile, which promotes CRC cells malignant skills and mediates their metastatic dissemination to the lung.

3. Research objectives

The global goal of this project is to disentangle the role of MAFs in CRC organ-specific dissemination to lung. Three specific aims are envisioned:

- **Aim 1** - To identify key mediators in MAFs-CRC cells lung metastasis-promoting communication.
- **Aim 2** - To elucidate the influence of MAF-specific molecules on CRC cells malignant abilities.
- **Aim 3** - To determine the contribution of MAF-specific molecules to lung metastatic colonization by CRC cells.

4. Research methodology and approach

4.1. Work Package 1 (WP1): Identifying key mediators in MAFs-CRC cells lung metastasis-promoting communication (→ Aim 1).

Approach: As mentioned above, in order to achieve lung colonization, CRC cells must undergo phenotypic adaptation, which may be facilitated by MAF-secreted molecules. These specific molecules may transform the lung microenvironment into a permissive niche suitable for tumor progression. However, there are no previous studies which define MAFs gene expression signature in colorectal lung metastasis. To characterize MAF-specific mediators, transcriptomes will be compared between MAFs and normal lung fibroblasts (NLFs) by transcriptomic microarray analysis of both cell types.

Methods (Fig.2):

Fibroblast isolation - MAFs and NLFs will be isolated from 8 patients with colorectal lung metastases subjected to complete pulmonary metastasectomy(21). These patients will show controlled primary tumors and no extrathoracic lesions, with the exception of hepatic lesions, according to the criteria for potentially curative operation(22). Before starting the procedure, written informed consent will be obtained from all the subjects. Tumor and normal lung tissue, which will be from as far from the tumor as possible, will be taken from the operating room, seared into small pieces and processed by collagenase digestion in order to obtain a single cell suspension. Then, NLFs and MAFs will be isolated by a method based on Fluorescence-Activated Cell Sorting (FACS) in which PDGFR α is used as a specific marker of both NFs and MAFs(23). After isolation and data processing by FACS sorter software (BD FACS Diva), cells can be either cultured (see 4.2) or lysed for RNA purification and further analysis.

Microarray analysis - After RNA extraction and integrity evaluation, complementary DNA will be synthesized from total RNA, then labeled with biotinylated nucleotides and assessed by hybridization to the Affymetrix U133A GeneChip. Data analysis will be performed using the Affymetrix Microarray Suite 5.0. Software. To find genes which define MAFs phenotype, we will discard those ones which expression do not change significantly between MAFs and NFs, and select those which are significantly overexpressed in MAFs samples. The statistical filters that will be used have been described by Kang et al.(24).

Histological validation - The expression of the MAF-specific molecule previously identified will be evaluated on human lung tissue by immunohistochemistry. Colorectal lung metastasis and normal lung samples will be obtained from a tumor bank and compared.

Expected outcome: From microarray data analysis we expect to identify few MAF-specific molecules. One of them will be selected for validation according to previous research evidence or database information. From histological validation we expect to find significantly higher expression of the assessed protein in lung metastatic lesions compared to normal lungs. If validation is successful, the molecule will be used for further experiments. If not, we will take into account this result and select another possible MAF-specific mediator from the microarray data analysis results.

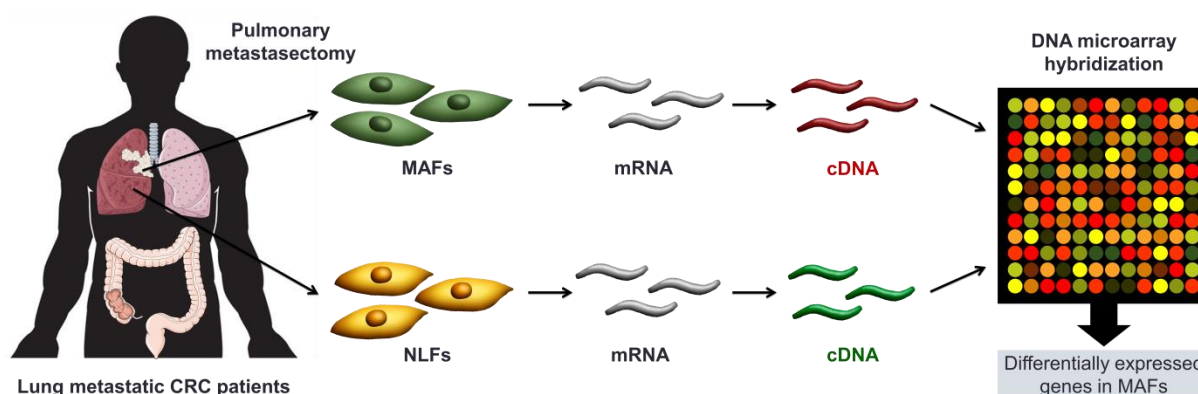


Figure 2. Work Package 1 summary. MAFs and NLFs will be isolated from tumor and normal tissue, respectively, from patients suffering from lung metastatic CRC disease. Total mRNA will be extracted from both cell types, complementary DNA will be synthesized and labeled, and subsequently hybridized to a DNA microarray chip. After statistical analysis, differentially expressed genes in MAFs will be identified. *Self-created figure.*

4.2. Work Package 2 (WP2): Elucidating the influence of MAF-specific molecules on CRC cells malignant abilities (→ Aim 2).

Approach: It is well known that CAFs play a crucial role in proliferation, invasiveness and angiogenesis of cancer. Thus, we hypothesize that MAFs are also relevant for malignant abilities acquisition by tumor cells. The effect of unaltered MAFs will be compared to the effect of siRNA-transfected MAFs (MAF*s), which do not express the protein identified in 4.1. To test our hypothesis we will characterize CRC cells phenotype by using various in vitro assays after MAF or MAF*-derived conditioned medium (MAF-CM/MAF*-CM) exposure of HCT 116 cells, a human colorectal adenocarcinoma cell line. The features that will be evaluated are the following: proliferation, invasion

and viability in presence of oxaliplatin, the main chemotherapeutic agent employed for the treatment of metastatic colorectal cancer(25).

Methods (Fig.3):

Primary culture of MAFs from patients: After fibroblast isolation by FACS (see 4.1), MAFs from 4 patients will be plated uniformly on Petri dishes and cultured as previously described(26) in DMEM (Dulbecco's Modified Eagle Medium, Gibco) culture medium supplemented with 10% fetal bovine serum at 37°C, 5% CO₂ atmosphere.

MAF siRNA transfection: Half of MAF cultures will be transfected with siRNA in order to silence the expression of the MAF-specific mediator identified in 4.1., by using Fibroblast Transfection Kit from Altogen Biosystems following manufacturer's instructions. Transfection and siRNA silencing efficiency will be assessed by western blot as previously described by Nolte et al.(27).

MAF-CM and MAF*-CM production: For the production of both types of conditioned media, MAFs and MAF*s will be cultured in 2% fetal bovine serum-containing DMEM until they reach 80-90% confluence. After washing twice with Phosphate-Buffered Saline (PBS), fetal bovine serum-containing medium will be changed by albumin-containing medium. Fibroblasts will be maintained under these conditions for 48 hours, allowing them to secrete soluble mediators into the environment. Eventually, both CM will be collected, centrifuged to remove dead cells and sterile filtered(28–31).

Assessment of CRC cells skills: Every one of the following assays will be carried out by using the well characterized human adenocarcinoma cell line HCT 116. HCT 116 cells will be maintained in McCoy's 5a medium (ATCC) supplemented with 10% fetal bovine serum, at 37°C, 5% CO₂ atmosphere. Before each experiment, HCT 116 cells will be treated with MAF-CM or MAF*-CM and incubated for 48 hours(30).

Proliferation assay: Proliferation of treated HCT 116 will be evaluated by the 5-bromo-2'-deoxyuridine (BrdU) proliferation ELISA kit (Roche Diagnostics) according to the manufacturer's instructions, as the most accurate method of measuring cell proliferation is an estimation of DNA-synthesizing cells(32). BrdU is a thymidine analogue which can be detected by fluorescently labeled antibodies and subsequent flow cytometry. Cells will be seeded in 96-well plates and incubated with BrdU(33). The optical density will be measured at 405 nm and results will be compared between MAF-CM and MAF*-CM treated HCT 166 cells.

Viability assay: HCT 116 cells viability will be assessed by the methyl thiazolyl tetrazolium (MTT) assay, a quantitative colorimetric method which evaluates metabolic activity or mitochondrial functionality. It consists on metabolic reduction of MTT to insoluble formazan by the mitochondrial enzyme succinate dehydrogenase(34). After incubation with MAF-CM or MAF*-CM, cells will be seeded in 96-well plates and treated with increasing oxaliplatin concentrations (0.0001-1000µM) previously established by Lu et al.(35). 48 hours later, 10 µl of 5 mg/ml MTT salt (Sigma Aldrich) will be added to the cells, which will be incubated for 4 hours more. In order to solve the formazan colored crystals, MTT will be replaced by 10% sodium dodecyl sulphate solution(28). Finally, absorbance will be quantified at 575 nm with a microtiter plate reader, taking 690 nm as the reference wavelength to remove background signals(31). Absorbance values will be transformed into cell viability percentages by comparing them to the absorbance values of untreated cells (100% viability).

Invasion assay: CRC cell invasion assay will be done by using the “chemoinvasion assay”, first described by Albin et al. more than 30 years ago(36). It uses Matrigel, a soluble basement membrane extract prepared from an epithelial tumor in order to measure the invasive skills of tumor cells(37). The assay is performed in Boyden migration chambers, which consist in upper and lower compartments separated by a porous filter. An “attractant” is administered in the lower well, so if tumor cells are malignant, they will degrade the Matrigel layer and then migrate to the lower compartment(38). In this project, the invasive ability will be tested according to the procedure described by Both et al.(39). Briefly, after incubation with different CM, the culture media will be replaced by RPMI 1640 (Roswell Park Memorial Institute, Gibco). Perforated membranes will be first coated with fibronectin to promote attachment and then covered with Matrigel (gelatinized at 37°C for 20 minutes). Membranes will be assembled in a Boyden chamber and fetal bovine serum will be used as attractant. The upper chamber will be filled with RPMI medium and HCT 116 cells previously treated with different CM, and the system will be incubated for 40 hours. After removing the cells which do not accomplish migration, the membranes will be stained with crystal violet and the cells on the lower surface of the filter will be counted manually in five different microscopic fields.

Expected outcome: We expect to find out that HCT 116 cells grown in the presence of MAF-CM have more robust tumor-promoting hallmarks than HCT 116 cells treated with MAF*-CM, as the second CM does not contain the mediator identified in 4.1. It will also be possible that such mediator confers specific abilities to CRC cells and so we will see significant differences in part of the experiments. If we do not find any differences between HCT 116 cells cultured in distinct conditions, the expression of the specific molecule by MAFs might be triggered by metastatic colorectal cancer cells as a consequence of lung colonization, and such protein might not exert any effect on tumor cells capabilities.

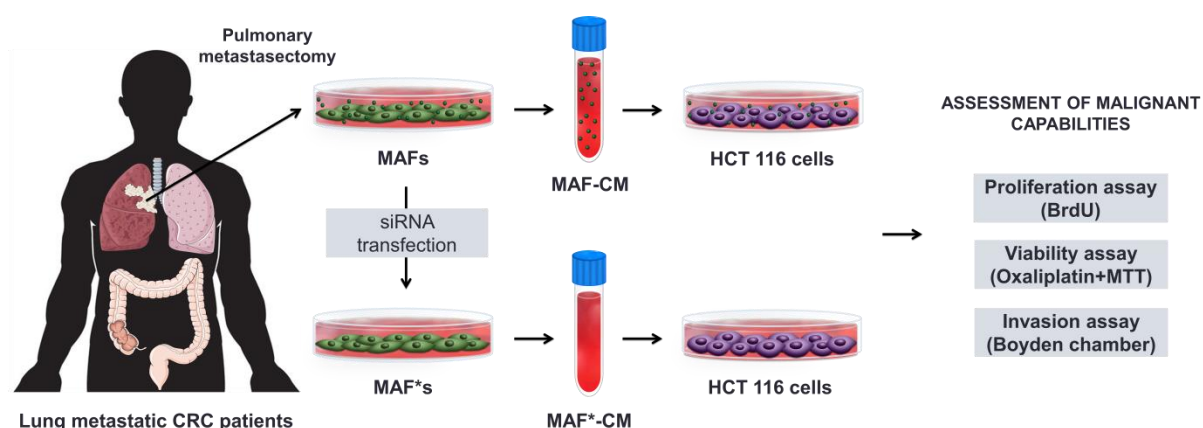


Figure 3. Work Package 2 summary. Previously isolated MAFs will be transfected with siRNA in order to silence the MAF-specific mediator (MAF*s). Conditioned medium (CM) will be obtained from unaltered MAFs and MAF*s, to treat HCT 116 cells. Subsequently, treated HCT 116 cells will be subjected to different assays in order to characterize their malignant capabilities. *Self-created figure.*

4.3. Work Package 3 (WP3): Determining the contribution of MAF-specific molecules to lung metastatic colonization by CRC cells (→ Aim 3).

Approach: As mentioned above, CRC cancer patients with advanced disease show a non-random metastatic spreading pattern, being liver and lung the most commonly affected distant sites, in that

order. In contrast to the liver, the leading position of the lung cannot be simply explained by the circulatory system anatomy, and so it might be due to particularities of the pulmonary microenvironment. In the last part of our project, we will try to elucidate if the presence of the mediator can explain at least partially the high frequency of pulmonary metastasis by playing a role in colonization. For this work, we will use a mouse model of metastatic CRC in which we will silence the expression of the mediator of interest in fibroblasts via conditional knockout technology. The occurrence and size of lung metastasis will be compared between ko mice and control mice, which fibroblasts express the mediator.

Methods (Fig.4):

Conditional knockout mice generation:

In order to generate mice whose fibroblastic cells do not express the identified mediator, we will use Cre-lox recombination technology. It consists in the expression of a site-specific DNA recombinase (Cre) and the introduction of two loxP (loci of recombination) sites, which will be recognized by Cre. Recombination results in deletion of the loxP-flanked sequence, and subsequently, in the inactivation of the gene of interest(40). For this purpose, two transgenic mouse strains are needed: one mouse strain with a loxP-flanked gene segment (identified mediator), known as floxed mouse, and a second mouse strain expressing Cre recombinase in a specific organ, tissue or cell type (fibroblasts). In our study, the progeny obtained by crossing both strains will express Cre recombinase only in fibroblasts, where recombination between both loxP sites will take place and the expression of the MAF-specific mediator will be silenced. All mice used will be athymic nude mice, as the immunodeficiency is essential to generate the CRC metastatic model explained below. The mouse strain harboring our mediator flanked by loxP sites will be created by gene targeting in embryonic stem cells as described by Meyers et al.(41). On the other hand, the second mouse strain, which will express the Cre recombinase under the influence of Fibroblast-Specific Protein promoter (Fsp-Cre) will be generated as previously reported by Bhowmick et al.(42). Conditional ko mice will be compared to unaltered athymic nude mice.

Metastatic CRC mouse model generation: Most genetically engineered CRC mouse models are not usually metastatic, fact that makes more difficult the study of this cancer stage. To solve this problem, Céspedes et al.(43) developed a technique based on orthotopic microinjection of human CRC cancer cells in nude mice, and they achieve metastatic foci generation in the most clinically relevant metastatic sites. Moreover, they characterized the metastatic potential of different human CRC cell lines, showing that HCT 116, which will be the cell line used in our project, generated lung metastasis in 50% of the mice. For our work, we will reproduce their orthotopic cell microinjection (OCMI) approach. Before starting the procedure, HCT 116 cells will be maintained as mentioned in 3.2. Briefly, four-week-old mice of both groups (ko and control) will be anesthetized with ketamine and xylazine, and their cecum will be exteriorized by laparotomy. HCT 116 cells will be suspended in DMEM and injected into the cecal wall by using an especially designed micropipette, described in their publication. The pipette must be introduced approximately 5 mm and with a 30° angle in order to obtain successful results. Finally, the intestine will be introduced into the peritoneal cavity and the incision will be closed with grapes. Mice will be maintained until death due to CRC or until the experiment finishes (120 days).

In vivo detection of colorectal tumors: Primary colorectal tumors apparition and growth will be assessed by manual palpation of the abdominal zone of the animals(44). In order to better characterize the primary tumors, once they have been detected, mice will be subjected to microcomputed tomography (microCT) colonography, as previously described by Pickhard et al.(45).

Mice will be anesthetized, and contrast material will be administered via the anal canal. Next, they will be scanned in the prone position by a microCT scanner (ImTek) by using the imaging parameters established in the publication. Images will be analyzed by employing AMIRA software (Thermo Scientific), which will provide us 2D sagittal, axial and coronal cross-sectional images, and then evaluated by a well-trained researcher, who will be uninformed about the type of mouse which is being analyzed. The frequency and the size of colorectal tumors will be compared between control mice and conditional ko mice.

In vivo detection of lung metastases: Mice which develop primary colorectal tumors will be next analyzed in order to detect the generation of lung metastases. In order to detect and measure lung metastatic masses, we will use microCT again, as it has been reported as a reliable non-invasive method of lung cancer progression monitoring in mice(46). Mice will be anesthetized and their chest area will be imaged by using a microCT scanner (ImTek). Images will be processed by AMIRA software and interpreted by an experienced researcher in a blinded manner.

Survival analysis: The cumulative survival of control and conditional ko mice will be estimated according to the Kaplan and Meier method(47) and plotted versus days after injection. The results will be analyzed by using Stata 7.0 software.

Expected outcome: We will expect that CRC incidence and primary tumor volume will not show significant differences between control and conditional ko mice, as the mediator is not expected to be involved in primary tumor formation but in metastatic colonization. Moreover, we will expect lung metastatic frequency and size to be higher in control mice, as they express the previously identified mediator. This result will mean that such molecule promotes lung colorectal cancer metastasis. In relation to the survival, we will expect it to be longer in ko mice, fact that will mean that the metastatic colonization process is partially impaired by the lack of the MAF-specific mediator.

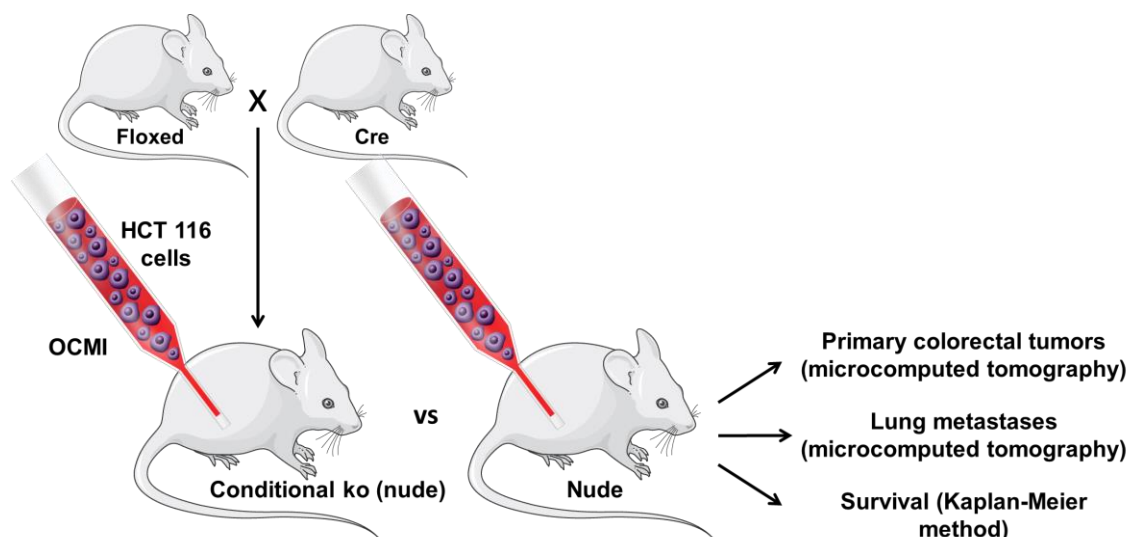


Figure 4. Work Package 3 summary. Conditional ko mice, whose fibroblasts do not express the previously identified mediator, will be generated by crossing a floxed mouse strain with a Cre mouse strain. Conditional ko mice and nude mice will be subjected to orthotopic cell microinjection (OCMI) of HCT 116 cells, directly into the cecal wall. Finally, primary colorectal tumor size and frequency, and lung metastases size and frequency will be assessed by microcomputed tomography. Survival will be analyzed according to Kaplan-Meier method. *Self-created figure.*

5. Coherence and effectiveness of the work plan

The project is structured in three work packages (WP), each related to its respective objective. All the WP are linked to each other, thus each has to be reached before starting the next one. The whole project has been planned to be carried out in 4 years by six researchers (three pre-doctoral students and three postdoctoral researchers) plus the principal investigator. Each pre-doc will work with a PhD, to favor the learning of the first ones and to increase the efficiency. Besides developing experimental procedures, all group members will attend various training activities, in order to speed the experimental set-up steps and get better quality results (Fig.5).

<u>Year 1</u>				<u>Year 2</u>		
Work Package 1 (→ Aim 1)				Work Package 2 (→ Aim 2)		
	Months 1-4	Months 5-8	Months 9-12	Months 1-4	Months 5-8	Months 9-12
EXPERIMENTAL PROCEDURES	CRC patients selection	Obtention of patient samples	Histological validation	MAF*s generation	Assessment of CRC cells malignant capabilities	
		Establishment of fibroblast primary cultures	Data analysis	CM production	Proliferation assay	Viability assay
		Microarray analysis		Establishment of HCT 116 cultures	Invasion assay	Data analysis
TRAINING ACTIVITIES	Establishment of primary cultures from patient samples			Boyden chamber assay training		
	Flow cytometry device handling					
	DNA microarray data analysis					

<u>Year 3</u>				<u>Year 4</u>		
Work Package 3 (→ Aim 3)						
	Months 1-4	Months 5-8	Months 9-12	Months 1-4	Months 5-8	Months 9-12
EXPERIMENTAL PROCEDURES	Conditional knockout mice obtention			HCT 116 cells injection		Data analysis
				In vivo monitoring of primary colorectal tumors and lung metastasis		
TRAINING ACTIVITIES	Laboratory animal manipulation	Orthotopic cell microinjection	MicroCT scanner manipulation			
			Radiographic image interpretation			
			Advanced image processing software			

Figure 5. Work plan for the proposed project. *Self-created figure.*

6. Dissemination plan

We will emphasize the spreading of the new knowledge generated by this project, as research dissemination is crucial for the progression of science. The primary ways of dissemination will be scientific publications in high impact international journals. Our work is likely to interest especially both doctors and biologists working on basic and translational cancer research; therefore we will select journals focused on these topics. Further, preliminary results will be presented by the group members at national and international conferences.

In addition to classic dissemination approaches, our outcome will be spread through press and social media releases, in order to reach the general public.

7. Discussion, conclusion and impact of the results

We consider that lung MAFs are specialized cells with their own characteristics. If we find transcriptomic differences between MAFs and normal pulmonary fibroblasts, this idea will be strengthened, as divergent expression profiles normally imply distinct functional phenotypes. It is widely accepted that CAFs differ functionally from normal fibroblasts(10–13), but the relationship between CAFs and MAFs has to be investigated in the future. However, the main pitfall of these types of studies lies in fibroblast heterogeneity in cancer patients(48), fact which means that our expected results might not be valid for all patients, and that further studies with a larger sample size must thus be performed.

Regarding the acquisition of malignant capabilities by CRC cells, we expect MAFs to act as enhancers, as CAFs do in primary tumors. If MAF-CM-treated tumor cells show higher proliferation rate, the mediator might facilitate the colonization of lung microenvironment in CRC patients, as transformation of pulmonary micrometastases into clinically detectable macrometastasis will occur in an easier way. What is more, it is known that chemoresistance to oxaliplatin happens to nearly all CRC patients(49). Therefore, if MAF*-CM-treated cells exhibit less viability when exposed to oxaliplatin than MAF-CM-treated cells, this could mean that MAFs contribute to resistance development. Finally, if MAF-CM-treated cells have a more invasive behavior, they might be more potentially metastatic in vivo, fact that would support the key role of the identified mediator in metastasis generation.

In relation to in vivo experiments, we expect to inhibit or decrease the generation of CRC lung metastases by silencing the identified mediator. These results will mean that such mediator is relevant for lung colonization by CRC cells and will shed some light on the puzzle of organ-specific metastasis development and “fertile soil” generation. Moreover, it could be tested as a target in lung disseminated CRC treatment and might contribute to the development of novel metastasis-specific therapeutic approaches which could improve the survival of patients suffering from a disease that nowadays is considered as almost incurable(50).

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