MMP14 empowers tumor-initiating breast cancer cells under hypoxic nutrient-depleted conditions

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ABSTRACT: Tumor-initiating cells (TICs) existing in breast cancer are thought to be involved in initiation, progression, and relapse of tumors. In these processes, the epithelial-to-mesenchymal transition (EMT) and proteases are crucial factors that also depend on the tumor milieu, including hypoxic nutrient-deprived, as well as normoxic nutrient-rich, environments. Therefore, we investigated EMT and proteases in TICs and their response to different environments by means of a newly generated immortalized TIC (iTIC) line. With the use of primary CD24⁺CD90⁺CD45⁻ TICs from the mouse mammary tumor virus-polyoma middle T mouse breast cancer model, iTICs were generated by single cell-initiated sphere and subsequent 2-dimensional monolayer culture. Our data demonstrate the possibility to generate iTICs that are highly tumorigenic in culture and in mouse mammary fat pad. Contrasting environmental conditions provide these cells with a phenotypic and molecular plasticity that has a growth-promoting character in nutrient-rich normoxia and a motile character in nutrient-deprived hypoxia. Expression profiling revealed partial and dynamically changing EMT states, as well as a significantly up-regulated proteolytic signature, including many metalloproteinases, such as matrix metalloproteinase 14 (Mmp14). Inhibitor treatment of metalloproteinases, as well as short hairpin RNA-mediated knockdown of Mmp14 strongly impacted TIC characteristics, including tumor initiation, cell growth, migration, and invasion, especially in starved environments. We conclude that metalloproteinases empower TICs to adapt to changing environments.

Key Words: mammary carcinoma • phenotypic plasticity • proteases

Tumor cells do not share an equal capacity to initiate tumor growth, perform cell–cell interactions, invade, metastasize, and cause relapse or acquire resistance to chemotherapeutics (1, 2). This intratumoral heterogeneity can be explained by the cancer stem cell model that proposes a hierarchical organization of tumor cells with cells possessing stem-cell characteristics at the top of this hierarchy (3). These cells, referred to as cancer stem cells or tumor-initiating cells (TICs), give rise to the bulk tumor cells with genetic and therefore, functional variation (1–3). First identified in hematologic malignancies, TICs also reside within solid tumors, specifically human breast cancers (4–6). In the transgenic mouse mammary tumor virus-polyoma middle T (MMTV-PyMT) mouse model for metastasizing breast cancer (7–9), we have recently documented the existence of a TIC subpopulation (10). This population was identified by the cell surface marker profile CD24⁺CD90⁺CD45⁻, which also identifies TICs of the MMTV-Wnt1 mouse model and metastasis-initiating...
cells (MICs) of the MMTV-PyMT mouse model (11, 12). These primary TICs (pTICs) were highly tumor initiating upon orthotopic transplantation (OT) of only 100 cells into the mammary fat pad. They also showed classic TIC characteristics, including metastasis formation upon OT and a propensity for anchorage-independent growth in vitro. Furthermore, a significantly up-regulated proteolytic signature was identified in these cells compared with non-CD24+ CD90+CD45+ cells. Therefore, it is important to analyze the specific role of proteases in TICs in more detail.

The TIC niche within a tumor is characterized by hypoxia and nutrient scarcity, which arise as a result of the expanding tumor tissue, concomitantly with an emerging inadequate vascularization (17–20). However, the oxygen and nutrient microenvironmental conditions are not static but change dynamically with tumor growth. Consequently, TICs are exposed to varying degrees of oxygen and nutrient environments. The impact environmental conditions have on TICs and their specific TIC characteristic functions remain poorly characterized (21).

The detailed functional characterization of TICs remains challenging as a result of their small cell population size in primary MMTV-PyMT cancers (10). In addition, commercially available breast cancer cell lines possess only limited stem cell-like characteristics, with serial passaging associated with differentiation of the cells. Therefore, they do not completely reflect TIC characteristics and are not a good alternative for pTICs (22–24). Accordingly, there is a strong need emerging for the generation of an immortalized TIC (iTIC) line faithfully representing TIC functionality.

In this study, we report the successful establishment of an iTIC line from CD24+CD90+CD45+ pTICs of MMTV-PyMT breast cancers. Whereas their tumor-initiating capacity was successfully assessed in vitro and in vivo, we have additionally found these iTICs to possess a phenotypic and molecular plasticity in distinct environmental conditions. In addition to typical epithelial and mesenchymal markers, proteases were differentially regulated upon changing environmental conditions and displayed an important, functional role in classic TIC characteristics, including anchorage-independent growth, tumor initiation, invasion, and metastasis. We have identified the transmembrane MMP14 to be crucial for the execution of these TIC characteristic processes in hypoxic, nutrient-deprived conditions.

**MATERIALS AND METHODS**

**Animal model**

iTICs, as well as primary whole tumor cells, were generated from primary tumors of FVB/N-TgN(MMTV-PyVT)634Mul/J mice (7) that were used as a model for invasive metastasizing breast cancer, as previously described (10). Female Rag2<sup>−/−</sup>γ<sup>−/−</sup> lymphocyte-deficient mice (25, 26) were used for OT and lung colonization assays. All animal studies and the maintenance of mice were performed in accordance with the German Law for Animal Protection, (July 28, 2014).

**Orthotopic tumor cell transfer into the mammary fat pad**

iTICs incubated in hypoxic stem cell conditions (HSCCs) or normoxic standard conditions (NSCs) for 7 d; iTICs harvested from tumors developing from first OT of 1000 iTICs, which were fluorescence-activated cell sorting (FACS) sorted for a DAPI<sup>−</sup>, CD45<sup>−</sup> population; or iTICs with short hairpin (sh) control (shCtrl) or shMMP14 were resuspended in 25 μl PBS (Thermo Fisher Scientific, Waltham, MA, USA) at dilutions of 40, 100, 1000, or 4000 cells, mixed with an equal volume of Cultrex (Treivigen, Gaithersburg, MD, USA), and transplanted into the fourth mammary gland of female Rag2<sup>−/−</sup>γ<sup>−/−</sup> lymphocyte-deficient mice via a 5-mm lateral incision. Tumor growth was monitored by palpation, twice per week for up to 4 mo, followed by tumor harvest at 1 cm³.

**Histology and immunohistochemistry**

Isolated tumors and lungs were paraffin embedded, processed, stained with hematoxylin/eosin (both MilliporeSigma, Burlington, MA, USA), as well as Ki67 (Santa Cruz Biotechnology, Heidelberg, Germany), E-cadherin (Cdh1; 610182; BD Biosciences, San Jose, CA, USA), keratin 8 (ab59400; Abcam, Cambridge, United Kingdom), or vimentin (Vim; 550513; BD Biosciences), and analyzed as previously described (10).

**Immunofluorescence**

iTICs, cultured in HSCC or 7 d NSC, were seeded on glass coverslips. After fixation and permeabilization with 4% paraformaldehyde, 0.2% Triton X-100, and acetone, unspecific antibody binding was blocked with blocking solution. Primary antibodies were applied overnight at 4°C. On the next day, coverslips were washed with PBS-Tween and incubated with the secondary antibody for 1 h at room temperature. After Hoechst staining for 5 min in the dark, coverslips were mounted with Permaflour onto a slide and dried for 4–5 h in the dark before imaging on a Zeiss Axiovert microscope with Apotome.2 (Carl Zeiss, Oberkochen, Germany). Primary antibody used was anti-mouse MMP14 (ab78738; Abcam); secondary antibody used was goat anti-mouse Alexa 488 (A11001; Thermo Fisher Scientific).

**Fluorescence-activated flow cytometry**

Cells were resuspended in cytometry buffer [PBS (Thermo Fisher Scientific), 2% fetal calf serum (FCS; Pan, Aidenbach, Germany), 5 mM EDTA] and stained at a concentration of 1 × 10⁶ cells/µl. After the blocking of unspecific antibody binding with CD16/32 (553142; 1:1000; BD Pharmingen, Franklin Lakes, NJ, USA) on ice for 10 min, cells were stained with conjugated first antibodies anti-mouse CD24-phycocerythrin (PE; 561079; BD Pharmingen), anti-mouse CD49d-PECy5 (15090082; eBioscience, San Diego, CA, USA), anti-mouse CD45-PECy7 (2045182; eBioscience), anti-mouse CD49f-FITC (555753; BD Pharmingen), or unconjugated first antibody anti-mouse MMP14 (ab51074; Abcam) at appropriate
illiciation and invasion. For those assays, 60,000 iTICs, cultured in HSCC or NSC (7 d), were added to the cells every other week, iTICs were transferred into a 2-dimensional (2D) cell culture containing a 2% Cultrex supplement (Trevigen, Gaithersburg, MD, USA) and in an atmosphere of 3% O₂, 5% CO₂, and 92% N₂ at 37°C, referred to as HSCCs. After 10 wk of passaging, pTICs were cultured in a single cell-initiated 3-dimensional (3D) cell culture, according to the protocol by Castro et al. (22), including a serum-free mammary stem cell medium with a 50% Cultrex supplement (Trevigen, Gaithersburg, MD, USA) and in an atmosphere of 3% O₂, 5% CO₂, and 92% N₂ at 37°C, referred to as NSCs. After 10 wk of passaging the cells every other week, iTICs were transferred into a 2-dimensional (2D) cell culture containing a 2% Cultrex supplement (Trevigen). Cultivation in NSCs included an atmosphere of 21% O₂ and 5% CO₂ at 37°C, as well as standard medium DMEM (Thermo Fisher Scientific), 10% FCS (Pan), 1% penicillin/streptomycin, and 1% l-glutamine (both Thermo Fisher Scientific) for 1–7 d.

Cell culture of primary whole tumor cells

Primary whole tumor cells were obtained by mechanical and enzymatic disruption of solid PyMT primary tumors, as previously described (27). They were grown either in NSC or HSCC, as previously described.

Annexin V-propidium iodide flow cytometric analysis

Single cells were obtained from 3D cell culture by Dispase (Thermo Fisher Scientific), as well as Accutase (MilliporeSigma) treatment for 30 and 10 min, respectively, at 37°C and from 2D monolayer culture by Accutase (MilliporeSigma) or Trypsin (Thermo Fisher Scientific) treatment for 5 min at 37°C. Cells were resuspended in 1 time Annexin V binding buffer and stained with Annexin V-FITC (6419; Santa Cruz Biotechnology) and propidium iodide, as previously described (10). Cells were analyzed on a FACSCalibur (BD Biosciences).

Cell growth, migration, and invasion

With the use of the xCelligence system (ACEA Biosciences, San Diego, CA, USA), iTICs or primary whole tumor cells were analyzed for their growth and migratory and invasive capacities. For growth analysis, E-Plate 16 were used. iTICs (20,000), cultured either in HSCC or NSC (7 d), were added to each well in triplicate. CIM-Plate 16 was used for analysis of migration and invasion. For those assays, 60,000 iTICs, cultured in HSCC or NSC (7 d), were seeded into the upper chamber and forced to move along an FCS gradient for migration. For invasion, the upper chamber was additionally coated with 30 μl Cultrex (1:15 in medium), forcing the cells to cross a layer of basement membrane. The impedance was measured every 15 min for 72 h at 37°C in an atmosphere of 21% O₂ and 5% CO₂ for growth, migration, and invasion. The broad spectrum metalloproteinase inhibitor TNP-α protease inhibitor 0 (TAPI-0; Enzo Life Sciences, Lausanne, Switzerland) was applied at a concentration of 10 μM.

Spheroid-sprouting assay

iTICs, cultured in HSCC or NSC (7 d), or iTICs with shCtrl or shMMP14 were suspended in appropriate medium with 0.24% (w/v) methylcellulose (MilliporeSigma) and cultured in hanging droplets (500 cells per droplet) overnight, as previously described (28). After spheroid formation, they were embedded into a collagen I (BD PharMingen) matrix with 0.6% methylcellulose for 6 h in a μ-slide, 8-well IbiDi slide (IbiDi, Martinsried, Germany). Invasiveness of tumor spheroids was assessed by analyzing sprout length and number of sprouts per spheroid in phase-contrast pictures taken with a Keyence BZ-9000 microscope (Keyence, Neu-Isenburg, Germany) and using Fiji/ImageJ software (National Institutes of Health, Bethesda, MD, USA) for analysis. TAPI-0 (Enzo Life Sciences) was applied at a concentration of 10 μM.

 Colony-formation assay

Up to 5000 iTICs, as well as 2 independent isolates of primary PyMT (pPyMT) whole tumor cells (annotated as pPyMT 2153 or pPyMT 2157), or iTICs with shCtrl or shMMP14 were seeded in a top agar of 0.7% noble agar (MilliporeSigma) onto a 1% agar base per well, as previously described (10). Cultures were kept at 37°C for 4 wk, either in HSCC or NSC. Subsequently, colonies were stained with 0.005% crystal violet and 0.2% methanol. Colonies were counted per well using an Eclipse TS 100 microscope (Nikon, Tokyo, Japan). TAPI-0 (Enzo Life Sciences) was applied at a concentration of 10 μM.

MTT assay

Whole cell lysate protein samples (20–40 μg) of iTICs, cultured in HSCC or NSC (1–7 d), or iTICs with shCtrl or shMMP14 were subject to SDS-PAGE and transferred to a PVDF membrane (GE Healthcare, Little Chalfont, United Kingdom) via a semidy system (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 3% nonfat milk in PBS-Tween (0.1%) and incubated for 3 h under appropriate conditions (HSCC or NSC). Reduction of MTT was measured at a wavelength of 570 nm with an EnSpire Multimode Plate Reader (MilliporeSigma).

Western blot

Whole cell lysate protein samples (20–40 μg) of iTICs, cultured in HSCC or NSC (1–7 d), or iTICs with shCtrl or shMMP14 were subject to SDS-PAGE and transferred to a PVDF membrane (GE Healthcare, Little Chalfont, United Kingdom) via a semidy system (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 3% nonfat milk in PBS-Tween (0.1%) and incubated with primary antibodies for mouse-MMP14 (ab51074; Abcam) and mouse-a-tubulin (T9026; MilliporeSigma) overnight at 4°C. Membranes were washed and subsequently probed with the corresponding secondary antibodies goat anti-rabbit horseradish peroxidase (172-1019; Bio-Rad) and goat anti-mouse peroxidase (A0168; MilliporeSigma) for 2 h at room temperature, washed again, and developed using a West Pico/Femto Chemiluminescent substrate (Thermo Fisher Scientific) and Fusion SL Detection System imager (Vilber Lourmat, Eberhardzell, Germany).

shRNA-mediated Mmp14 knockdown and luciferase construct

Lentiviral shRNAs for Mmp14 were purchased from Dharmacon (The RNAi Consortium, Broad Institute, Cambridge, MA, USA).
The shRNA construct (shMMP14: TRCN0000031266 TTGC-TTCCTAAAGGGACACAG-3') included hairpins consisting of a 21-bp length stem (sense and antisense strands), separated by a 6 base noncomplementary loop. Hairpin sequences were cloned into the pLKO.1 vector. Stable iTICs were selected using Puromycin (MilliporeSigma). The plasmid construct for the introduction of luciferase into iTICs was kindly provided by Prof. Dr. Robert Zeiser (University Medical Center Freiburg, Germany). iTICs were stably transduced using the pMission transduction system (MilliporeSigma), as previously described (28).

Microarray analysis

Total RNA was isolated from iTICs cultured in HSCC and 1–7 d in NSC using thepeqGOLD Total RNA Kit (Peqlab, Erlangen, Germany). RNA quality control was performed using the RNA 600 Pico Assay Kit and Agilent Bioanalyzer 2100 (both Agilent Technologies, Santa Clara, CA, USA). Samples with an RNA integrity number > 8 and a final concentration of 100 ng/μl were subject to microarray analysis. HSCC and 7 d NSC samples were submitted as biologic triplicates. Analysis was performed at the Genomic Core Facility of the German Cancer Research Center (Heidelberg, Germany). Labeled total RNA was hybridized to a Mouse WG-6 v2.0 BeadChip (Illumina, San Diego, CA, USA), following the manufacturer’s protocol. Raw bead count data were analyzed using the R/Bioconductor package bead array package (29), followed by quantile normalization with prior background correction. Fold-change values of the expression of analyzed genes in NSC were calculated relative to HSCC. The appropriate P value was negatively adjusted by the false-discovery rate using the Benjamini-Hochberg method. Gene set enrichment analysis was applied using the R/Bioconductor package Gage (30), which tests for significant differential regulation of a gene set instead of individual genes. Pathways were considered significant with an adjusted P < 0.01 value (Benjamini-Hochberg) and were connected by an edge if they share 30% of their genes. Raw data were deposited in the Gene Expression Omnibus repository under the access identification GSE113826 (National Center for Biotechnology Information, Bethesda, MD, USA; https://www.ncbi.nlm.nih.gov/geo).

qRT-PCR

RNA was isolated from iTICs using the RNeasy Mini Kit (Qiagen, Hamburg, Germany) and transcribed to cDNA using the iSCRIPT cDNA synthesis system (Bio-Rad). The PyMT oncogene, as well as a panel of epithelial-to-mesenchymal transition (EMT) markers, was detected in quantitative RT-PCR (qRT-PCR) with the following primers: PyMT, forward 5'–TCCAACAGATACACCCGCCAC-3', reverse 5'-GTGCTTTGTGCTTTCTGGA-3'; Cdhl, forward 5'-GTCATCCAAAGTGAGCCTGGA-3', reverse 5’–CGGTGATGCTGAGAACCCTT-3'; N-cadherin (Cd2h), forward 5'-TTATGCCCAAGACAGAAGCC-3', reverse 5’–TTGCCAAGTGTCTAGGGAATAC-3'; Vim, forward 5’–TCCCTTGACGTTTCTGCC-3', reverse 5’–GGATGAGGAATAG-3'; Twist1, forward 5’–TTGGCAAGTTGTCTAGGGAATAC-3', reverse 5’–CCTGCTGAAAGCC-3'; b-actin, forward 5’–GCCAGCTTAGGACAGGATGG-3', reverse 5’–GGAAGCTGAGGACCAGGATGG-3' and normalized to β-actin.

Data presentation and statistical analysis

Quantitative values are represented as means ± SEM. For all statistical analysis, OriginPro 2016 and 2018 software (Microcal; OrigenLab Corp., Northampton, MA, USA) was used, using a 2-sided Student’s t test for comparison of 2 groups or a paired t test for comparing paired groups. For categorical data, the χ² test was performed. The Kaplan–Meier plot and log-rank test were used for tumor-free survival and tumor onset analysis. Principal component analysis (PCA) was used to describe EMT marker expression as linearly uncorrelated variables [principal components (PCs)]. iTIC frequencies were calculated using L-Calc by Stemcell Technologies (Vancouver, BC, Canada).

RESULTS

CD24+CD90+ derived, iTICs display phenotypic plasticity and tumor-initiating characteristics in vitro

In previous work, we examined the CD24+CD90+CD45− cancer cell population derived from breast tumors of MMTV-PyMT mice and found that those cells were highly tumorigenic in vitro and in vivo and showed a distinct expression signature of various proteolytic enzymes, including Mmp14. For closer investigation of this interesting TIC population, we set out to generate an iTIC line from primary-sorted CD24+CD90+CD45− cells following the protocol of Castro et al. (22). In brief, CD24+CD90+CD45− cells obtained from MMTV-PyMT breast cancers by FACS were cultured in a single cell-initiated sphere culture in Cultrex (Fig. 1A). Importantly, these cultures were kept under HSCCs (described in Materials and Methods). In 3D Cultrex culture, single cells started to form round and compact spheres of which some extended protrusions into the surrounding matrix after 6 d (Fig. 1B). Subsequent to 3D culture, iTICs were propagated in 2D culture (Fig. 1A). In the HSCC monolayer, iTICs displayed epithelial characteristics with cell islands forming close cell–cell contacts surrounded by scattered cells (Supplemental Fig. S1A).

Oxygenation and nutrient supply varies within a tumor (17, 20). Therefore, TIC characteristics under normoxic, nutrient-rich environments were additionally investigated by transferring iTICs into NSCs (described in Materials and Methods) for periods of 1–7 d (Fig. 1A). In NSC, contact-dependent growth of iTICs in a 3D Cultrex matrix resulted in smaller spheres, which did not extend strands into the surrounding matrix (Fig. 1B). Monolayer cultures in NSC induced extensive morphologic transformations in iTICs (Supplemental Fig. S1A), including increased cytoplasm, polygonucleation, and a spindle shape, with close contacts to adjacent cells. Therefore, epithelial, as well as mesenchymal, features were observed upon exposure to NSC.

In 3D Cultrex culture, the percentage of apoptotic cells reached 15 vs. 24% early apoptotic cells (HSCC vs. NSC, P < 0.05) and 32 vs. 24% late apoptotic cells (HSCC vs. NSC). However, this high cell death could be a result of the harsh conditions during retrieval of spheres from the Cultrex matrix. Therefore, we measured cell death in 2D monolayer culture. Here, the number of early apoptotic cells was 10 vs. 6% (HSCC vs. NSC) and 13 vs. 11% for late apoptotic cells (HSCC vs. NSC) with no significant difference between
Figure 1. Phenotypic plasticity and tumorigenic properties of iTICs in vitro. A) Scheme of the generation and culture of iTICs from primary-isolated CD24^+CD90^+CD45^- TICs. B) Anchorage-dependent growth of iTICs in a 3D Cultrex matrix. C) Cell-surface marker analysis of generated iTICs in HSCC and 1–7 d in NSC. Cells were stained for CD24 and CD90 and depleted for DAPI. D) Histogram plot of CD24 and CD90 cell-surface markers in iTICs cultured in HSCC and for 7 d in NSC. E) Cell-surface marker analysis of CD24 and CD90 in iTICs cultured in HSCC and iTICs cultured for 7 d in NSC that were reintroduced into HSCC for 7 d. F) Quantification of CD24^+CD90^+ double-positive cells, cultured either in HSCC, 1–7 d in NSC or 7 d NSC and reintroduction into HSCC for 7 d (all n = 4). G) Colony-forming capacity of iTICs cultured in HSCC (n = 9) and 7 d in NSC (n = 4).
HSCC and NSC (Supplemental Fig. S1B). We conclude that HSCC or NSC conditions do not have differential effects on iTIC cell death.

Next, the cell-surface marker profile of iTICs cultured in 2D monolayer was analyzed (Fig. 1C, F). Surprisingly, the surface-marker profile of CD24+CD90−-isolated iTICs changed in HSCC to a mainly CD24+ single-positive profile (93%). Exposure to NSC, however, led to an increase in CD24+CD90− double-positive cells, with 42% CD24+CD90− cells after 7 d of NSC compared with 2% double-positive cells in HSCC. In addition, iTICs in NSC (7 d) displayed a higher abundance of CD24 and CD90 on the cell surface compared with iTICs in HSCC, as indicated by the higher geometric mean of fluorescence (Fig. 1D). When iTICs cultured 7 d in NSC were re-exposed to HSCC for 7 d, the surface marker profile was reversed, with 1.3% of the cells remaining CD24+CD90− double positive (Fig. 1E, F). Interestingly, iTICs were almost 100% positive for the marker CD49f, which was used in the characterization of an MMTV-PyMT TIC population by Ma et al. (31) (Supplemental Fig. S1C). The marker remained stable between the 2 culture conditions and was therefore not further used for investigation of iTICs. Moreover, the morphology of the cells re-exposed to HSCC reverted partially back to a dense-clustered layer of cells typical for HSCC, which are interspersed by mesenchymal cells (Supplemental Fig. S1A). Taken together, these data suggest a phenotypic plasticity of iTICs that is dependent on environmental conditions.

To explore the functionality of iTICs in HSCC and NSC in more detail, anchorage-independent growth of iTICs in soft agar was analyzed (Fig. 1G). In HSCC, iTICs formed numerous defined, round, and dense colonies after 4 wk, with a size of ~300 μm in diameter that became clearly visible upon crystal violet staining (Fig. 1G, H and Supplemental Fig. S1D). In comparison, iTICs in NSC formed a significantly decreased number of colonies with a diameter only one-third the size (~100 μm; Fig. 1G, H), which resembles the colony-forming efficiency of CD24+CD90− CD45− cells freshly isolated from MMTV-PyMT breast cancers (10). Nevertheless, iTICs in both conditions formed more colonies than primary whole tumor cells from the MMTV-PyMT mouse model (pPyMT cells), either cultured in HSCC or NSC (Fig. 1G, H). Compared with iTICs in HSCC, pPyMT cells formed <0.05% colonies in HSCC and <0.13% colonies in NSC. Moreover, the assembled colonies were very small, with an average diameter of 50 μm. Hence, iTICs exhibit a high ability for anchorage-independent growth, especially in HSCC.

**iTICs are highly tumorigenic in vivo, which is influenced by environmental conditions**

To test the tumor-initiating ability of iTICs in vivo, cell dosages of 40, 100, 1000, or 4000 iTICs, cultured in HSCC or NSC, were transplanted into the mammary fat pad of female Rag2−/−γc−/− immunodeficient mice. The PyMT oncogene expression was not affected by the growth conditions (Supplemental Fig. S2A). Tumor initiation, detected as the first palpable tumor (diameter ~2 mm), ranged from 1 to 15 wk after transplantation (Fig. 2A). As expected, there was a longer latency of tumor detection in mice that received fewer cells. In addition, iTICs, cultured in NSC for 7 d before transplantation into the mammary fat pad, showed a trend for earlier tumor onset than HSCC-cultured cells, as well as faster tumor growth that started earlier for 4000 and 100 iTICs and later for 1000 and 40 iTICs (Fig. 2A and Supplemental Fig. S2B). This apparent difference for cell numbers and culture conditions, however, did not reach statistical significance. Remarkably, except for 4 mice that developed an abscess at the beginning of the study and had to be euthanized, all mice that received a transplant developed tumors, even those that had only been transplanted with 40 iTICs. Following the theory of limiting dilution, the tumor-initiating frequency was 1 in 23 cells for iTICs in HSCC, as well as in NSC (95% confidence interval of 12–47; Table 1). Interestingly, analysis of tumor volumes 3 wk after initial tumor detection revealed a higher volume of tumors derived from iTICs in NSC. Specifically, tumor volume was significantly larger in the group of tumors derived from 100 iTICs cultured in NSC, with an average volume of 1.63 cm^3 compared with tumors derived from 100 iTICs cultured in HSCC that had an average volume of 0.4 cm^3 (Fig. 2B).

To investigate the long-term, self-renewal ability of iTICs, which is a characteristic of stem cells and cancer stem cells (1), a serial passage of iTICs through a second round of OT was performed. Therefore, tumors derived from the first OT of 1000 iTICs, either cultured in HSCC or NSC, were harvested and sorted for a living CD45+ population of cells, of which 1000 were retransplanted into the mammary fat pad. Four mice were used per condition, and all mice rapidly developed tumors. In detail, after only 8 or 10 d, tumors derived from iTICs in NSC were palpable, whereas from iTICs in HSCC, all mice showed tumors after 14 d (Fig. 2C). As observed in the first round of OT, tumors were initiated significantly faster with iTICs derived from NSC (Fig. 2C). Three weeks after first detection, NSC-derived tumors were significantly larger compared with the tumors derived from HSCC (Fig. 2D).

Therefore, we decided to examine the ability of iTICs to grow and be metabolically active in vitro. iTICs in NSC showed an 8 times higher growth rate compared with iTICs in HSCC (Fig. 2E). In addition, iTICs in NSC reduced MTT significantly more efficient than iTICs in HSCC, indicating an increased viability and metabolic activity (Fig. 2F). Together, these results show enhanced growth and cell viability when iTICs are cultured in NSC. This could explain the earlier tumor onset and increased growth upon OT of NSC-treated cells.

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4) compared with pPyMT cells (2155, 2157) from the MMTV-PyMT mouse model cultured in HSCC (n = 2) or NSC (n = 4, **P < 0.01**).
Figure 2. NSCs promote tumorigenic properties of iTICs. A) Tumor onset (in weeks) after transplantation of 4000 (black; n = 6), 1000 (gray, n = 10), 100 (blue, n = 6), or 40 (green, n = 10) iTICs cultured in HSCC (diamond) or 7 d in NSC (circle) into the mammary fat pads of immunodeﬁcient mice. B) Tumor volumes 3 wk after transplantation of iTIC cultured in HSCC (black) and NSC (gray). Dashed lines, mean volume of all tumors per cell dosage. C) Kaplan–Meier plot of tumor onset of iTIC-derived tumors after second OT of 1000 iTICs derived and sorted (DAPI^− and CD45^−) from tumors from the ﬁrst transplantation of 1000 iTICs cultured either in HSCC (black) or 7 d NSC (gray) in days (all n = 4). D) Tumor volumes of iTIC-derived tumors after second transplantation. HSCC, black; NSC, gray. Dashed lines, mean volume of all tumors per condition. E) Cell growth of iTICs cultured in HSCC (black) and 7 d in NSC (gray; n = 3). The slope cell index was analyzed between 5 and 40 h. Values are relative to HSCC in percent, including a representative image of the cell growth curves (HSCC, blue; NSC, red). F) Metabolic activity of iTICs in HSCC (black) and 7 d in NSC (gray) measured by the reduction of MTT (n = 5). *P < 0.05, ***P < 0.005.
HSCC promotes cell motility and lung metastasis of iTICs

At the end of the transplantation experiment, the lungs were analyzed for the presence of lung metastases, originating from the engrafted tumors. The metastatic burden was increased in HSCC iTIC-derived transplants, with 19 metastases-bearing mice compared with 10 metastases-bearing mice in NSC iTIC-derived tumors (Fig. 3A and Table 2). Mice receiving iTICS cultured in HSCC developed, on average, 0.6, 0.8, 1.2, and 1.2 metastases upon the transplantation of 4000, 1000, 100, and 40 iTICS, respectively, whereas mice receiving iTICS cultured in NSC before transplantation developed, on average, 0.5, 0.6, 0, and 0.9 metastases upon the transplantation of 4000, 1000, 100, and 40 iTICS, respectively (Fig. 3B). This indicates an advantage for iTICS kept in HSCC to colonize the lung.

Next, we explored the effect of HSCC and NSC cultivation on iTIC motility. The system looked at migration through a porous membrane and invasion through a basement membrane mimic (Cultrex). In HSCC, iTICS showed ~1.7 times enhanced migration compared with iTICS cultured in NSC (Fig. 3C). Interestingly, the invasion of iTICS through the ECM-mimic was even more enhanced (3.5 times) in HSCC compared with iTICS in NSC (Fig. 3D). To reflect the in vivo situation of a growing tumor, iTIC spheres were embedded into a collagen I matrix to analyze their collective cell migration and ECM invasion by studying the protraction of multicellular strands (sprouts) into the matrix. Spheroid sprouting was compared between HSCC and NSC iTIC-generated spheroids. The results confirmed and strengthened the data of the orthotopic implantation. Whereas iTIC-derived spheroids were significantly longer than the sprouts under the in vitro conditions, sprout formation was almost completely absent in iTICs cultured in NSC prior to transplantation developed, on average, 0.5, 0.6, 0, and 0.9 metastases upon the transplantation of 4000, 1000, 100, and 40 iTICS, respectively (Fig. 3B). This indicates an advantage for iTICS kept in HSCC to colonize the lung.

A partial EMT state characterizes iTICS in changing environmental conditions

We next performed a transcriptome analysis to determine whether the observed phenotypic differences of iTICS, cultured in HSCC or NSC regarding growth, invasiveness, migration, and metastatic characteristics, also go in hand with changes in gene expression. Microarray analysis was carried out for iTICS cultured in HSCC and 1–7 d culture in NSC. An average expression level could be determined for a total number of 20,105 genes. To gain insight into the biologic processes of iTICS cultured under the 2 environmental conditions, we performed a gene set enrichment analysis using the gene ontology terms (Supplemental Table S1). Supplemental Fig. S3A, B shows the significant (q < 0.0001) Gene ontology terms belonging to “biological processes” (Supplemental Table S1). Up- or down-regulated gene sets in iTICS, cultured for 7 d in NSC compared with iTICS in HSCC, are represented as network plots, of which a selection is presented for the down-regulated genes sets as a result of illustration purposes. Enriched gene sets for iTICS growing 7 d in NSC include growth, mitochondrial processes, and energy metabolism, as well as an immune response gene set (Supplemental Fig. S3A), supporting the enhanced growth capacity and metabolic activity observed for iTICS cultured in NSC. Gene sets up-regulated in HSCC or down-regulated in NSC indicate growth suppression, lysosomal processes, and enhanced cell motility, thereby supporting the proinvasive and promigratory phenotype of iTICS in HSCC (Supplemental Fig. S3B).

Tumor growth was initiated by iTICS, independent of the culture conditions. However, tumor onset and growth rate, as well as the ability to disseminate and form metastases, were distinct between the conditions. Furthermore, functional differences were observed in vitro for cell growth and motility. Therefore, the effect of the culture conditions on the expression of EMT genes was assessed. A panel of frequent EMT genes, as described by Zhao et al. (32), as well as common stem-related genes (Gene Set-RNA Profiler; Qiagen), was analyzed in iTICS cultured in HSCC and 7 d NSC (Supplemental Fig. S3C and Supplemental Table S2). Typical EMT markers, including Snai1, Zeb1, and the mesenchymal marker Vim were significantly up-regulated on a transcriptional level in NSC compared with HSCC. At the same time, the typical constituent of epithelial desmosomes Cdh1 and the IGF receptor (Igf1r) was significantly down-regulated in NSC. Typical stem cell factors, such as sex determining region Y-box 2 (Sox2), octamer-binding transcription factor 4 (Oct4), Nanog homeobox (Nanog), and B lymphoma Mo-MLV insertion region 1 homolog (Bmi-1), were either not detected or showed very low, not dynamically regulated mRNA levels (Supplemental Table S2). In addition, we established that iTIC-derived orthotopic tumors expressed both epithelial proteins (Cdh1, keratin 8) and the mesenchymal marker Vim (Supplemental Fig. S3D). In summary, these data indicate that iTICS possess an EMT signature rather than a typical stem-cell expression profile, which corroborates our data on pTICS from MMTV-PyMT tumors (10). The interesting finding of iTICS expressing both epithelial and mesenchymal markers at the same time is in line with recent findings on EMT transitional states of cancer cells (33, 34).

To explore this further, we addressed whether, in addition to the micro milieu, the cell-surface markers CD24+ and CD90+ influence the expression of EMT markers and cell phenotypes. Therefore, mRNA expression of EMT
markers were compared in sorted CD24+ single-positive and CD24+CD90+ double-positive iTICs kept in HSCC or 7 d NSC and analyzed by PCA (Fig. 4A). Along the PC 1, which accounts for 75.9% of the expression variance between cell types and growth conditions, a clear separation of iTICs, according to their culture conditions, was found. Furthermore, PC 2, describing 17.1% of the variances, separated iTICs in NSC in accordance to their cell-surface marker profile, whereas iTICs in HSCC clustered together, regardless of their CD24+CD90+ marker profile. With the comparison of the culture conditions, iTICs in NSC displayed increased expression levels of Vim, Zeb1, Twist1, and Cdh2, whereas iTICs in HSCC display increased expression of Cdh1 (Fig. 4B). These results are in line with the previous transcriptome comparison of HSCC- and NSC-cultured iTICs.

To investigate the PC 2 variance seen for iTICs in NSC, their expression of EMT markers, as well as cell appearance and growth, was analyzed more closely. Whereas CD24+ iTICs in NSC displayed higher levels of Cdh1, CD24+CD90+ iTICs in NSC expressed increased Vim, Zeb1, and Twist (Fig. 4C). The epithelial character of CD24+ iTICs and the more...

Figure 3. Hypoxic stem-cell conditions enhance cell motility of iTICs. A) Ki67 lung staining demonstrating iTIC-derived tumor macro- and micrometastasis. Original scale bars, 100 μm. B) Average number of metastases per mouse for 4000, 1000, 100, and 40 orthotopically transplanted iTICs in HSCC (black) and 7 d NSC (gray). C, D) Migration (n = 3) (C) and invasion (n = 3) (D) of iTICs cultured 7 d in NSC (gray) and HSCC (black). Calculated are the slopes of the cell index between 25 and 70 h, including representative images of the migration and invasion curves (HSCC, blue; NSC, red). E) Average number of sprouts per spheroid and length of sprouts (in micrometers) from a 3D collagen spheroid-sprouting assay of iTIC-generated spheroids 7 d in NSC (gray) and HSCC (black). F) Representative image of sprouting spheroids. Original scale bars, 100 μm. *P < 0.05, **P < 0.01.
mesenchymal character of CD24+CD90+ iTICs were also evident from their morphologies (Fig. 4D). In line with this argument is the result of CD24+ iTICs being more proliferative than CD24+CD90+ iTICs (Fig. 4E). Again, these results reinforce the idea of partial and transitional EMT states in iTICs that vary upon changing microenvironments (34–37).

Comparative transcriptome analysis reveals Mmp14 as the most abundant metalloproteinase

We have recently shown that protease/peptidase genes and, in particular, metalloproteinases play a major role in TICs (10). Thus, we focused on metalloproteinases and compared their expression between HSCC and NSC (Supplemental Table S3). Mmp14 displayed the highest expression levels in HSCC with an about 2-fold higher expression, followed by Mmp15 (Fig. 5A). Interestingly, in contrast to mRNA expression, MMP14 showed higher abundance at the membrane of iTICs cultured in NSC (Fig. 5B, C). This result was supported by MMP14 Western blotting (Fig. 5D), whereas nonquantitative immunofluorescence detection showed a uniform MMP14 staining among iTICs cultured in HSCC or NSC (Supplemental Fig. S4A). Because many proteases showed diverse mRNA expression levels between HSCC and NSC, the regulation

### Table 2. Number of tumor-bearing mice, with or without metastases, after OT

| Condition                  | Metastases in tumor-bearing mice |
|----------------------------|----------------------------------|
| Mice with metastases      | 19 10                            |
| Mice without metastases   | 12 21                            |
| Number of tumor-bearing mice | 31 31                           |

χ²: 5.24, P = 0.022.

**Figure 4.** Partial EMT states in iTICs. A) PCA in accordance with EMT factors of iTICs in HSCC (red shades) and NSC (blue shades), CD24+ (darker red and blue), and CD24+CD90+ (lighter red and blue) sorted cells in triplicates. B) Heatmap of EMT factor mRNA expression levels as percentage of β-actin for iTICs in HSCC- and NSC- and CD24+- and CD24+CD90+-sorted cells in triplicates. C) qRT-PCR of EMT factors in CD24+CD90+ iTICs in NSC (n = 3; bars), normalized to CD24+ iTICs in NSC (dashed line; n = 3). D) Morphology of CD24+ and CD24+CD90+ iTICs in NSC. Original scale bars, 100 μM. E) Cell growth of CD24+ iTICs (black) and CD24+CD90+ iTICs (gray) in NSC (n = 3). The slope cell index was analyzed between 10 and 28 h. Displayed is the slope cell index, including a representative image of the cell growth curves (CD24+, red; CD24+CD90+, blue). Akt, PKB; Met, mesenchymal-to-epithelial transition. *P < 0.04.
of proteases appears to be dynamic and dependent on the environmental conditions in which iTICs are kept (Fig. 5E and Supplemental Table S4).

**Inhibition of metalloproteinases affects TIC characteristics mainly in HSCC**

To investigate if high metalloproteinase expression is associated with a functional impact on the phenotypic plasticity of iTICs, an in-depth analysis of metalloproteinase function in iTICs was performed. For this purpose, iTICs were treated with the broad-spectrum metalloproteinase inhibitor TAPI-0 at nontoxic concentrations and analyzed for their anchorage-independent and -dependent growth in vitro.

In HSCC, TAPI-0 treatment resulted in a 4-fold reduction in colony formation compared with control-treated iTICs in an anchorage-independent growth assay (Fig. 6A, B). Additionally, the size of colonies was decreased. In NSC, iTICs formed significantly fewer colonies compared with iTICs in HSCC. Nevertheless, TAPI-0 treatment abolished colony formation completely. In comparison, primary whole tumor cells in HSCC or NSC, treated with TAPI-0, also showed a 50% reduced colony-forming efficiency. However, colony size was not reduced compared with control-treated colonies, and colonies were 50 μM or smaller (Supplemental Fig. S5A, B). In anchorage-dependent growth assays, TAPI-0 treatment greatly reduced the protrusions that were extending from control-treated iTICs in HSCC (Fig. 6C). Additionally, iTIC spheres generated in a 3D matrix appeared with diffuse borders upon TAPI-0 treatment and were much smaller than control-treated spheres (Fig. 6C, upper). Sphere formation in NSC without inhibitor treatment was greatly impaired (Fig. 1B). Although some spheres appeared to be on the verge of extending protrusions into...
the surrounding matrix, this aspect was completely abolished upon TAPI-0 treatment in NSC. Spheres were reduced in size and did not show any invasive characteristics (Fig. 6C, lower). Next, migration and invasion were analyzed in a Boyden chamber set up upon TAPI-0 treatment under both environmental conditions. Migration was strikingly impaired by TAPI-0 treatment in HSCC, with a reduction of ~70%, whereas in NSC, the migration was decreased by 28% (Fig. 6D). TAPI-0 treatment reduced the invasion of iTICs cultured in HSCC by 56%, whereas in NSC, the reduction was only 16% (Fig. 6E). Interestingly, TAPI-0 treatment did not significantly influence the motility of primary whole tumor cells in migration and invasion (Supplemental Fig. S5C, D), indicating a specific role for proteases in the motility of iTICs. Next, the effect of TAPI-0 on collective cell migration and ECM invasion was analyzed by treating iTIC-derived spheroids with the metalloproteinase inhibitor. As invasive sprout formation was not observed in spheroid cultured in NSC (Fig. 3E, F), the TAPI-0 assay was performed in HSCC only. Control-treated, iTIC-derived spheroids produced an average of 18 invasive sprouts per spheroid. TAPI-0 significantly impaired sprout formation, with an average of 6.7 sprouts per spheroid (Fig. 6F, H). Once sprouts had formed, their lengths were similar between both conditions (Fig. 6G). These results show that metalloproteinases are involved in anchorage-independent and -dependent growth, as well as in invasion and collective cell migration of iTICs, indicating a specific role for proteases in these cell types. Remarkably, the effect of the broad-spectrum metalloproteinase inhibitor was always more pronounced in HSCC.

**MMP14 significantly influences TIC characteristics in HSCC**

Our results show that the metalloproteinase inhibitor TAPI-0 reduced growth and motility of iTICs, especially in HSCC. We chose the highly abundant MMP14, which is a key enzyme of the MMP family, to study the specific impact of this protease on iTICs by targeted silencing of *Mmp14* through shRNA-mediated RNA interference.

Figure 6. Metalloproteinase influence on iTIC characteristics. A) Impact of the broad range metalloproteinase inhibitor TAPI-0 on colony-forming efficiency of iTICs in HSCC and 7 d in NSC (*n* = 4). B) Representative images of colonies treated with DMSO as a control or TAPI-0 cultured in HSCC or NSC. Original scale bars, 100 μm. C) 3D Cultrex culture of iTICs in HSCC and NSC for 6 d in the presence of DMSO as a control or TAPI-0. Original scale bars, 100 μm. D, E) Migration (*D*) and invasion (*E*) of iTICs cultured in HSCC and 7 d in NSC in the presence of DMSO (*n* = 4) as a control or TAPI-0 (*n* = 3). Calculated are the slopes of the cell index between 25 and 70 h. Values are relative to DMSO-treated iTICs in HSCC, including representative images of the migration and invasion curves (HSCC DMSO, blue; HSCC TAPI-0, turquoise; NSC DMSO, red; NSC TAPI-0, orange). F) Average number of sprouts per spheroid of iTICs in HSCC treated with DMSO as a control or TAPI-0. *n* = 4). G) Average length of sprouts of iTICs treated with DMSO (black) as control or TAPI-0 (gray). H) Representative images of a 3D spheroid-sprouting assay of iTICs in HSCC treated with DMSO as a control or TAPI-0. Original scale bars, 100 μm. **P < 0.01.
Knockdown of the protease was accomplished at the protein level, with reduced abundance of MMP14 in the whole cell lysate (Fig. 7A). Subsequently, the role of MMP14 on TIC characteristics was analyzed. Reduced expression of Mmp14 resulted in a significant 57% decrease in colony-forming efficiency (Fig. 7B). Morphologically, the colonies were compact, with defined borders, such as those of the control colonies, but with an overall decrease in size (Fig. 7C). On real-time monitoring, cell growth was detected to be 74% decreased by shMMP14 (Fig. 7D). Likewise, migration and invasion decreased significantly, by 69 and 80%, respectively, compared with iTICs with shCtrl (Fig. 7E, F). The shRNA-mediated knockdown of Mmp14 further influenced collective cell migration and ECM invasion in a 3D spheroid-sprouting assay. The number of sprouts was decreased to 7 invasive strands per spheroid compared with 18 sprouts per control spheroid, as well as an overall shortening in length from an average of ~69 μm in control strands and 48 μm in Mmp14 knockdown strands (Fig. 7G). Together, these results show a great functional impact of MMP14 on TIC characteristics, including anchorage-independent growth, cell growth, migration, and collective cell invasion, under HSCC in vitro.

The impact of MMP14 on tumor initiation was further investigated in vivo by the transplantation of 1000 iTICs treated with shMMP14 or shCtrl into the right and left mammary fat pad of the same mice, respectively (Fig. 7H). Whereas tumors derived from shCtrl cells developed between 2 and 3 wk, the knockdown of Mmp14 significantly delayed tumor onset to more than 4 wk (Fig. 7I). Tumor volumes were analyzed 5.5 wk after transplantation. A significantly greater tumor volume for tumors derived from shCtrl cells, averaging 1.2 cm³, was observed, whereas tumors derived from iTICs with reduced Mmp14 expression exhibited a volume of only 0.3 cm³ (Fig. 7J). These results show that reduced expression of Mmp14 in iTICs delays tumor onset, resulting in decreased tumor volume compared with iTICs with normal Mmp14 expression. Therefore, we found MMP14 to have a strong and significant impact on the tumor-initiating and tumor growth characteristics of MMTV-PyMT iTICs, not only in vitro but also in an in vivo setting.

**DISCUSSION**

Investigation of iTICs in breast cancer is primarily dependent on human breast cancer cell lines, of which only a limited number possess TIC properties, or on transgenic mouse models (22, 31). Furthermore, cultivation and functional analysis of human and murine mammary stem–initiating cells iTICs is challenging (22–24). Although we have demonstrated a significant proteolytic signature in pTICs of the MMTV-PyMT model (10), the functional impact of proteases on TIC characteristics is largely unknown.

Here, we report on the generation of iTICs derived from the MMTV-PyMT mouse model for breast cancer, which retained high colony-forming capacity in vitro and a high tumorigenicity in vivo. Additionally, this study identified a phenotypic plasticity in iTICs that is directed by nutrient-rich normoxia or nutrient-deprived hypoxia and describes partial and dynamic EMT states of these cells, as well as a high expression of proteases, specifically MMP14, as important players in TIC characteristics.

iTICs were derived from primary CD24⁺CD90⁺CD45⁻ TICs, which were demonstrated to be highly tumorigenic (10). Nevertheless, serial passage of stem cells or cancer stem cells in culture often leads to differentiation (22–24). With the use of a protocol by Castro et al. (22), using 3D and 2D Cultrex-supplemented culture and an oxygen content of 3%, differentiation could be avoided. Furthermore, despite elevation of oxygen levels to 21% and use of general NSC, these CD24⁺CD90⁺CD45⁻–derived iTICs maintained high tumorigenicity in vivo. Importantly, their colony-forming capacity in vitro is correlated with their tumor-initiating capacity in vivo. Compared with their primary equivalents, iTICs showed increased tumorigenicity, which might arise through serial passage and single cell-initiated sphere culture.

Although tumorigenicity of iTICs was maintained in both environmental conditions, phenotypic and molecular plasticity was observed for iTICs in NSC and HSCC. Naturally, different environments coexist within the tumor tissue, with fluctuations in oxygen levels and nutrient supplies as a result of expanding tumor mass and a resulting insufficiency in vascularization (19, 21, 38). Whereas TICs have been shown to reside in hypoxic stem cell niches within the tumor, just as hematopoietic stem cells in the bone marrow (39), they will eventually encounter higher oxygen levels, as microenvironmental conditions are not static (21), and they are able to attract their own vessels by the secretion of proangiogenic factors (40). By mimicking this change in the environment, the cultivation of iTICs in HSCC or NSC led to a reversible change in morphology, cell-surface marker expression, EMT marker profiles, and functional TIC hallmarks, including tumor initiation, growth, metabolic activity, and motility. Such plasticity was also found in primary colorectal tumors, where a cancer stem-cell phenotype is dependent on the environment and activation of different gene programs, including EMT genes (41). Nevertheless, our iTICs in HSCC were able to proliferate and initiate tumor growth in vitro and in vivo, reflecting their extraordinary capabilities under changing conditions. Notwithstanding these results, these cells very likely display phenotypic plasticity in the in vitro experiments, as after extravasation into the lung stroma, the iTICs grow out into macro metastases, under conditions modeled by NSC. Hence, the cell-culture conditions imprint different cell states onto iTICs, resulting in smaller tumors but a higher metastatic burden in HSCC and larger tumors but fewer metastases in NSC. In this work, we demonstrate an adaptability of iTICs to different environments, which activates programs necessary for their versatile functions of initiating tumors, tumor growth, and metastasis formation, without differentiating into noncancerous cell populations. The environment-dependent expression of typical EMT markers, as well as the changing of morphology and functionality of iTICs, which describes their cellular plasticity, is quite puzzling. Instead of interpreting...
Figure 7. MMP14 promotes iTIC characteristics and tumorigenicity in vitro and in vivo. A) Protein abundance of MMP14 in iTICs with shCtrl or shMMP14. B) Colony-forming efficiency of iTICs with shCtrl or shMMP14. Values are relative to shCtrl. C) Representative images of colonies derived from iTICs with shCtrl or shMMP14 cultured in HSCC. Original scale bars, 100 μm. D) Cell growth of iTICs with shCtrl and shMMP14 measured by the xCelligence system (n = 6). Calculated is the slope of the cell index between 11 and 30 h, including representative images of cell growth curves (shCtrl, red; shMMP14, blue). E, F) Migration (n = 6) (E) and invasion (n = 5) (F) of iTICs with shCtrl or shMMP14. Calculated are the slopes of the cell index between 20 and 40 h for migration and between 12 and 30 h for invasion, including representative images of migration and invasion curves (shCtrl, red; shMMP14, blue). G) Representative image of 3D spheroid-sprouting assay of iTICs with shCtrl and shMMP14. Original scale bars, 100 μm. Average number of sprouts per spheroid and length of sprouts. H) Scheme of iTIC shCtrl and iTIC shMMP14 OT into the mammary fat pad. I) Tumor onset, in weeks, after the injection of 1000 iTICs with shCtrl or shMMP14 into the mammary fat pad of immunodeficient mice (n = 5). Dashed lines of the same color connect matching shCtrl and shMMP14 (continued on next page)
EMT as a binary switch, our results support a recent concept of intermediate hybrid states of EMT in cancers, as described for skin and mammary primary tumors in vitro and in vivo (34–37).

The phenotypic plasticity was additionally reflected in the expression profile of proteases, which have never been profoundly investigated in TICs before. Proteolytic processing is of great importance at all tumor stages. It can influence post-translational modifications of chemokines and cytokines in the tumor microenvironment, modulate the availability of growth and angiogenic factors, alter cell-cell contacts, and cleave the ECM, supporting tumor development and progression (14, 15, 42–44). The transcriptome analysis of iTICs revealed a protease signature that is differentially and dynamically regulated as a function of NSC and HSCC, thereby implicating a role for proteases in the phenotypic plasticity of iTICs. Other reports have shown changes in gene expression in breast cancer and hypoxic conditions (45, 46). The altered gene-expression patterns include regulators of invasion and metastatic processes in which proteases, such as MMPs, are extensively involved (47, 48). In our work, distinct proteases involved in growth processes, as well as motility, were significantly up-regulated in NSC or HSCC. Of those proteases, MMPs were the most abundant group, which is similar to what has been seen in MICs of the MMTV-PyMT model. MICs showed high expression levels of MMP2, -9, -13, and -14, which have been shown to promote the migratory capacities of these cells (49). In our study, Mmp14 mRNA was the most significantly up-regulated metalloproteinase in iTICs, showing an increase in expression in HSCC compared with NSC. However, at the protein level, MMP14 was more abundant in NSC but still more abundant in both conditions compared with primary whole tumor cells of the MMTV-PyMT model. Hence, the effects of a broad-spectrum inhibition of metalloproteases were not significant on primary whole tumor cells, indicating that these proteases play a distinct role in TICs. Physiologically, MMP14 is necessary for the -proteolytic activation of MMP2 and -13, which might be key events in tumor invasion and metastasis (50, 51). In our previous work, Mmp14 was identified as the protease with the highest absolute expression in pTICs compared with primary non-TICs, corroborating and underlining its importance in TICs (10). The expression of Mmp14 might be regulated by the low oxygen levels of HSCC, therefore displaying higher levels in HSCC. Whereas in a study by Miyoshi et al. (52), Mmp14 expression was up-regulated by hypoxia in hepatoma cells in a hypoxia-inducible factor (Hif)-1α-independent manner, Petrella et al. (48) showed Mmp14 transcriptional expression to be regulated by the binding of the hypoxia-regulated transcription factor Hif-2α, as a result of a Hif-binding site in the promoter region of the protease. Our data show a higher protein abundance of MMP14 in NSC, and yet, the functional impact of MMP14 was more pronounced in HSCC. This indicates that the transcriptome does not reflect the abundance of protein, as a result of post-transcriptional modifications and different scenarios in changing environmental conditions, such as HSCC and NSC (53). Additionally, the protein abundance of MMP14 does not always seem to mirror the functional necessity of this protease. One explanation could be an enhanced conversion from zymogen to active MMP14 by the protease furin in HSCC compared with NSC. Furin was found to be up-regulated upon hypoxia (54) and higher expressed in HSCC compared with NSC in iTICs. However, the precise molecular mechanisms of Mmp14 upregulation and functionality upon HSCC in iTICs remain to be determined. A functional importance of MMP14 in TIC characteristics was confirmed, specifically in HSCC. By shRNA-mediated knockdown of Mmp14, the impact on self-renewal, invasiveness, and migration in HSCC

| Cell type and condition | pTIC NSC | Primary tumor cell NSC | HSCC | iTIC | NSC |
|-------------------------|----------|------------------------|------|------|-----|
| Cell-surface marker profile | CD24+CD90+ (100%) | Not CD24+CD90+ | CD24+CD90+ (95%) | CD24+CD90+ (50%) | CD24+CD90+ (50%) |
| In vitro colony formation | 1.44 colonies/1000 seeded cells | 0.06–0.2 colonies/1000 seeded cells | 292 colonies/1000 seeded cells | 1 in 23 | 1 in 23 |
| In vivo tumor-initiating frequency | 1 in 91 | 1 in 3976 | | | |
| Up-regulated proteases (transcriptome level) | Metalloproteases (Mmp3, Mmp13, Mmp14, Adam12, Adam5, Adam2) | Reference for pTIC | Metalloproteases (Mmp11, Mmp13, Mmp14, Mmp15) | Metalloproteases (Mmp2, Mmp3, Mmp10, Mmp12) |

Adam, a disintegrin and metalloproteinase. *Hillebrand et al. (10).
became evident by impaired colony-forming efficiency, invasiveness, and collective cell migration. Whereas the elimination of Mmp14 expression is able to reduce invasiveness and migration in breast cancer cells (50), and a transmigration program induced by Snail acts in a MMP14-dependent manner in these cells (55), our study identifies the promotile function of MMP14, specifically in iTICs of the mammary gland. Clearly, MMP14 has an important function in the trafficking of cancer cells through the ECM (56). Moreover, the reduced expression of Mmp14 in iTICs had a significant effect in vivo. Upon the transplantation of 1000 iTICs with shMMP14 into the mammary fat pad, tumor initiation was notably delayed and tumor volumes significantly reduced compared with control.

In summary, the successful generation of iTICs has aided in determining a significant phenotypic plasticity, transitional EMT states, and the additional feature of a dynamic protease expression profile in TICs that exhibits a highly motile character in nutrient-deprived hypoxia and growth capacity in nutrient-rich normoxia. Furthermore, a functional role of proteases was revealed, aiding the intermediate EMT characteristics of iTICs. Specifically, MMP14 empowers breast cancer TICs to initiate tumors and activate motile programs under hypoxic nutrient-deprived conditions. Our model of iTICs closely reflects pTICs of the MMTV-PyMT mouse model (Table 3). Therefore, our cell model can be applied as a model for murine pTICs to study their phenotypic characteristics but could also be applied to generate human iTICs to study the relevance of TICs in a human context.

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AUTHOR CONTRIBUTIONS

L. E. Hillebrand and T. Reinheckel designed the study and wrote the manuscript; L. E. Hillebrand, S. M. Wickberg, and A. Gomez-Auli performed experiments and analyzed data; H. Busch and M. Boerries performed microarray and bioinformatics analysis; M. Follo provided FACS and analysis; J. Maurer provided expertise for the cultivation of TICs and scientific input on the project strategy; and all authors read, critically revised, and approved the final version of the manuscript.

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