A high-throughput in vivo screening method in the mouse for identifying regulators of metastatic colonization

A high-throughput *in vivo* screening method in the mouse for identifying regulators of metastatic colonisation

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Abstract

We describe a sensitive, robust, high-throughput method for quantifying the ability of metastatic tumour cells to colonise a secondary organ. Metastasis is the leading cause of death in cancer patients and successful colonisation of the secondary organ is the rate-limiting step in the metastatic process, thus experimental methods that can be used to interrogate the key factors required for this critical step are of great importance. The experimental metastasis assay involves tail vein injection of cancer cells into the mouse and determination of the resulting secondary organ colonisation, primarily in the lung. This assay can be used to investigate factors that regulate metastatic colonisation both at the tumour cell intrinsic level (via manipulation of the tumour cells prior to injection) and tumour cell extrinsic level (such as the tissue microenvironment, via the use of genetically modified mice or agents such as antibodies, drugs, etc). With this method we have robustly screened more than 950 genetically-modified mouse lines to identify novel microenvironmental regulators of metastatic colonisation. Details are given on the appropriate cell numbers, handling of the cells, recipient animals, and injection techniques. Further, we discuss key experimental design considerations, including the choice of method used to determine metastatic burden and statistical analysis of the results, as well as providing trouble shooting tips and identification of the factors that contribute to experimental variability.

Introduction

Metastasis, a major contributor to cancer-related deaths, is a complex multi-step process, which involves the invasion of adjacent tissues, intravasation, transport through the circulatory system, arrest at a secondary site, extravasation and growth in a secondary organ (**Figure 1**). Tumour cells that have undergone intravasation are able to survive in the circulation with high efficiency and extravasate equally as efficiently, with >80% of cells successfully completing this process [reviewed in Chambers et al., 2000 [1]], Indeed, the ability to extravasate *in vivo* is not necessarily predictive of subsequent metastasis formation (with metastatic ras-transformed and control fibroblasts extravasating equally well [2]. Thus is it the post-extravasation steps that are much less efficient and more variable with *in vivo* videomicroscopy studies demonstrating that only a small proportion of extravasated cells begin to grow (~2% of injected cells), and only a subset of micrometastases continue to form macrometastases (~0.02% of injected cells) [3]. Therefore, the ability to grow and 'conquer' a foreign environment, i.e., to 'colonise' a secondary site, is the rate-limiting step in the metastatic cascade.

The colonisation of a secondary organ is dependent upon both tumour cell intrinsic and extrinsic factors. The importance of tumour cell intrinsic factors is highlighted by exome sequencing of primary and matched metastatic tumour tissues from patients to identify the genetic divergence of metastases from the primary tumour [4], as well as the identification of metastasis-suppressor genes, such as *NM23* and *KISS1* [reviewed in Hurst & Welch, 2011 [5]]. The importance of extrinsic factors, i.e., the microenvironment (or 'host'), which includes fibroblasts, immune cells, and endothelial cells, is highlighted by reports identifying constitutional genetic polymorphisms that substantially influence the metastatic efficiency of tumors (such as *Sipa1* [6]) and a recent study in which we used an experimental metastasis assay to screen 810 mutant mouse lines and identified 23 genes as potential host regulators of metastatic colonisation [7].

Tools for studying metastatic colonisation

Much *in vitro* work has been performed to assess the metastatic propensity of tumour cells by examining their abilities in terms of adhesion and invasion. However, whilst these characteristics are necessary for metastasis, by themselves they are not sufficient as cells that lack the ability to complete other steps of the metastatic cascade, such as successful growth at the secondary site ('colonisation'), may not form macroscopic metastatic lesions *in vivo* [8-10]. In addition, *in vitro* assays do not take into account tumour cell extrinsic factors, such as the surrounding stroma, and the haematopoietic system. Thus the study of metastasis is ideally performed *in vivo*. The chorioallantoic membrane of the developing chick embryo is rich in blood vessels and tumour cell xenografts can be injected onto the membrane and their ability to intravasate and disseminate can be monitored [11]. More recently, a high-throughput system for *in vivo* assessment of tumour cell intrinsic or microenvironmental modifiers of metastasis was described using zebrafish [12]. However, the mouse remains the most widely used *in vivo* system for modeling metastasis, due to their physiological and genetic similarities to humans.

Modelling metastasis in the mouse can be achieved through the use of autochthonously arising metastatic lesions or transplantation models (**Figure 1**) [reviewed in Khanna and Hunter, 2005 [13]]. Autochthonous ('endogenous') tumours capable of completing the entire metastatic process can be generated by the use of genetically-modified (GM) mice (such as *MMTV-PyMT* mice which develop mammary tumours that metastasize to the lung) and/or chemically-treated mice (such as 7,12-dimethylbenz(a)anthracene (DMBA)/12-O-tetradecanoyl-phorbol-13-acetate (TPA)-treated mice that form squamous cell carcinomas skin tumors that metastasize to the lymph nodes and lungs). However, it can take many months for the metastatic lesion to arise, and it can happen in a very asynchronous fashion (with great variability from mouse to

mouse), so is not amenable to high-throughput screening. In addition, it does not allow modification of tumour cell intrinsic factors to determine how they affect the ability of the tumour cell to metastasize. An alternative is the spontaneous metastasis assay, in which tumour cells are orthotopically transplanted into the mouse. However, it can take weeks for metastasis to occur, and unless subcutaneously/intradermally injecting cells, this technique comes with the added complication that it involves surgery (such as administration of tumour cells into the mammary fat pads or brain) or use of anaesthetics and an imaging apparatus (such as administration of cancer cells directly into the colon, kidney or lung). The alternative is the experimental metastasis assay, in which cells are injected directly into the circulation, can take as little as 7-10 days, does not require any imaging or surgical equipment or skills, and allows assessment of both tumour cell intrinsic and extrinsic factors of metastatic colonisation.

Development and applications of the method

The experimental metastasis assay determines the colonisation ability/potential of tumour cells by the measurement of the tumour burden in specific organs of the animal after injection of the tumour cells directly into the haematogenous circulation. It has been a long-standing technique in the metastasis field, with studies in the early 1950's demonstrating that the injection of rabbit V_2 carcinoma cells into either the portal vein/hepatic artery or a systemic vein led to the development of tumors in the liver or lung, respectively [14]. In support of the "seed and soil" concept that Stephen Paget hypothesized in 1889 to explain the non-random pattern of cancer metastasis [15], seminal work by Fidler and co-workers in the late 1970's used the experimental metastasis assay to demonstrate that the organ selectivity of metastatic cells is dependent on both tumour cell properties and host factors [16,17].

Here we have taken the fundamentals of the experimental metastasis assay, and generated a method that is robust enough to be performed as a bespoke or high-throughput assay, to identify both tumour intrinsic and extrinsic factors that regulate metastatic colonisation. Our protocol involves intravenous injection of tumour cells into the lateral tail vein of mice, followed by determination of metastatic burden of the secondary organ, primarily the lung, 10 days later (**Figure 2**). It can be performed using either murine or human tumour cell lines, in both immunodeficient and/or immunocompetent mice.

This method can be used to examine tumour cell intrinsic and extrinsic factors that can regulate metastatic colonisation. Tumour cell intrinsic factors can be examined via prior manipulation of the tumour cell line *in vitro* (such as by CRISPR/Cas9). This could be done using both a targeted approach, in which guide RNAs are used to target a single gene to determine the metastatic capabilities of that modified cell line, or as a screening approach, in

which a library of guide RNAs are used, to determine which gene(s) confers metastatic potential and enable successful colonisation of a secondary organ. This method can also be used to compare the metastatic potential of tumour cell lines that have undergone different selection processes *in vitro* or *in vivo* (**Figure 3a**). The cell lines can be either of murine origin, which allows injection into syngeneic wildtype or genetically modified mice and has the advantage of an immunocompetent host, or of human origin, if injected into immunodeficient mice (such as NOD-SCID mice), to avoid immunological rejection.

The role of tumour cell extrinsic factors can be examined by the use of GM mice carrying specific genetic modifications, such as null alleles, point mutations, gain-of-function alleles or conditional alleles. Using this protocol we have screened 950 genetically-modified mouse lines to discover novel genes that contribute to microenvironmental regulation of metastatic colonisation (**Figure 3b**; the results of 810 mutant lines screened has been previously described [7]). Examples of such genes include *Slc9a3r2* (*Slc9a3r2* mutant mice showed increased colonisation) and *Arhgef1* (*Arhgef1* mutant mice showed decreased colonisation; **Figure 3c**).

The use of bone marrow chimaeras, where the host animal is irradiated to eliminate the stem cells in their bone marrow so they can be replaced with donor bone marrow, allows examination of the role of the haematopoietic (immune) system in metastatic colonisation. For example, the decreased metastatic colonisation phenotype observed in *Arhgef1* mutant mice is mediated, at least in part, by the haematopoietic system, as bone marrow chimaeras given *Arhgef1* mutant bone marrow also showed a decreased metastatic colonisation phenotype (**Figure 3d**). Similarly, when used in combination with *in vivo* depletion techniques, in which animals are depleted of specific components of the haematopoietic system via administration of antibodies, the role of individual haematopoietic cell types in metastatic colonisation can be interrogated. For example, administration of anti-asialo ganglio-N-tetraosylceramide (ASGM1) antiserum to deplete natural killer (NK) cells, results in increased metastatic colonisation, confirming the key role of NK cells in controlling metastasis (**Figure 3e**). Finally, this method can also be used in a pre-clinical setting, to assess the effectiveness of potential therapeutic drugs on metastatic colonisation [reviewed in Steeg, 2016 [18]].

Limitations of the method

As the experimental metastasis assay involves injection of cancer cells directly into the bloodstream, it bypasses the early steps of the metastatic pathway, specifically the invasion of the cancer cells into the surrounding tissue and subsequent intravasation. However, it can be argued that these early steps are not the ‘critical’ ones as many tumour cells break away from

the primary tumour mass and can be found in the blood stream as ‘circulating tumour cells’ (CTCs). The ability to generate CTCs is however, not indicative of their metastatic potential as preclinical models have demonstrated that within 24 hours of intravenous administration, less than 0.1% of the tumour cells remain viable and that less than 0.01% of these surviving CTCs can produce metastases [19].

After tail vein injection, the first capillary bed that the tumour cells encounter are those of the lung (if they pass through the lungs, they enter the arterial system and eventually pass into the portal circulation) and thus the experimental metastasis assay using the tail vein route tends to favour colonisation of the lung. However, it can be argued that metastases in the lung are found in many cancer types (breast cancer, colon cancer, bladder cancer and melanoma, to name a few [20]), so it is a highly clinically relevant organ for investigation of colonisation factors. Furthermore, although not commonly used, there are some tumour cell lines that have been shown to colonise other organs after tail vein injection; EL-4 cells (a C57BL/6 dimethylbenzanthracene-induced thymoma line [21]), ESb cells (a DBA/2 methylcholanthrene-induced lymphoma line) [22] and RAW117-H10 cells (a BALB/c large-cell lymphoma line [23] all colonise the liver after tail vein injection. In contrast, other cells line colonise multiple organs, such the C57BL/6J methylcholanthrene-induced pleomorphic myofibrosarcoma ER 15-P cell line, that colonises the lungs, mediastinal lymph nodes, liver, kidneys and brain (with repeated administration of tumor cells from liver metastases into the tail vein leading to selection of a tumor cell line with a tendency to liver metastasis) [24]. Alternatively, injection of tumour cells into the portal vein or left cardiac ventricle leads to colonisation of the liver and bone/brain, respectively.

Experimental design

Mice should be matched by sex, age and genetic background as all of these factors can influence metastatic colonisation (**Box 1** and **Figure 4**). The experimenter should ensure that the experiment is performed in accordance with local animal research regulations. The tumour cell line should be thawed and passaged as minimally as possible prior to injection, to ensure they maintain their potency *in vivo*. In this protocol we provide details for the B16-F10 mouse melanoma cell line that is widely used, however, many other tumour cell lines can be used. After administration of the tumour cells, the metastatic burden of any tissue can be determined in three ways, depending upon the cell line administered (**Box 2** and **Figure 5**): specifically visually counting the number of lesions (if a pigmented cell line is used), counting of the number of lesions from H&E-stained sections of the tissue (applicable for all cell lines) or qPCR for the level of mCherry expression relative to vimentin expression (once the cell line has been

transfected with an mCherry-expressing plasmid; use of a fluorescent tag also means the metastatic lesions can be counted under a fluorescence microscope). Wherever possible, the individual performing the assessment of metastatic burden should be blinded to the identity of the samples (e.g., which samples came from the mutant/drug-treated mice versus the control/vehicle-treated mice, or which samples were from mice dosed with modified cells versus mice dosed with unmodified cells).

Statistical analysis

To avoid temporal effects confounding the statistical analysis, test and control samples should always be run concurrently. If a low sample size within a single experimental run, we recommend the use of the non-parametric Mann-Whitney to identify significant differences between the groups before assessing the biological significance using the 'metastatic ratio'. We suggest both a statistical significance test and biological effect size cut off to select a treatment for further screening (for instance in our screen we used a Mann-Whitney P value of ≤ 0.0175 with a metastatic ratio of ≤ 0.6 or ≥ 1.6 [7]). If significant in the initial cohort assayed, the experiment should be independently repeated an additional three times and the data from all cohorts processed through an integrative data analysis to determine those lines that were statistically significant. This two-step process ensures robust reproducible findings are identified.

Integrative Data Analysis [25] assesses for treatment effect across multiple experiments by treating each experiment as a fixed effect. We recommend implementing an iterative top down modeling strategy, starting with the most comprehensive model (either Eq. [1], or [2]) appropriate for the collection strategy implemented and ensuring the model only includes terms where the terms can be independently assessed [full details including code is published elsewhere: van der Weyden et al., manuscript submitted].

$$Y=\beta_0 + \beta_1 Sex + \beta_2 Experiment + \beta_3 Treatment + \beta_4 Sex*Treatment \quad [Eq.1]$$

$$Y=\beta_0 + \beta_2 Experiment + \beta_3 Treatment \quad [Eq. 2]$$

The optimisation process first selects a covariance structure for the residual, then the model reduced by removing non-significant fixed effects, and finally the treatment effect is tested and model diagnostics visualised. The issue of multiple testing can be managed by adjusting the calculated p values to either control the family wise error rate (FWER) or the more sensitive false discovery rate (FDR).

MATERIALS

REAGENTS

- Cells (typically established tumour cell lines, of human or mouse origin). **! CAUTION** Ensure the cells have been tested and found to be free of mycoplasma and any mouse pathogens.
- Cell culture medium (with serum and supplements) and reagents for growing, passaging and collecting the cells (depending on the specific requirements for those cells).
- Recipient mice (age-, sex- and strain-matched adult mice, ideally 6-8 weeks of age). **! CAUTION** All animal studies should be carried out according to all relevant governmental and institutional animal care and research legislation and regulations.
- Wet-ice.
- Phosphate buffered saline (without Ca^{2+} and Mg^{2+}).
- *Optional* (if performing analysis of metastatic burden by qPCR): reagents to extract genomic DNA from tissue homogenates (we use Qiagen Puregene Core A) qPCR kit for probe based assays (we use Qiagen QuantiNova probe PCR kit); qPCR consumables (96 or 384 well plate and seals); Vimentin (VIC conjugated primer limited) and mCherry (FAM conjugated) from Life Technologies (as shown below).

    Mouse vimentin (request primer limited format) - primer and probe sequences
    AGC TGC TAA CTA CCA GGA CAC TAT TG
    CGA AGG TGA CGA GCC ATC TC
    VIC - CCT TCA TGT TTT GGA TCT CAT CCT GCA GG – TAMRA

    mCherry- primer and probe sequences
    GAC CAC CTA CAA GGC CAA GAA G
    AGG TGA TGT CCA ACT TGA TGT TGA
    6FAM - CAG CTG CCC GGC GCC TAC A - TAMRA

EQUIPMENT

- Cell culture incubator (humidified, and supplied with CO_2, depending on the requirements of the cell line).
- Class II laminar flow hood for cell culture work.
- Pipette aid with sterile pipettes (10 mL plugged pipettes with marked graduations recommended).
- Centrifuge with swing-bucket rotor.

- Centrifuge tubes (15 mL and 50 mL conical recommended).
- Cryo-vials/microcentrifuge tubes.
- Pipette and pipette tips.
- Cell counter (electronic or haematocytometer).
- PPE for handling mice (as required by institutional regulations).
- Heated rodent chamber.
- Rodent restrainer.
- 1 mL syringes and 27' gauge needle.
- Necropsy equipment (scissors and tweezers, exact type depends upon personal preference).
- *Optional*: light microscope (if determining metastatic burden by H&E-stained paraffin sections) or method to homogenise tissues and qPCR instrument and analysis software (if determining metastatic burden by mCherry qPCR)

PROCEDURE

Passaging of cells to be injected • TIMING 30 min (2-3 d before animal injection)

1. Use standard procedures recommended for the specific cell line in use and passage the cells such that on the day of the experiment the cells are ~80% confluent. Ideally, the cells should be passaged no sooner than 2-3 days prior to the experiment, to ensure they are in log-phase growth.

   ☐**CRITICAL STEP** The cells should be passaged at least once after being removed from liquid nitrogen before being used in the assay, but no more than five times. Prolonged culture *in vitro* or passaging can lead to reduced tumourigenicity *in vivo*.

Preparation of cells to be injected • TIMING 30 min (day of injection)

2. Harvest cells as required in a culture hood, and determine their concentration using a cell counter (either electronic or haematocytometer).
3. Remove the appropriate amount of cell suspension required and centrifuge at 300 g for 3 min at room temperature.

   ☐**CRITICAL STEP** You should always prepare approximately 20% extra cell suspension as some cells will be lost in the void volume of the needle and syringe.

4. Discard the supernatant and resuspend the pellet in the appropriate volume of PBS (100 uL administered per mouse). Aliquot the cell suspension into cryo-vials/eppendorfs and place on wet-ice. Bring this to the animal house.

Preparation of the recipient mice • TIMING 10 min (day of injection)

5. In the animal room, turn on the hotbox and allow it to reach the desired temperature (38 ± 1°C).
6. Place mice inside the hotbox (each cage can go into a compartment of the hotbox, no need to separate the mice from one cage into individual compartments of the hotbox).
7. Have the rodent restrainer in position, as well as the required number of syringes and needles (depending on the number of mice to be dosed).

Injection of the mice • TIMING 10-40 min, depending on the number of mice

CRITICAL STEP You should be as efficient as possible, as the cells will deteriorate sitting in the PBS for long periods and you want to avoid a significant difference in the number of metastases between mice dosed at the beginning of a dosing session and the end of the session.

8. Mix the gently cells (by inverting the tube several times), fill the syringe and place the needle on the end. **! CAUTION** Remove all air bubbles before placing the needle on the end of the syringe and then again be sure to push the plunger till the cell suspension comes out the end of the needle – air bubbles can lodge in the small capillaries of the mouse and cause immediate death.
9. Immediately place a mouse from the hotbox into the restrainer and locate one of the two lateral tail veins (either by turning the tail 90° or rotating the whole restrainer 90°).
10. Inject the tail vein with the desired volume (typically 100-200 uL) and then remove the mouse from the restrainer and house as before the injection.

    CRITICAL STEP Ideally inject no more than ~5 mice per syringe to ensure that the cells are not sitting in the syringe for too long and have a chance to settle out of suspension. Use a fresh needle/syringe every ~5 mice to ensure the needle doesn't become blunt (making it more painful for the mice) and no cells that have come out of suspension are mixed in with fresh cells suspension being drawn into the syringe. **! CAUTION** Never draw up cells into the syringe with the needle attached as it may lyse them.

Determination of metastatic burden • TIMING 2 min to 2 days, depending on the number of mice and particular method used (3 methods listed below)

11. Humanely sacrifice the mice as required by your local authority and remove the organ(s) of interest. Typically this will be the lung thus all steps hereafter only mention “lung”, however depending on the cell line used, this may also be the liver and/or other organs.
12. Rinse the organ in PBS and dissect out all 5 lobes of the lung. □**CRITICAL STEP** Be sure to collect (and count) all 5 lobes, as a single lobe will not necessarily be representative of the metastatic burden of the entire organ (since metastatic burden is never equally distributed between the lobes).

(i) macroscopic counting • TIMING 2 min per mouse, depending on the number metastatic colonies (10-30 days post-injection, depending on the cell line used)

13. Either by eye or with the use of a dissecting microscope, count the number of metastatic colonies on the surface of all 5 lobes of the lung, on both sides of the lungs to obtain the total metastatic burden for that mouse. **! CAUTION** Care needs to be taken if Bouin’s solution is used to fix and stain the lungs to aid visualization of the metastatic colonies as this reagent is toxic (thus needs to be done in a ventilated fume hood).

(ii) H&E-stained paraffin sections • TIMING 2 days for processing of the tissues and 5 min counting per mouse, depending on the number metastatic colonies

14. Re-hydration, paraffin-embedding, sectioning and H&E-staining of the lungs can be performed by routine histopathology methods, and the number of metastatic colonies on the section of all 5 lobes of lung can be counted to obtain the total metastatic burden for that mouse.

(iii) mCherry qPCR • TIMING 2 days for DNA extraction of the tissues and 1-2 hours for qPCR

15. Homogenise all 5 lobes of the lung in 1 mL TBSTx (1x Tris-buffered saline with 0.5% Triton X-100).
16. Use 100 uL of the homogenate to extract genomic DNA (we use Puregene Core A, Qiagen) with the remainder of the homogenate being stored at -20°C for future use if desired.

17. Perform qPCR to determine the relative tumour burden – this can be achieved in a duplex reaction using a FAM-conjugated mCherry probe and VIC-conjugated Vimentin probe that is primer limited.
18. The qPCR reaction is performed in triplicate per tissue sample loading 50 ng of genomic DNA or 1 ng genomic DNA from the tumour cell line (positive control) or water and undosed tissue (both negative controls).
19. Setup the qPCR according to the manufacturer's instructions (we use Quantinova probe PCR kit) and perform 50 cycles of amplification.
20. Once complete determine the cycle threshold for each well for both the mCherry and vimentin probe.
21. Derive the relative tumour burden using the following equation:
    - Relative tumour burden = $100000 \times 2^{-\Delta CT}$
    - ΔCT = CT mCherry – CT vinculin (where there is no mCherry amplification, the CT value is set to 50 or the number of cycles used for amplification).

TIMING

Step 1, passaging of the cells to be injected: 30 min (2-3d before animal injection)

Step 2, preparation of cells to be injected: 30 min (day of injection)

Step 5, preparation of recipient mice for dosing: 10 min (day of injection)

Step 8, injection of the mice: 10-40 min (depending on the number of mice to be dosed)

Step 11, determination of metastatic burden: variable (depending on method used)

? TROUBLE SHOOTING

Trouble shooting advice can be found in Table 1.

ANTICIPATED RESULTS

The anticipated results with the experimental metastasis assay depend on several factors. The number (and tissue site) of metastatic colonies formed will be dependent upon the cell line used to dose the mice, the dose of cells used, and the mouse genetic background, sex, age and mutant status. For large-scale mouse phenotyping of metastatic colonisation using a dose of $4x10^5$ mouse melanoma B16-F10 cells, wildtype C57BL/6 mice typically show 100 or 150 pulmonary metastatic colonies at 10 days post-dosing when analysed by macroscopic counts (for males and females, respectively; values ≤ 35 counts indicate the entire dose was not

successfully administered into the vein). The successful procedure is dependent upon accuracy in locating and administering the full dosing volume to the tail vein, in a rapid and reliable manner.

BOX 1: Sources of variation in the experimental metastasis assay

Below we highlight factors that can significantly affect the metastatic colonisation rates and thus need to be considered in the experimental design.

Sex

We found that female mice had significantly higher rates of pulmonary metastatic colonisation (increased number of metastatic colonies on the lungs) compared to males (**Figure 4a**). Thus it is important to use sex-matched mice.

Genetic background

We found that the genetic background of the mouse can affect the number of pulmonary metastases (**Figure 4b**), which is most likely due to potential MHC (*H2*) haplotype differences between the strain of the mouse and the strain from which the tumour cell line was derived. For example, the B16-F10 mouse melanoma cell line is derived from a C57BL/6 mouse, and we find significant differences pulmonary metastatic colonisation of these cells between C57BL/6 and 129S5 mouse strains (which are both $H2^b$) and the BALB/c and CBA mouse strains, which are $H2^d$ and $H2^k$, respectively. Thus it is important to ensure that the control and mutant mice used in any experimental metastasis assay are strain-matched.

Age

We found that the age of the mouse can affect pulmonary metastatic colonisation ability, with younger mice having a higher tumour burden (**Figure 4c**). This may be a due to a relatively immature immune system and/or decreased blood volume in younger mice relative to older mice. Thus the age of the mouse is an important fact to consider and we recommend using mice at 6-8 weeks of age wherever possible.

Assay date

By performing the experimental metastasis assay as part of a large-scale phenotyping pipeline, we found that similar to other phenotyping variables [Karp et al., 2012; [26]], assay date is a major source of variation (**Figure 4d**). This variation arises from multiple sources, including

the researcher, culture and preparation of tumour cells and the cage. As this temporal variation has significant impact on data analysis, with counts done on the same day being more similar to themselves than counts done on another day, the solution is to compare mice assayed on different days using the 'metastatic ratio' (MR). The MR is calculated by dividing the counts for the experimental mice by the counts for the control mice dosed on the same day (this can be either mutant mice versus control mice (**Figure 3b**) or mice dosed with modified cells versus control cells).

Statistical power

Using C57BL/6 wildtype data, we estimated that the average metastatic burden for mice dosed with $5x10^5$ B16-F10 cells was 186 with standard deviation of 44 for males and 256 with a standard deviation of 87 for females (from a study comparing male and female animals, n=13/group). Where statistical power (or sensitivity) is the probability of making the correct decision when the biological effect actually exists, a power analysis for a two-tailed Mann-Whitney test, using G*Power 3.1.9.2, assuming the underlying distribution is normal, found a 60% change in mean (equivalent to the metastatic ratio filter we routinely use) had a statistical power of 0.921 and 0.724 for males and females, respectively, at 0.05 significance threshold.

BOX 2: Methods to determine metastatic burden of a tissue

Below we detail the different techniques that are available to determine the relative metastatic burden of a tissue after administration of tumour cells to the mouse.

Macroscopic

Macroscopic counting of the number of metastatic lesions by eye (or under a low-powered/dissecting microscope) immediately after dissection has the advantage that it is quick, easy and no specialized skills or equipment are required. The disadvantage of this, however, is that it is only applicable to pigmented cell lines, such as melanomas. Alternatively, it is possible to fix organs in Bouin's solution for 24 hours, followed by 100% ethanol washes, which makes the tumour cells appear white, and thus able to be counted.

Histopathology

Fixation of the relevant tissue, followed by embedding in paraffin, sectioning and haematoxylin and eosin (H&E) staining is a method that can be used to determine metastatic colonisation of any organ, by any tumour cell line administered. This technique has the

advantage that the tissue can be serially sectioned and thus the tumour burden of the whole tissue/organ can be determined. It can also allow for measurement of the individual metastatic lesions, thus can provide both quantitative and qualitative information about the metastatic colonisation phenotype of that mouse. It can also inform as to the presence of other cell types present in the organ, such as immune infiltrates. The disadvantage is that it depends on the researchers access to histology tools/services and/or a pathologist, and is not as quick as the macroscopic method.

Quantitative polymerase chain reaction (qPCR)

The use of qPCR to determine metastatic colonisation of an organ can be achieved once the tumour cell line has been transfected with a marker, typically mCherry. In this case, the tissue can be collected for DNA extraction and qPCR performed to assess the level of mCherry (from the tumour cells) relative to the level of vimentin (from both the tumour cells and the tissue; an internal control). The advantage of this technique is that the total tumour burden of the tissue can be assessed, rather than only what appears on the surface of the tissue (macroscopic method) or on the particular section (histopathology method). The disadvantage of this technique is that it requires transfection/drug selection of the cell line, which may be difficult depending on its amenability to be transfected and may also alter the *in vivo* properties of the cell line. This method also requires access to a qPCR machine.

FIGURE LEGENDS

Figure 1. Schematic of the metastatic process and experimental procedures to model metastasis in the mouse. A healthy cell acquires the necessary mutations to become malignant and proliferates to form a primary tumour mass. (1) Metastasizing tumour cells invade the basement membrane, and (2) undergo intravasation into the circulation (or lymphatics). (3) The circulating tumour cells must then survive, arrest and (4) extravasate the circulatory system. (5) Survival after arriving at the secondary site is the rate-limiting step of the metastatic process, as the newly arrived tumour cell can undergo apoptosis, remain as a single cell or occult micrometastasis which can remain dormant for years, or (6) proliferate and progressively colonise the organ. There are 3 ways to models of metastasis in the mouse, involving the autochthonous model, the spontaneous metastasis assay or the experimental metastasis assay. The steps of the metastatic pathway that these techniques mimic is indicated by the arrows, with the boxes detailing the nature and timeframe of each of these procedures.

Figure 2. Schematic of the experimental metastasis assay using tail vein injection. The tumour cells are thawed and subsequently passaged before being harvested for tail vein injection into recipient mice. Ten days later, the tissue(s) can be collected (lungs are the predominant colonisation organ after tail vein administration) and examined for their metastatic tumour burden either by eye (if the cells are pigmented, such as the B16-F10 mouse melanoma cell line shown), by light microscopy examination of haematoxylin and eosin-stained paraffin-embedded tissue sections (for all cell types, such as the MC-38 mouse colorectal adenocarcinoma cell line shown; x50 magnification) or by quantitative polymerase chain reaction (qPCR) of the tissue for mCherry expression levels relative to endogenous vimentin expression levels (for all cell types once transfected with an mCherry-expressing plasmid, such as the EO771.LMB mouse breast cancer cell line).

Figure 3. Applications for the experimental metastasis assay. a, Female mice were tail vein dosed with $1x10^5$ of either metastatic B16-F10 melanoma cells or highly metastatic B16-BL6 melanoma cells and the number of pulmonary metastatic foci counts at day 10. **b,** Mutant mouse lines (assayed with sex-, age-, and background matched wildtype controls) were tail vein dosed with B16-F10 cells and 10 days later the number of pulmonary metastatic foci counted by eye, with the result represented as a 'metastatic ratio' (mean number of metastases for the mutant line divided by the mean number of metastases for concurrently assayed wildtype controls, thus allowing comparison of mutant lines tested on separate days). **c,** Examples of mutant mouse lines, with respective controls (+/+), that show an ability to regulate metastasis with homozygous *Arhgef1* mutant (*Arhgef1*$^{tm1a(EUCOMM)Wtsi}$) mice showing decreased metastatic colonisation and homozygous *Slc9a3r2* mutant (*Slc9a3r2*$^{tm2a(EUCOMM)Hmgu}$) mice showing increased metastatic colonisation. **d,** Bone marrow chimaeras (irradiated female wildtype mice transplanted with donor bone marrow) were tail vein dosed with $4x10^5$ of B16-F10 melanoma cells and 10 days later the number of metastatic foci counted by eye. The donor bone marrow came from either wildtype (+/+) or homozygous *Arhgef1* mutant (*Arhgef1*$^{tm1a(EUCOMM)Wtsi}$) mice. **e,** Wildtype female mice were *in vivo* depleted for natural killer (NK) cells by intra-peritoneal (i.p.) administration of ASGM1 antibody at -3 days, -1 hour and +5 days, with tail vein dosing of $4x10^5$ of B16-F10 melanoma cells at day 0, and counting of the number of metastatic foci at day 10. Squares represent individual mice with error bars shown as SD, and statistical analysis performed using Mann-Whitney test (for a, c, d, e). Circles represent individual mutant mouse lines (b). Details on the cell lines and housing and husbandry of the mice used in these experiments are given in Supplementary File 1.

Figure 4. Effect of sex, genetic background and age on the experimental metastasis assay metastatic colonisation and variation of results of the assay over time. **a,** Male and female mice tail vein dosed with $5x10^5$ B16-F10 cells. Squares represent individual mice with error bars shown as SD. Statistical analysis was performed using Mann-Whitney t test. **b,** female mice tail vein dosed with $5x10^5$ B16-F10 cells, squares represent individual mice with error bars shown as SD. Statistical analysis was performed using a Kruskal-Wallis test with post hoc testing using a Dunn's multiple comparisons test. **c,** Female mice of different ages were tail vein dosed with $4x10^5$ B16-F10 cells. Squares represent individual mice with error bars shown as SD. Statistical analysis was performed using a Kruskal-Wallis test with post hoc testing of all groups against other using a Holm-Sidak's multiple comparisons test (the P value shown is comparing the 3 week age with each of the other age groups). **d,** Graph showing the variation in the metastasis count over a 46-month period (within the dates shown) of wildtype mice on a B6N background, aged 7.5 to 9.4 weeks, administered $4x10^5$ B16-F10 cells. Vertical ticks indicate individual assay dates, solid horizontal line indicates the mean and dashed horizontal line indicates the 95% confidence interval. The boxplot represents a five point summary of minimum, first quartile, mean, third quartile and maximum, which excludes outliers if they are >1.5 times the interquartile range (Q3-Q1) from the relevant quartile, and these are shown as circles on that day. Details on the cell lines and housing and husbandry of the mice used in these experiments are given in Supplementary File 1.

Figure 5. Methods for determining metastatic burden of a tissue. a, Representative macroscopic image of lungs from three individual female mice, tail vein dosed with $4x10^5$ B16-F10 mouse melanoma cells (the black spots on the pink lobes of the lung are the metastatic colonies that can be counted by eye). **b,** Representative image of H&E-stained lungs from a male mouse, tail vein dosed with $1x10^6$ LL/2 mouse lung carcinoma cells (x50 magnification; the box shows a large metastatic colony on a haematoxylin and eosin-stained, paraffin-embedded section of mouse lung). **c,** Representative image of Bouin's stained lungs from a male mouse, tail vein dosed with $1x10^6$ mouse LL/2 lung carcinoma cells (the white spots on the yellow lobes of the lung are the metastatic colonies that can be counted by eye). **d,** Comparison of two methods of determining pulmonary metastatic colonisation (counting from H&E-stained paraffin sections versus counting by eye) on female and male wildtype mice dosed with $4x10^5$ B16-F10 cells and the number of metastatic foci on the lungs of each mouse at day 10 first counted by eye, then put into formalin for histopathology processing (R^2 value determined using a second order polynomial (quadratic) non-linear fit). **e,** Wildtype female mice

tail vein dosed with $4x10^5$ of either B16-F10 mouse melanoma cells or F10CS cells (B16-F10 cells stably-expressing mCherry) and pulmonary metastatic foci counted at day 10, show no difference in metastatic potential between the two cell lines (i.e., stable transfection of mCherry has not altered the metastatic properties of the cells). Squares represent individual mice with error bars shown as SD, and statistical analysis performed using a Mann-Whitney test. **f,** Wildtype female mice tail vein dosed with different numbers of F10CS cells and 'metastatic burden' determined at day 10 by qPCR on DNA extracted from the lungs (expression level of mCherry relative to endogenous vimentin expression), demonstrate the sensitivity of the assay. **g,** Comparison of two methods of determining pulmonary metastatic colonisation (qPCR versus counting by eye) on female wildtype and mutant mice dosed with $4x10^5$ F10CS cells and the number of metastatic foci on the lungs of each mouse at day 10 first counted by eye, then DNA extracted from the lungs (R^2 value determined using a second order polynomial (quadratic) non-linear fit). **h,** Female wildtype (+/+) and homozygous *Arhgef1* mutant (*Arhgef1*$^{tm1a(EUCOMM)Wtsi}$) mice tail vein dosed with $4x10^5$ EO771.LMB cells and the lungs collected at day 10 for DNA extraction and qPCR (mCherry/vimentin). Squares represent individual mice with error bars shown as SEM, and statistical analysis performed using an unpaired two-tailed t test with Welch's correction. Details on the cell lines and housing and husbandry of the mice used in these experiments are given in Supplementary File 1.

AUTHOR CONTRIBUTIONS

L.v.d.W. and D.J.A. conceived the idea of screening mice from large-scale mouse phenotyping pipelines for metastatic colonisation. L.v.d.W, A.S. and A.O.S. performed the experiments. N.A.K. performed statistical analysis. M.J.A. performed histopathology analysis. L.v.d.W. and A.O.S. wrote the manuscript, with contributions from all authors.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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