1 Original Article

2 Spatial transcriptomics reveals the two cancer stem cell-like populations in
3 triple-negative breast cancer

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20

21 Abstract

22 Gene expression analysis at the single-cell scale by next generation sequencing has

23 revealed the existence of clonal dissemination in cancer metastasis. The current spatial

24 analysis technologies elucidate the heterogeneity of cell-cell interactions *in situ*;
 25 however, further analysis is needed to elucidate the nature of tumor heterogeneity. To
 26 reveal the expressional heterogeneity and cell-cell interactions in primary tumors and
 27 metastases, we performed transcriptomic analysis of microtissues dissected from a
 28 triple-negative breast cancer (TNBC) cell line MDA-MB-231 xenograft model by our
 29 automated tissue microdissection punching technology. This multiple-microtissue
 30 transcriptome analysis revealed that there were existed three cell-type clusters in the
 31 primary tumor and axillary lymph node metastasis, two of which were cancer stem
 32 cell-like clusters (CD44/MYC-high, HMGA1-high). The CD44/MYC-high cluster showed
 33 aggressive proliferation with MYC expression. The HMGA1-high cluster exhibited
 34 HIF1A activation and upregulation of ribosomal processes. Furthermore, we developed
 35 a cell-cell Interaction (CCI) analysis to investigate the ligand-receptor interactions
 36 (cancer cell to stroma and stroma to cancer cell) in each spot. The CCI analysis
 37 revealed the interaction dynamics generated by the combination of cancer cells and
 38 stromal cells in primary tumors and metastases. Two cancer stem cell-like populations
 39 were also detected by the scRNA-seq analysis of TNBC patients. In addition, the gene
 40 signature of the HMGA1-high cancer stem cell-like cluster has the potential to serve as
 41 a novel biomarker for diagnosis. The mixture of these multiple cancer stem cell-like
 42 populations may cause differential anticancer drug resistance, increasing the difficulty
 43 of curing this cancer.

44 **Introduction**

45 Breast cancer cells metastasize to multiple distant organs, such as the axillary lymph
 46 nodes, lungs, bone, liver, and brain (Nakayama *et al*, 2021; Obenauf & Massague,
 47 2015). In particular, metastasis to axillary lymph nodes is an indicator of cancer grade in
 48 breast cancer patients (Giuliano *et al*, 2011). Most breast cancer tissues, including
 49 distant metastases, exhibit genetic heterogeneity (McGranahan & Swanton, 2017).
 50 Single-cell analyses have revealed that cancer cells evolve through the acquisition of
 51 genomic mutations in the primary tumor and metastases (Echeverria *et al*, 2018; Yates
 52 *et al*, 2017). Most previous analyses have been performed using isolated cancer cells
 53 and stromal cells from cancer tissues. Thus, the cell-cell interactions between cancer
 54 cells and stromal cells remains to be analyzed. In recent studies, the current single-cell
 55 analysis and spatial transcriptome technologies reveal the heterogeneity of cell-cell
 56 interactions between cancer cells and stromal cells *in situ* (Andersson *et al*, 2021; Wu *et*
 57 *al*, 2021); however, further analysis is needed to elucidate the nature of tumor
 58 heterogeneity.

59 Comprehensive gene expression analysis of metastases harvested from
 60 approximately 500 specimens in a various cancer types and metastatic organs
 61 (MET500 cohort) have suggested that metastatic tissues could be divided into several
 62 categories (e.g. proliferative or EMT-like/inflammatory) (Robinson *et al*, 2017). In
 63 particular, some samples were found to show signatures of more than one category,
 64 suggesting that these samples had micro intraheterogeneity. To clarify such
 65 heterogeneity, microtissue sectioning using the laser capture microdissection has often

been performed (Civita *et al*, 2019). This method has several disadvantages, however, including the laborious and time-consuming nature of sample handling and a high risk of RNA degradation. Thus, in previous work, we developed a system involving automated tissue microdissection punching followed by transcriptomic analysis of the tumor microtissue (Yoda *et al*, 2017). To analyze the expressional heterogeneity in microtissues from the primary tumor and axillary lymph node metastases, we performed analysis of the spatial microtissue transcriptome in the triple-negative breast cancer (TNBC) cell line MDA-MB-231 xenograft model. We focused on the expression profiles of known metastasis-promoting genes and cancer stem cell markers in dissected microtissues.

Results

Sampling microtissues from primary tumors and axillary lymph node metastases in MDA-MB-231 xenografts

Primary tumors and axillary lymph node metastases were harvested from NOD-SCID mice with MDA-MB-231-parent-*Venus* cell line xenografts. We subjected the sliced tissues to microtissue dissection by an automated tissue microdissection punching system (Figure 1A). RNA was successfully recovered from the microtissues collected at 93 spots in the primary lesion and 44 spots in axillary lymph node metastases using a microtissue automatic sampling device (Figure 1B). In samples of this size, although the number of cells present in the tumor tissue varies, it can be inferred that several to

approximately 10-30 cells are present in each spot (Yoda *et al.*, 2017). RNA-seq analysis was performed on the total RNA extracted from each spot. We checked the quality of the fastq files by FASTQC. Total RNA samples contained RNA from human cancer cell lines and RNA from mouse stromal cells in the tumor microenvironment. Therefore, the obtained sequences were mapped to both the human reference genome and the mouse reference genome by HISAT2 (Kim *et al.*, 2019). Protein-coding genes (human: 19961 genes, mouse: 22050 genes) were extracted as transcripts per million (TPM) for spatial transcriptome analysis with Seurat (Butler *et al.*, 2018; Stuart *et al.*, 2019) (Figure 1C, Supplementary Figure S1A, S1B and S1C).

97

98 **Analysis of microtissue transcriptomes**

99 The clustering analysis and UMAP plots showed 3 clusters of cancer cells (transcripts
100 mapped to the human reference genome) and 4 clusters of stromal cells (transcripts
101 mapped to the mouse reference genome) in the microspots dissected from primary
102 tumors and axillary lymph node metastases (Figure 2A and B). Next, we evaluated the
103 expression of cancer stem cell markers to focus on cell–cell interactions in the
104 metastatic stem cell niche (Oskarsson *et al.*, 2014). We found that human cancer
105 clusters showed specific gene expression patterns for high mobility group AT-Hook1
106 (HMGA1), CD44, and MYC (Figure 2C). Consequently, these human clusters were
107 named HMGA1-high, CD44/MYC-high, and Marker-low. Mouse stromal clusters
108 showed specific gene expression patterns for transthyretin (Ttr), Cd3d (T-cell marker),
109 membrane spanning 4-domains a1 (Ms4a1, B-cell marker), and inhibin subunit beta A

110 (Inhba; a subunit of both activin and inhibin) (Figure 2D). Ttr and Inhba were highly
 111 expressed in their respective specific clusters (Supplementary Table S2). Therefore,
 112 these mouse clusters were named Ttr-high, Tcell-like, Inhba-high, and Bcell-like. CD44
 113 was broadly expressed in all human clusters; however, HMGA1 was expressed in only
 114 HMGA1-high clusters (Figure 2E). CD44 and HMGA1 are well-known markers of cancer
 115 stem cells in breast cancer (Liu *et al*, 2010; Pegoraro *et al*, 2013). These results
 116 suggested that 2 types of cancer stem cell-like populations existed in MDA-MB-231
 117 xenograft models.

118 The heterogeneity of each cluster was demonstrated by calculation of network
 119 topology using the normalized closeness centrality (Watanabe *et al*, 2020). The
 120 centralities showed the correlation of gene expression in each cluster. These results
 121 showed that the cancer cells in the HMGA1 clusters had a the expanded diverse of
 122 expressional heterogeneity compared with CD44/MYC clusters (Supplementary Figure
 123 S1D and S1E). Interestingly, although 3 human clusters were present in both the
 124 primary tumors and the lymph node metastases (Figure 2F), mouse stromal clusters
 125 showed a site-specific pattern. Most of the Ttr-high clusters were observed in the
 126 primary tumors. On the other hand, most Tcell-like clusters and B-cell-like clusters were
 127 found in the lymph node metastases (Figure 2G).

128 Next, we performed spot analysis with the spatial information to determine the
 129 spatial heterogeneity in the xenografts (Figure 3A, 3B, and 3C). Human
 130 CD44/MYC-high cancer cells tend to localize the outside of the primary tumor. However,
 131 lymph node metastases did not show such a tendency (Figure 3A). Cell cycle analysis

132 of cancer cells showed that cell proliferation occurred outside of the primary tumor and
 133 at sparse sites among the lymph node metastases (Figure 3B). Approximately 50 % of
 134 the cells in CD44/MYC-high clusters and 30% of the cells in HMGA1-high clusters were
 135 actively undergoing cell division (Supplementary Figure S2A, 3D and Supplementary
 136 Table S3). These results suggested that the cells divided from two cancer stem cell-like
 137 clusters, leading to cancer expressional heterogeneity. Mouse stromal cell localization
 138 showed that most Tcell-like clusters were present throughout the entire lymph node
 139 metastases; on the other hand, Tcell-like clusters also existed outside of the primary
 140 tumor. Most Ttr-high clusters were present at the sparse primary tumor (Figure 3C). The
 141 mouse Tcell-like clusters and Inhba-high clusters were recruited into the cell cycle
 142 (Supplementary Figure S2B, S2C, S2D and Supplementary Table S3). Next, a
 143 comparative analysis of the cell cycle in the primary tumor and lymph node metastasis
 144 showed that the two cancer stem cell-like clusters (HMGA1-high and CD44/MYC-high)
 145 increased the cell division index in lymph node metastases (Figure 3E). In contrast,
 146 Marker-low clusters did not change the cell cycle index in either location. This result
 147 suggested that the cancer stem-like cells in metastatic tissues proliferated aggressively.

148

149 **Enrichment analysis of microspots**

150 The differentially expressed genes (DEGs) in human clusters and mouse
 151 clusters were extracted and visualized in heatmaps (Figure 4A, Supplementary Figure
 152 S51, S5B, Table S2 and S4). The human HMGA1-high cluster showed that high
 153 expression of TMSB10 (Zhang *et al*, 2017), CTSD (Ashraf *et al*, 2019) and LGALS1

154 (Balestrieri *et al*, 2021; Jung *et al*, 2007), which are correlated with poor prognosis in
155 breast cancer. The human CD44/MYC-high cluster expressed SENPK, SENPN and
156 PTK2 (focal adhesion kinase: FAK), which regulate the cell cycle and cell division. The
157 human Marker-low clusters showed that low expression levels of these genes. The
158 mouse Tcell-like clusters expressed cytokines and immune receptors. To determine the
159 biological function of DEGs, we performed upstream analysis, GO enrichment analysis
160 and pathway enrichment analysis using Metascape (Zhou *et al*, 2019). DEGs from each
161 human cluster showed the upregulated DEGs in the HMGA1-high clusters and
162 CD44/MYC-high clusters, and downregulated DEGs in the Marker-low clusters (Figure
163 4A, Supplementary Table S2 and S4). Upstream analysis of DEGs showed that HIF1A
164 downstream genes were upregulated in the HMGA1-high cluster, and MYC
165 downstream genes were upregulated in the CD44/MYC-high cluster. In the Marker-low
166 clusters, E2F1 downstream genes were downregulated (Figure 4B). We performed
167 pathway and GO enrichment analyses focused on the two cancer stem cell-like clusters
168 that had upregulated DEGs. Amide metabolites, VEGFA-VEGFR signaling, and
169 apoptosis regulation were enriched in both clusters. On the other hand, the DEGs in
170 CD44/MYC-high clusters were enriched in many terms related to the cell cycle and cell
171 division, namely, cell division, cell cycle, and telomeres. In HMGA1-high clusters,
172 ribosome assembly and ribosome biogenesis were significantly enriched (Figure 4C, 4D
173 and Supplementary Figure S3). The results of enrichment analysis using DEGs in the
174 Marker-low clusters showed that metabolism of RNA, translation, and mitochondrial
175 organization were enriched (Supplementary Figure S4).

176 In mouse clusters, enrichment analysis of the DEGs was performed by using
177 Metascape. Enrichment analysis of the DEGs in the Ttr-high clusters showed no
178 enriched terms. The Tcell-like clusters and Inhba-high clusters had two common
179 enriched terms; glycolysis and neutrophil degradation (Supplementary Figure S5).

180

181 **Cell-cell interaction (CCI) analysis of microspots**

182 To estimate the CCI score, we utilized a cell-cell interaction database
183 (<https://baderlab.org/>) and extracted a total of 115,900 interactions (3,209 ligands,
184 4,364 receptors and 433 extracellular matrix) (Watanabe *et al.*, 2020). Highly expressed
185 genes (expression level > 2) were selected for CCI analysis, and ligand-receptor
186 interactions (human to mouse and mouse to human) were estimated in each spot
187 (Figure 5A). We extracted 2,432 interactions and 7 clusters of “mouse (stromal) to
188 human (cancer)” CCI patterns (Figure 5B and Supplementary Table S7). In particular,
189 Cluster 2 was constituted by only lymph node metastasis spots, while Cluster 6 was
190 constituted by only primary tumor spots. We thus focused on these clusters: the CCI
191 heatmap showed lymph node metastasis-specific CCI (Figure 5C and 5D) and primary
192 tumor-specific CCI (Figure 5E and 5F). The “human (cancer) to mouse (stromal)” CCI
193 pattern showed 741 interactions (Supplementary Figure S6, Supplementary Table S7
194 and S8).

195 Next, we focused on cell type-specific CCI. Ten sets of cancer cell-stromal
196 cell interactions were observed (Figure 6A and 6B). The PT-1, PT-2 and PT-3 groups
197 existed in only the primary tumors; in contrast, the Mix-1~7 groups were present in both

the primary tumors and lymph node metastases (Figure 6C). As a result, 4,126 interactions “cancer to stromal” (Supplementary Table S8) and 4,165 interactions of “mouse (stromal) to human (cancer)” (Supplementary Table S9) were estimated from the cell type-specific CCI analysis. The ligand-receptor interaction with the highest CCI score was shown for each group (Figure 6D-G). Among cancer cell-to-stromal interactions, annexin A2 (ANXA2) and heat shock protein 90 alpha (HSP90AA1) had high CCI scores in various groups. On the other hand, from stromal to cancer cell interactions, B2m interactions were estimated in various groups. Alb interaction was estimated in the Mix5 and Mix6 groups. The interactions of the Tcell-like cluster (Mix-1, Mix-3 and Mix-5) changed depending on the type of cancer cell (Figure 6E and 6G). Similarly, the Ttr-high (PT-1, PT-2 and Mix-4) cluster interactions also changed depending on the type of cancer cell. On the other hand, in view of the stromal to cancer interaction, the Mix5 and Mix6 clusters have specific interactions with proteins such as integrin B1 (ITGB1) and cystatin C3 (CST3) (Figure 6G).

TNBC patients showed two cancer stem cell-like populations

To confirm our findings in clinical samples, we reanalyzed the public single-cell RNA-seq (scRNA-seq) dataset (Karaayvaz *et al*, 2018) and the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) (Pereira *et al*, 2016). First, we analyzed the scRNA-seq dataset of 5 TNBC patients (Figure 7A). We extracted 546 cancer cells from the dataset with UMAP visualization (Figure 7B). Module analysis was performed using the HMGA1 signatures and CD44/MYC signatures from DEG analysis

220 to detect the HMGA1-high cluster and CD44/MYC cluster in TNBC patients. TNBC
 221 patients had the two cancer stem cell-like populations and a double-positive population
 222 (Figure 7C, 7D, Supplementary Tables S10 and S11). Next, survival analysis was
 223 performed using the HMGA1 signatures and CD44/MYC signatures with METABRIC
 224 claudin-low subtype (TNBC) cohorts. High expression of HMGA1 signatures correlated
 225 with poor prognosis in the claudin-low subtype (Figure 7E). In contrast, CD44/MYC
 226 signatures did not correlate with prognosis in these cohorts (Supplementary Figure S7).

227

228

229 Discussion

230 Spatial transcriptomics technologies (Rodrigues *et al*, 2019; Yoda *et al.*, 2017)
 231 have enabled us to reveal the *in situ* expressional profiles and microheterogeneity of
 232 cancer. In particular, in a xenograft model, both human-derived RNA and
 233 mouse-derived RNA can be analyzed both simultaneously and individually by mapping
 234 the sequence reads to a human genome reference or mouse genome reference
 235 (Bradford *et al*, 2013; Callari *et al*, 2018). In this study, by combining microtissue
 236 sampling and the isolation of human-mouse gene expression by mapping, we revealed
 237 the expressional heterogeneity of cancer cells and stromal cells, and the heterogeneity
 238 of cancer-stroma interactions in MDA-MB-231 primary tumors and the axillary lymph
 239 node metastases.

240 Interestingly, we observed two types of cancer stem cell-like populations in
 241 both the primary tumors and lymph node metastases. One of the cancer stem cell-like

242 populations expressed CD44 and MYC. The CD44 gene is a well-known cancer stem
 243 cell marker in breast cancer (Liu *et al.*, 2010; Marotta *et al.*, 2011; Sheridan *et al.*, 2006).
 244 Most MDA-MB-231 cells expressed high levels of CD44 (Burdick *et al.*, 2012), although
 245 the expression levels varied (Figure 2E). The cluster with the highest CD44 expression
 246 was MYC, and these cells proliferated aggressively *in vivo*. The other cancer stem
 247 cell-like population, the HMGA1-high cluster, was observed in both the primary tumors
 248 and lymph node metastases. HMGA1 promotes tumor initiation, cancer stemness and
 249 metastasis in TNBC (Huang *et al.*, 2015; Pegoraro *et al.*, 2013; Shah *et al.*, 2013). The
 250 DEGs enrichment analysis indicated that HIF1A activation and ribosomal-related
 251 bioprocesses were enriched in the HMGA1-high cancer stem cell-like population. HIF1A
 252 is a well-known regulator of hypoxia that activates stemness, glycolysis, angiogenesis,
 253 and invasion/metastasis (Choudhry & Harris, 2018; Petrova *et al.*, 2018). In terms of
 254 ribosomal-related processes, the upregulation of translation and ribosomal processes
 255 may promote distant metastasis in breast cancer (Ebright *et al.*, 2020). In the clinical
 256 scRNA-seq analysis, both types of cancer stem cell-like populations were observed in
 257 single-cell analysis of TNBC patients (Karaayvaz *et al.*, 2018). Our results showed that
 258 the mixture of these multicancer stem cell-like populations makes curative treatment
 259 difficult and causes the anticancer drug resistance in the clinic. In addition, the HMGA1
 260 signatures has the potential to be a novel biomarker for diagnosis, and HMGA1-high
 261 cancer stem cells may contribute to poor prognosis.

262 Our results showed Ttr-high and Inhba-high stromal populations in the
 263 xenograft model. High expression of transthyretin (Ttr) enhances tumor proliferation and

264 growth (Lee *et al*, 2019). *Inhba* is a member of the TGF-beta superfamily (Bloise *et al*,
265 2019). *Inhba* is upregulated in breast tumors, and induces epithelial-mesenchymal
266 transition (EMT), tumor growth and distant metastasis (Bashir *et al*, 2015; Kalli *et al*,
267 2019). Most *Inhba*-high populations also existed in the primary tumor. Our results
268 suggested that stromal expression of *Ttr* and *Inhba* enhanced tumor growth in the
269 primary tumors of MDA-MB-231 xenografts.

270 CCI analysis of each the spot and group was performed to understand the
271 interaction dynamics of different combination of cancer cell and stromal cell types. This
272 analysis will be helpful for discovering the cancer stem cell niche and metastatic niche
273 (Oskarsson *et al.*, 2014). *B2M* is a gene that presents self-antigens on the plasma
274 membrane. Cancer cells present self-antigens to immune cells in the tumor tissue
275 (Popat *et al*, 2020). *B2M* has a different molecular regulatory mechanism in ER-positive
276 and ER-negative breast cancer, and it controls the proliferation of cancer cells (Chai *et*
277 *al*, 2019). Our results suggested that MDA-MB-231 cells interact with immune cells
278 through *B2M* antigen presentation, which controls cancer cell proliferation in the
279 xenograft model. In addition, *HSP90AA1* and *ANXA2* expressed by cancer cells had
280 high CCI scores in the spot CCI analysis. High expression of *HSP90AA1* in TNBC or
281 *HER2-/ER+* breast cancer patients is correlated with poor prognosis, and the
282 *HSP90AA1* gene is often amplified (Cheng *et al*, 2012). *HSP90AA1* is secreted in
283 extracellular vesicles under hypoxia and enhances the migration of cancer cells and
284 stromal cells in breast cancer (Santos *et al*, 2017). *HSP90AA1* may contribute to the
285 involvement of extracellular proteins in the cell-cell interactions. High expression of

286 ANXA2 is correlated with poor prognosis in TNBC patients (Gibbs & Vishwanatha,
287 2018) and regulates drug resistance to EGFR1-targeted therapy (Fan *et al*, 2019;
288 Zhang *et al*, 2018). ANXA2 controls angiogenesis in TNBC xenografts and has the
289 potential to be a novel therapeutic target in TNBC (Sharma & Jain, 2020). ANXA2 is a
290 regulator of endocytosis and exocytosis on the plasma membrane (Bharadwaj *et al*,
291 2013; Grindheim *et al*, 2017). This interaction has the potential to mediate cell-cell
292 communication via exosomes, and thereby promotes the migration of cancer cells and
293 immune cells.

294 Several spots did not contain enough RNA for analysis or exhibited bias
295 toward either human RNA or mouse RNA (Supplementary Figure S1A). Thus, one
296 limitation of this sampling method is that some spots have a biased cell type or no cells.
297 Read counts of each cluster showed lower counts for the human Marker-low cluster,
298 mouse Ttr-high cluster, mouse Inhba-high cluster, and mouse B-cell like cluster than for
299 other clusters (Supplementary Figure S1B and S1C). The limitations of cell type bias
300 and low RNA extraction efficiency caused these low transcript counts.

301 Our results showed that HMGA1 signatures correlated with poor prognosis in
302 TNBC patients in the METABRIC cohort; on the other hand, CD44/MYC signatures did
303 not correlate with progression (Supplementary Figure S7). Previous research reported
304 that the diagnosis of invasive breast cancer on the basis of CD44 expression alone is
305 difficult and that it is necessary to examine the expression of other genes (Mylona *et al*,
306 2008; Wang *et al*, 2017). Our study supports that CD44 signatures are not sufficient for
307 diagnosis. In contrast, HMGA1 signatures is useful for the diagnosis of TNBC patients.

Our results showed that these spatial transcriptomics methods will be helpful for the diagnosis, further identification of biomarkers, and elucidation of the essential characteristics of cancer.

Materials and Methods

Cell culture

The MDA-MB-231-*luc2-Venus* cell line was cultured in RPMI-1640 (Fujifilm Wako, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Fujifilm Wako), 100 µg/ml streptomycin (Meiji Seika Pharma Co. Ltd. Tokyo, Japan) and 100 U/ml penicillin (Meiji Seika Pharma) at 37°C with 5% CO₂.

Animal studies

A. breast cancer xenograft model was established in NOD.CB-17-Prkdc^{scid}/J mice (NOD-SCID; Charles River Laboratories Japan, Inc., Kanagawa, Japan) by orthotopic transplantation as previously described (Nakayama *et al*, 2017). A total of 1.0 x 10⁶ cells were injected into the 4th fat pad of NOD-SCID mice. The primary tumor was removed 8 weeks after transplantation. An axillary lymph node metastasis was sampled 2 weeks after removing the primary tumor. The growth of the primary tumors and metastases were monitored by bioluminescence using an in vivo imaging system (IVIS-XRMS, PerkinElmer, MA, USA). For bioluminescence monitoring by IVIS, mice were anesthetized with 2.5% isoflurane (Fujifilm Wako) and intraperitoneally injected with

330 3 mg D-luciferin (Gold Biotechnology Inc., MO, USA) in 200 µl PBS as previously
 331 described (Han *et al*, 2020; Nakayama *et al*, 2020). The harvested organs were placed
 332 in ice-cold PBS (Fujifilm Wako) and embedded in SCEM (Super Cryoembedding
 333 Medium, SECTION-LAB, Japan) using liquid nitrogen and stored at -80 degree until
 334 sectioning.

335

336 **Microtissue dissection and RNA-seq analysis**

337 Microtissue sampling was performed by an automated tissue microdissection punching
 338 system as previously described (Yoda *et al.*, 2017). Frozen sections were sliced at a
 339 thickness of 20 µm and transferred on an LMD film II (SECTION-LAB). Microspots were
 340 sampled with a 100 µm needle in the dissection instrument. RNA-seq was performed by
 341 Illumina HiSeq as previously described (Yoda *et al.*, 2017).

342

343 **Mapping and quality check**

344 Transcriptome analysis was performed with HISAT2 version 2.0.5 (Kim *et al.*, 2019) and
 345 RSEM version 1.3.0 (Li & Dewey, 2011). The gene expression of cancer cells was
 346 obtained by mapping RNA-sequence reads to the human reference genome or mouse
 347 reference genome. We subjected 'protein_coding' genes to spatial transcriptome
 348 analysis.

349

350 **Clustering and UMAP visualization**

351 Data mining analyses such as clustering, UMAP analysis, and DEG extraction were
 352 performed with the functions 'runPCA', 'FindNeighbors', 'FindClusters', and 'runUMAP'
 353 and 'FindAllMarkers' in 'Seurat' version 3.2. (Stuart *et al.*, 2019). Cell cycle detection
 354 was performed by the function 'CellCycleScoring'. Heatmap drawing was performed
 355 using 'ComplexHeatmap'(Gu *et al.*, 2016). These packages and functions were run in R
 356 version 3.6.3.

357

358 **Network analysis for expressional heterogeneity**

359 Correlational network analysis for calculation of the expressional heterogeneity was
 360 performed by the 'igraph' package as previously described (Nakayama *et al.*, 2017;
 361 Watanabe *et al.*, 2020). We calculated Pearson's correlational coefficients between the
 362 spots classified into the same clusters. Next we calculated the normalized closeness
 363 centrality using the correlational network.

364

365 **Enrichment analysis using DEGs**

366 Pathway and GO enrichment analyses were performed by the Metascape
 367 (<https://metascape.org/gp/index.html#/main/step1>) (Zhou *et al.*, 2019). DEGs from each
 368 cluster were subjected to the Metascape interface. Differential enrichment terms were
 369 analyzed by multiple gene list mode. The results of enrichment analysis were visualized
 370 as heatmaps and networks.

371

372 **Cell-Cell Interaction (CCI) analysis**

Ligand-receptor interactions between human cancer cells and mouse stromal cells were performed using the interaction database of the Bader laboratory from Toronto University (https://baderlab.org/CellCellInteractions#Download_Data) in R software version 3.6.3. For spot CCI analysis, we extracted the genes whose expression value was greater than 2. We selected the combinations representing ligand-receptor interactions, in which both ligand genes and receptor genes were expressed in the same spot. Hierarchical clustering was performed by 'hclust' and it was visualized as a circular clustering plot by the 'circlize' package in R (Gu *et al*, 2014).

In the CCI analysis of the group with containing both human cancer cells and mouse stromal cells, we calculated the number of spots with expression values greater than 2. Only groups whose expression cell ratio exceeded 10% were extracted for CCI analysis, and the CCI score between each group was calculated as previously described (Watanabe *et al.*, 2020).

Analysis of the public single-cell RNA-seq dataset

To confirm the cancer stem cell signatures in MDA-MB-231 xenografts, we performed reanalysis of scRNA-seq of TNBC patients from the public cohort (Karaayvaz *et al.*, 2018). The normalized scRNA-seq dataset was downloaded the GSE138390 dataset from the Gene Expression Omnibus (GEO) and analyzed with the annotation metadata. The dataset was analyzed and visualized by the UMAP plot with Seurat in R. Module analysis was performed using the function 'AddModuleScore' with gene signatures (Supplementary Table S10) in 'Seurat'.

395

396 **Clinical dataset analysis**

397 Survival analysis of the Molecular Taxonomy of Breast Cancer International Consortium
 398 (METABRIC) cohort (Ali *et al*, 2020; Curtis *et al*, 2012; Pereira *et al*, 2016) was
 399 performed by the Kaplan-Meier method using the 'ggplot2', 'survminer' and 'survival'
 400 packages with R as previously described (Kuroiwa *et al*, 2020; Murakami *et al*, 2019;
 401 Nishiyama *et al*, 2021)

402

403 **Code availability**

404 The source code of spatial transcriptome analysis is available on GitHub
 405 (<https://github.com/JunNakayama/Spatial-Transcriptomics-of-MDA-MB-231-xenografts>)

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407

408 **Data availability**

409 Spatial expression data (RNA-seq of each microspot) were deposited at GEO
 410 accession number GSE184720.

411

412

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425

426 **Authors' Contributions**

427 JN designed the study, performed the animal experiments and analysis of spatial
428 transcriptomics, and wrote the manuscript. HM and TY performed the dissection of
429 micro-tissues. KA performed analysis of NGS data. MH designed the experiment of
430 micro-tissue dissection and interpreted the results. HT, YY and KS interpreted the
431 results. All authors reviewed the manuscript.

432

433 **Competing Interests**

434 The authors declare that no conflict of interest exists.

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639
640

641 **Figure Legends**

642 **Figure 1. Microtissue sectioning from the primary tumors and the axillary lymph** 643 **node metastases in the TNBC xenograft model.**

644 (A) Experimental flowchart of spatial transcriptomics. The human TNBC cell line
645 MDA-MB-231-Parent-Venus was transplanted orthotopically into a female NOD-SCID
646 mouse. After 8 weeks, the primary tumor was harvested, and the center of the tumor
647 was sectioned. After 4 weeks, axially lymph node metastases were harvested and
648 sectioned from the same mouse. Sectioning was performed by an automated tissue
649 microdissection punching system with a 100 µm needle. (B) In total, 93 microspots were
650 sectioned from the primary tumor, and 43 microspots were sectioned from axillary
651 lymph node metastases. RNA was extracted from a total of 137 spots. (C) A flowchart of
652 the transcriptome analysis. Quality check was performed by FASTQC. The reads were
653 mapped to the human genome reference and the mouse genome reference by HISAT2.
654 Protein-coding genes were selected for analysis with Seurat.

655

656 **Figure 2. Transcriptome profiling of the microspots.**

657 (A) UMAP plot of human (cancer cell) spot clustering. (B) UMAP plot of mouse (stromal
658 cell) spot clustering. (C) Violin plot of cancer marker genes. (D) Violin plot of stromal
659 marker genes. (E) Heatmap of CD44, HMGA1, and MYC expression in each human
660 cluster. (F & G) Bar plot of spot counts in the primary tumor and lymph node
661 metastases: (F) human (G) mouse.

662

Figure 3. Spatial transcriptome with cell cycle detection.

(A) Spatial transcriptomics of human (cancer cell) clusters at the primary tumors and the lymph node metastases. (B) Cell cycle phase of cancer cells at the primary tumor and lymph-node metastases. (C) Spatial transcriptomics of mouse (stromal cell) clusters at the primary tumors and lymph node metastases. (D) Sunburst plot of the cell cycle in human cancer cell clusters. (E) Bar plot of cell cycle phases in the primary tumors and lymph node metastases.

Figure 4. Enrichment analysis of two cancer stem cell-like populations.

(A) Heatmap of cluster marker genes in human cancer cell DEGs. (B) Heatmap of enrichment scores in upstream analysis. (C) Heatmap of enrichment analysis in the two cancer stem cell-like populations (HMGA1-high and CD44/MYC-high). (D) Enrichment network in two cancer stem cell-like populations.

Figure 5. Spot cell-cell interaction analysis in 'stromal to cancer interaction'.

(A) A flowchart of the CCI analysis. (B) Circular clustering plot of hierarchical analysis of spot CCI analysis (stromal cell to cancer cell). (C & D) Heatmap of CCI in the spots.

Figure 6. Cell-cell interaction analysis with cell type combinations.

(A) A table of the combinations of cell types. (B) UMAP plot of the combination group. (C) Bar plot of counts of the group. (D) Circular bar plot of the top CCI (cancer-to-stromal cell interaction) in each group. (E) Bubble chart of the

685 cancer-to-stromal CCI in each group. (F) Circular bar plot of the top CCI
686 (stromal-to-cancer cell interaction) in each group. (G) Bubble chart of the
687 stromal-to-cancer CCI in each group.

688

689 **Figure 7. Reanalysis of clinical scRNA-seq and cohorts with cancer stem cell-like**
690 **signatures.**

691 (A) A flowchart of the reanalysis of a public scRNA-seq dataset. We downloaded
692 GSE118389 (scRNA-seq data of 5 TNBC patients) and analyzed it with Seurat. Log
693 normalization, scaling, PCA and UMAP visualization were performed following the basic
694 protocol in Seurat. To extract the cancer cells, cells expressing EPCAM (epithelial
695 marker) were filtered. (B) UMAP plot of cancer cell from 5 TNBC patients. (C&D)
696 Module analysis of HMGA1-high signatures and CD44/MYC-high signatures with UMAP
697 plots. The pie chart showed the ratio of cells that expressed the signatures. (E) Survival
698 analysis of claudin-low (TNBC) patients in METABRIC cohorts by the Kaplan-Meier
699 method. Survival analysis with the expression of the HMGA1 signatures.

700

701

702

703 **Supplementary Figure Legends**

704 **Supplementary Figure S1. Read counts and heterogeneity per spot.**

705 (A) Read counts of human and mouse transcripts in each spot. (B) Read counts of
706 human cancer clusters. (C) Read counts of mouse stromal clusters. (D) Normalized
707 closeness centrality in each human cancer cluster. (F) Normalized closeness centrality
708 in each mouse stromal cluster.

709

710 **Supplementary Figure S2. Cell cycle phase in mouse clusters.**

711 (A) The ratio of the cell cycle phase of human cancer clusters. (B) Cell cycle phase of
712 mouse stromal cells at the primary tumor and lymph-node metastasis. (C) Sunburst plot
713 of the cell cycle in mouse stromal cell clusters. (D) The ratios of the cell cycle phases of
714 mouse stromal clusters.

715

716 **Supplementary Figure S3. Enrichment network of two cancer stem cell-like** 717 **populations.**

718 (A) Circos plot of DEGs in cancer clusters. The edges showed the overlap of DEGs
719 between each cluster. (B) Network visualization of p values in enrichment analysis.

720

721 **Supplementary Figure S4. Enrichment analysis of Marker-low clusters.**

722 (A) Heatmap of enrichment analysis in Marker-low clusters. (B) Enrichment network in
723 Marker-low clusters.

724

725 **Supplementary Figure S5. Enrichment analysis in mouse stromal clusters.**

726 (A) Circos plot of DEGs. The edges showed the overlap of DEGs between each cluster.

727 (B) Heatmap of DEGs in mouse stromal cell. (C) Heatmap of enrichment analysis in

728 Ttr-high, Tcell-like, and Bcell-like clusters (D) Enrichment network in Ttr-high, Tcell-like,

729 and Bcell-like clusters

730

731 **Supplementary Figure S6. Spot Cell-Cell Interaction analysis in ‘cancer to stromal**

732 **interaction’**

733 (A) Circular clustering plot of hierarchical analysis of spot CCI analysis

734 (cancer-to-stromal cell). (B & C) Heatmap of CCI in the interactive spots.

735

736 **Supplementary Figure S7. Survival analysis with the expression of the CD44/MYC**

737 **signatures**

738 Survival analysis of claudin-low patients in METABRIC cohorts by Kaplan-Meier method.

739 Survival analysis with the expression of the CD44/MYC signatures.

740

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

A

Cell-Cell Interaction Database

3,209 Ligands
4,364 Receptors
433 Extracellular Matrix

Total 115,900 interactions

Choose expressed genes
Expression > 2

Estimation of
ligand-receptor interaction
in the same spot

B

- LYMPH
- PT

C

② Lymph specific interaction

E

⑥ Primary tumor specific interaction

D

F

Figure 6

Figure 7

Supplementary Figure S1

Supplementary Figure S2

Supplementary Figure S3

A

B

Supplementary Figure S4

A

B

Supplementary Figure S5

A Cancer to Stromal Interaction

● LYMPH
● PT

B 2 Lymph-PT interaction

C 5 PT specific interaction

Supplementary Figure S7