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The metastatic spread of breast cancer accelerates during sleep

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Abstract

The metastatic spread of cancer is achieved by the haematogenous dissemination of circulating tumour cells (CTCs). Generally, however, the temporal dynamics that dictate the generation of metastasis-competent CTCs are largely uncharacterized, and it is often assumed that CTCs are constantly shed from growing tumours or are shed as a consequence of mechanical insults[1]. Here we observe a striking and unexpected pattern of CTC generation dynamics in both patients with breast cancer and mouse models, highlighting that most spontaneous CTC intravasation events occur during sleep. Further, we demonstrate that rest-phase CTCs are highly prone to metastasize, whereas CTCs generated during the active phase are devoid of metastatic ability. Mechanistically, single-cell RNA sequencing analysis of CTCs reveals a marked upregulation of mitotic genes exclusively during the rest phase in both patients and mouse models, enabling metastasis proficiency. Systemically, we find that key circadian rhythm hormones such as melatonin, testosterone and glucocorticoids dictate CTC generation dynamics, and as a consequence, that insulin directly promotes tumour cell proliferation in vivo, yet in a time-dependent manner. Thus, the spontaneous generation of CTCs with a high proclivity to metastasize does not occur continuously, but it is concentrated within the rest phase of the affected individual, providing a new rationale for time-controlled interrogation and treatment of metastasis-prone cancers.

Access options

Data availability

RNA-seq data have been deposited in the Gene Expression Omnibus (GEO, National Center for Biotechnology Information; accession number GSE180097). Processed transcriptomics data, large datasets and other files required for reproducibility are available from the Zenodo data repository (https://doi.org/10.5281/zenodo.6358987). The human reference genome (GRCh38), mouse reference genome (GRCm38), human gene annotation (release 35) and mouse gene annotation (release M25) files were downloaded from GENCODE (https://www.gencodegenes.org). Gene sets were downloaded from the Molecular Signatures Database (MsigDB, v7.4, http://www.gsea-msigdb.org/gsea/msigdb/collections.jsp). All data are available from the corresponding author upon reasonable request. Source data are provided with this paper.

Code availability

Code related to the data analysis of this study has been deposited to GitHub (https://github.com/TheAcetoLab/diamantopoulou-ctc-dynamics) and archived at Zenodo

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Contributions

Z.D. and N.A. designed the study, carried out the experiments and wrote the manuscript. F.C.-G. carried out the computational analysis. M.S. generated the 4T1-CD90.1 cells and carried out the immune cell analysis. S.B. carried out immunofluorescence staining. K.S. contributed to mouse experiments and processed mouse tissues. I.K. processed blood samples. F.D.S., C.F., B.S., M.V., C.R., W.P.W., C.K. and V.H.-S. provided patient samples. All authors have read, commented on and approved the manuscript in its final form.

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Ethics declarations

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Additional information

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Extended data figures and tables

Extended Data Fig. 1 Tumor size and CTCs intravasation rates during different phases of the circadian rhythm.

a, Pie charts displaying the mean percent of total CTCs, single CTCs, CTC clusters and CTC-WBC clusters detected during the rest or active phase in breast cancer patients ($n = 30$), in NSG-LM2 mice ($n = 6$), NSG-CDX-BR16 mice ($n = 6$), NSG-4T1 mice ($n = 4$) or BALB/c-4T1 mice ($n = 6$). **b**, Time kinetic analysis showing mean CTC counts in the NSG-LM2 ($n = 3$), NSG-4T1 ($n = 3$) and BALB/c-4T1 (ZT0, ZT4, ZT20 $n = 3$; ZT12, ZT16 $n = 4$) breast cancer mouse models over a 24-h time period. Data are presented as mean ± s.e.m. **c**, Plots showing the size of the primary tumors dissected at different timepoints from NSG-CDX-BR16 mice (ZT0, ZT4, ZT16, ZT20 $n = 4$; ZT12 $n = 3$), NSG-LM2 ($n = 3$), NSG-4T1 mice ($n = 3$) and BALB/c-4T1 (ZT0, ZT4, ZT20 $n = 3$; ZT12, ZT16 $n = 4$) mice. Data are presented as mean ± s.e.m. **d**, Representative bioluminescence images of lungs from NSG-CDX-BR16, NSG-LM2 and NSG-4T1 mice taken at different timepoints (ZT0, ZT4, ZT12, ZT16, ZT20) ($n = 3$). **e**, Box plots showing the distribution of the

number of single CTCs ($P = 0.0043$), CTC clusters ($P = 0.0087$) and CTC-WBC clusters ($P = 0.0130$) collected at ZT4 or ZT16 in the NSG-CDX-BR16 mouse model ($n = 6$). Center lines in the box represent the median; box limits represent first and third quartile; extremes of the whisker lines represent the minimum and maximum observed values. Data are presented as mean ± s.e.m.; *$P < 0.05$, **$P < 0.01$ by two-sided Mann-Whitney test. **f**, Plots showing the size of primary tumors from NSG-CDX-BR16 ($n = 6$), NSG-LM2 ($n = 6$), NSG-4T1 ($n = 4$) and BALB/c-4T1 ($n = 5$) mice dissected at ZT4 or ZT16. Data are presented as mean ± s.e.m. **g**, Plots showing the mean fold change increase of CTC counts isolated at ZT4 or ZT16 from NSG-LM2, NSG-CDX-BR16, NSG-4T1 and BALB/c-4T1 mice. **h**, Pie charts displaying the mean percentage of single CTCs, CTC clusters and CTC-WBC clusters detected during the rest or active phase in patients ($n = 7$), NSG-LM2 ($n = 6$), NSG-CDX-BR16 ($n = 6$), NSG-4T1 ($n = 4$) or BALB/c-4T1 ($n = 6$) mice. For all panels, n represents the number of biologically independent mice

Source data

Extended Data Fig. 2 The abundance of CTCs during the rest phase is due to increased intravasation.

a, Box plots showing the distribution of the number of CTCs collected at ZT4 or ZT16 via cardiac puncture or tumor draining vessel (TDV) in the NSG-CDX-BR16 breast cancer mouse model ($n = 4$; $P = 0.0286$ for all). **b**, Representative bioluminescence images of lungs from NSG-CDX-BR16 mice taken at different timepoints (ZT0, ZT4, ZT12, ZT16, ZT20) over a 24-h time period ($n = 4$). **c**, Box plots showing the distribution of the number of CTCs collected at ZT4 or ZT16 via cardiac puncture or TDV in the NSG-LM2 breast cancer mouse model ($n = 4$; $P = 0.0286$ for all). **d**, Representative bioluminescence images of lungs from NSG-LM2 mice taken at ZT4 or ZT16 ($n = 4$). **e**, Plot showing the size of primary tumors dissected from NSG-LM2 mice at ZT4 or ZT16 ($n = 4$). **f**, Time kinetic analysis showing fold change differences in the number of LM2 cells detected in the circulation after their intravascular inoculation at different time points of the circadian rhythm (ZT0, ZT4, ZT12, ZT16) ($n = 3$ except ZT4 where $n = 4$). **g**, Plots showing the percentage of CTC clearance at different time points of the circadian rhythm (ZT0, ZT4, ZT12, ZT16) 5 min after intravascular inoculation of LM2 cells ($n = 3$ except ZT4 where $n = 4$). For panels "e", "f" and "g", data are presented as mean ± s.e.m. For panels 'a' and "c", center lines in the box represent the median; box limits represent first and third quartile; extremes of the whisker lines represent the minimum and maximum observed values. *$P < 0.05$ by two-sided Mann-Whitney test. For all panels, n represents the number of biologically independent mice

Source data

Extended Data Fig. 3 Circadian rhythm and melatonin regulate the intravasation of CTCs.

a, Illustration of the experimental design for "b" and "e". **b**, Box plots showing the mean number of CTCs isolated from NSG-LM2 ($n = 5$; single CTCs $P = 0.0079$, CTC clusters $P = 0.0079$, CTC-WBC clusters $P = 0.0317$) and NSG-4T1 ($n = 4$; $P = 0.0286$ for all) mice that were kept in standard light cycle conditions (12:12, LD) or being jet-lagged. The blood draw was performed at ZT4. **c**, Plots showing the mean fold change decrease of CTC counts upon jet lag in NSG-LM2 ($n = 5$) and NSG-4T1 ($n = 4$) mice shown in "b". **d**, Plots showing the size of primary tumors dissected from NSG-LM2 ($n = 5$) and NSG-4T1 ($n = 4$) mice shown in "b". Data are presented as mean ± s.e.m. **e**, Box plots showing the distribution of the number of CTCs isolated from NSG-LM2 mice that were being jet-lagged (*left*) or kept in standard light cycle conditions (*right*) and were treated with melatonin alone or in combination with its antagonist luzindole. The blood draw was performed at ZT4 or ZT0. ($n = 4$, except control and melatonin-treated mice in combination with luzindole at ZT4 where $n = 5$; ZT4 $P = 0.0159$ except CTC-WBC clusters treated with melatonin in combination with lunzindole where $P = 0.0317$; ZT0 $P = 0.0286$ except single CTCs treated with melatonin in combination with lunzindole where $P = 0.0091$). **f**, Plots showing the size of primary tumors dissected from mice shown in "e". Data are presented as mean ± s.e.m. **g**, Representative bioluminescence images of lungs from NSG-LM2 mice that were kept in standard light cycle conditions (12:12, LD) and were treated with melatonin alone or in combination with luzindole. For panels 'b' and "e", center lines in the box represent the median; box limits represent first and third quartile; extremes of the whisker lines represent the minimum and maximum observed values. * $P < 0.05$, ** $P < 0.01$ by two-sided Mann-Whitney test. For all panels, n represents the number of biologically independent mice

Source data

Extended Data Fig. 4 Light exposure impacts CTC intravasation.

a, Time kinetic analysis showing mean CTC counts (single CTCs, CTC clusters and CTC-WBC clusters) in the NSG-LM2 mice kept in altered light-dark (LD) cycles (LD 14:10, $n = 3$; LD 10:10, $n = 4$, except ZT10 and ZT20 where $n = 3$; LD 14:14, $n = 4$, except ZT14 where $n = 3$). **b**, Scatter dot plots showing the distribution of the number of single CTCs, CTC clusters and CTC-WBC clusters isolated from NSG-LM2 mice that were kept in altered light cycles (LD 14:10, $n = 3$; LD 10:10, $n = 4$; LD 14:14, $n = 4$; $P = 0.0286$ for all). * $P < 0.05$ by two-sided Mann-Whitney test. **c**, Plots showing the size of primary tumors dissected from NSG-LM2 mice shown in "a". **d**, Representative bioluminescence images of lungs from NSG-LM2 mice shown in "a". **e**, Graphs showing time kinetic analysis of CTC counts (single CTCs, CTC clusters and CTC-WBC clusters)

in the BL/6-EO771.lmb (ZT4, ZT12, ZT16 n = 4; ZT0 n = 3) and BL/6-$Bmal1^{-/-}$-EO771.lmb (n = 3) breast cancer mouse models collected via tumor draining vessel (TDV) over a 24-h time period. **f**, Plots showing the size of the primary tumors dissected from BL/6-EO771.lmb and BL/6-$Bmal1^{-/-}$-EO771.lmb mice shown in Fig. 1d. **g**, Plotted actograms showing the running activity of the BL/6-EO771.lmb and BL/6-$Bmal1^{-/-}$-EO771.lmb mice with dark and light areas representing low and high activity, respectively. For all panels, data are presented as mean ± s.e.m. n represents the number of biologically independent mice

Source data

Extended Data Fig. 5 Rest-phase CTCs have increased metastatic potential.

a, Representative immunofluorescence images for Pan-CK in lungs of mice injected with single CTCs, CTC clusters and CTC-WBC clusters collected at ZT4 or ZT16 from NSG-LM2 mice (ZT4 n = 3 except CTC-WBC clusters n = 2; ZT16 n = 4 for all). Scale bar = 100 μm. **b**, Plot showing the size of the metastatic lesions detected in the lungs of mice injected with single CTCs, CTC clusters or CTC-WBC clusters collected at ZT4 or ZT16 of NSG-LM2 mice (ZT4 n = 3 except CTC-WBC clusters n = 2; ZT16 n = 4 for all; P = 0.0007). **c**, Representative bioluminescence images of bones from mice injected with single CTCs, CTC clusters or CTC-WBC clusters collected at ZT4 or ZT16 from NSG-CDX-BR16 mice. Mice were not injected with CTC-WBC clusters collected during the active phase, due to their rarity. **d**, Plot showing normalized bioluminescence signal obtained from bones of mice shown in panel "c" (single CTCs n = 4; CTC clusters n = 5; P = 0.006). **e**, Representative bioluminescence images of livers from mice injected with single CTCs or CTC clusters, collected at ZT4 or ZT16 from NSG-CDX-BR16 mice. Mice were not injected with CTC-WBC clusters collected during the active phase, due to their rarity. **f**, Plot showing normalized bioluminescence signal obtained from liver of mice shown in panel "e" (n = 5 except single CTCs collected at ZT16 where n = 4; P = 0.0301). For all panels, data are presented as mean ± s.e.m.; unpaired two-sided t-test * P < 0.05, ** P < 0.01, *** P < 0.001. n represents the number of biologically independent mice

Source data

Extended Data Fig. 6 Time point of CTC isolation is the main determinant of gene expression heterogeneity in CTCs.

a, Heatmap showing the Pearson's correlation coefficient of PC1-7 eigenvectors from gene expression with technical and biological variables in BR16-CDX CTCs. P values by two-sided Pearson's correlation test (*P < 0.01, **P < 0.001, ***P < 0.0001). **b**, Scatter plot showing the correlation of the fold-change between active and rest phase in single CTC (Y-axis) versus CTC clusters and CTC-WBC (X-axis), using genes with $FDR \le 0.05$ in any of the two sets (two-sided

Pearson's correlation coefficient 0.57, *P* value ≤ 2.22e-16). Points are colored according to the dataset where they were found with a *FDR* ≤ 0.05 (both, single CTC or CTC clusters and CTC-WBC clusters). The dashed *red* line represents the linear regression line using all the points in the plot. **c**, Bar plot showing the number of differentially expressed genes (absolute $\log_2$ fold change ≥ 0.5 and *FDR* ≤ 0.05) using all the samples ('All'), using clustered CTCs (CTC clusters and CTC-WBC clusters) and using single CTCs. **d**, Heatmap showing the pair-wise similarity matrix of enriched gene sets (gene set enrichment analysis (GSEA) adjusted *P* value ≤ 0.001) using differential expression between CTCs of rest and active phase from NSG-CDX-BR16 mice. Heatmap colors represent the Jaccard similarity coefficient using the set of core genes in each gene set. The heatmap on the *right* represents the GSEA adjusted *P* value. **e**, Plots comparing the normalized enrichment score (NES) and adjusted *P* value (dot size) obtained using GSEA for gene sets shown in "d". Gene sets with an adjusted *P* value ≤ 0.05 in each sample set are highlighted in *red*. **f**, GSVA score for translation (*yellow*, $n = 5$) and cell division (*blue*, $n = 17$) gene sets in CTCs obtained from the NSG-LM2 time-kinetics experiment. *Yellow* and *blue* lines represent the average at each time point. Individual points represent the enrichment score for each CTC sample. The *white* and *grey* backgrounds represent environmental light (rest period) and dark conditions (active period), respectively. Adjusted F-test *P* values as obtained from limma are shown for each individual gene set.

Extended Data Fig. 7 The proliferation status of primary tumours changes in a circadian rhythm dependent manner.

a, Representative immunofluorescence images of Ki67 and Pan-CK in primary tumors from NSG-CDX-BR16 mice, dissected at different timepoints (ZT0, ZT4, ZT12, ZT16, ZT20) over a 24-h time period (ZT4, ZT12, ZT16 $n = 3$; ZT0, ZT20 $n = 2$; $P = 0.0270$; scale bar = 100 μm) (*left*). The plot shows the intensity of Ki67 in tumors of NSG-CDX-BR16 mice during different timepoints (*right*). Data are presented as mean ± s.e.m.; unpaired two-sided *t*-test * $P < 0.05$. **b**, Representative immunofluorescence images of Ki67 in CTCs collected at ZT4 and ZT16 from NSG-LM2 and NSG-CDX-BR16 mice. Scale bar = 10 μm. **c**, Plots showing the distribution of Ki67 intensity in single CTCs (NSG-LM2 $P = 0.0495$; NSG-CDX-BR16 $P = 0.0001$) and CTC clusters (NSG-LM2 $P = 0.0223$; NSG-CDX-BR16 $P = 0.0045$) collected at ZT4 and ZT16 from NSG-LM2 and NSG-CDX-BR16 mice ($n = 3$). Box center lines in the box represent the median; limits represent first and third quartile; extremes of the whisker lines represent the minimum and maximum observed values. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by two-sided Mann-Whitney test. For all panels, *n* represents the number of biologically independent mice

Source data

Extended Data Fig. 8 Breast cancer derived CTCs lack a functional circadian clock.

a, Plot showing the expression distribution of core circadian genes in CTCs from NSG-CDX-BR16 mice. The fold change (FC, in $\log_2$ scale) and adjusted P value from the global differential expression analysis are shown for each gene. **b**, Density plot showing the distribution of the average expression ($\log_2$ counts per million) of genes in CTCs from NSG-CDX-BR16 mice. Core circadian genes are labeled in the X-axis. **c**, qPCR for *Bmal1* expression in the liver, adrenal glands and primary tumor of NSG-LM2 mice ($n = 3$ for all the time points of the adreanal glands; $n = 3$ for all the time points of the liver and tumor, except ZT4 and ZT20 where $n = 4$). Data are relative to the time point with the lowest expression levels of *Bmal1* (ZT16; set as 1) and are presented as mean ± s.e.m. n represents the number of biologically independent mice

Source data

Extended Data Fig. 9 Assessment of interestial pressure, immunosurveillance and apoptosis in CTCs at different timepoints of the circadian rhythm.

a, Representative immunofluorescence images of Taz and Yap in CTCs collected at ZT4 and ZT16 from NSG-LM2 and NSG-CDX-BR16 mice. Plots showing the distribution of Taz and Yap intensity in CTCs shown in the same panel ($n = 3$). Scale bar = 10 μm. **b**, Plot showing the expression distribution of TEAD genes in CTCs from NSG-CDX-BR16 mice. The fold change (FC, in $\log_2$ scale) and adjusted P value from the global differential expression analysis are shown for each gene. **c**, Gating strategy to determine the frequency of the indicated cell populations in panels "d" and "e". The percentage values refer to the parental population considered in each panel. **d**, Plot showing the frequency of white blood cells (WBCs) from peripheral blood (PB) isolated during the rest ($n = 8$) or active phase ($n = 10$) from BALB/c-4T1 mice. **e**, Plot showing the frequency of tumor-infiltrated WBCs isolated during the rest ($n = 8$) or active phase ($n = 10$) from BALB/c-4T1 mice. **f**, Representative immunofluorescence images of cleaved caspase-3 in CTCs collected at ZT4 and ZT16 from the NSG-LM2 and NSG-CDX-BR16 mice. Plots showing the distribution of cleaved caspase-3 intensity in CTCs shown in the same panel ($n = 3$). Scale bar = 10 μm. For panels "a" and "f", center lines in the box represent the median; box limits represent first and third quartile; extremes of the whisker lines represent the minimum and maximum observed values. ns: non statistically significant by two-sided Mann-Whitney test. For panels "d" and "e", data are presented as mean ± s.e.m.; ns: non statistically significant by unpaired two sided t-test. For all panels, n represents the number of biologically independent mice

Source data

Extended Data Fig. 10 Expression of receptors activated by circadian rhythm regulated ligands.

a, Density plots showing the distribution of the average expression ($\log_2$ counts per million) of genes encoding for receptors of circadian-regulated hormones, growth factors or molecules in CTCs from NSG-CDX-BR16 mice, NSG-LM2 mice and patients with breast cancer. Genes for the glucocorticoid receptor, androgen receptor and insulin receptor are labeled in the X-axis. **b**, Time kinetic plot showing the pharmacokinetic profile of dexamethasone-treated mice ($n = 2$). **c**, Plots showing the size of the primary tumors dissected from dexamethasone-treated or control NSG-LM2 mice ($n = 4$). **d**, Plots showing the size of the primary tumors dissected from testosterone-treated ($n = 5$) or control NSG-LM2 mice ($n = 4$). **e**, Representative bioluminescence images of lungs from untreated or testosterone-treated NSG-LM2 mice (*left*). Plot showing normalized bioluminescence signal obtained from lungs of testosterone-treated or control NSG-LM2 mice ($n = 3$; $P = 0.0005$). **f**, Plot showing plasma concentration of testosterone in control and testosterone-treated mice ($n = 3$; $P = 0.0237$). **g**, Plots showing the primary tumors dissected from control or insulin-treated mice at ZT4 and ZT16 ($n = 8$, except insulin-treated mice at ZT16 where $n = 6$.). **h**, Plot showing plasma concentration of insulin in control and insulin treated mice ($n = 5$; $P = 0.0321$). For all panels, data are presented as mean ± s.e.m.; For panels "e", "f" and "h", * $P < 0.05$, *** $P < 0.001$ by unpaired two sided *t*-test. For all panels, n represents the number of biologically independent mice

Source data

Supplementary information

Reporting Summary

41586_2022_4875_MOESM2_ESM.xlsx

Supplementary Table 1 **Clinical features of enrolled patients with breast cancer**. For each patient, the table shows patient age, initial AJCC stage, date of first diagnosis, ER, PR, HER2 and Ki67 status of the primary tumour. The table also shows the presence of metastatic disease at the time of blood donation, as well as the number of single CTCs, CTC clusters and CTC–WBC clusters detected during the rest (04:00 am) or active period (10:00 am) per 7.5 ml of peripheral blood.

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Supplementary Table 2 **Genes differentially expressed in CTCs of NSG-CDX-BR16 mice during the rest phase versus active phase**. Table listing the differentially expressed genes comparing CTCs obtained in the rest phase ($n = 65$) versus the active phase ($n = 73$) of NSG-

CDX-BR16 mice. All genes evaluated are included in the table ($n = 12{,}261$). Fold change and P values were computed with the quasi-likelihood approach from edgeR using robust dispersion estimates. For fold-change calculation, active-phase samples were used in the denominator.

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Supplementary Table 3 **GSEA from differentially expressed genes in CTCs of NSG-CDX-BR16 mice during the rest phase versus active phase**. Table listing the enriched gene sets ($n = 138$, adjusted P value < 0.05) in CTCs obtained in the rest versus active phase from NSG-CDX-BR16 mice. The GSEA was carried out using ranking genes as input, according to fold change as shown in Supplementary Table 2. P values were obtained using the fast GSEA method with an eps parameter of 1×10^{-10}.

41586_2022_4875_MOESM5_ESM.xlsx

Supplementary Table 4 **List of receptors activated by circadian-regulated hormones, growth factors or molecules**. Table listing the expression levels of genes for receptors activated by circadian-rhythm-regulated hormones, growth factors or molecules in CTCs obtained from NSG-CDX-BR16 mice. Fold change, P values and FDR were extracted from the global differential expression analysis results and were computed with the quasi-likelihood approach from edgeR using robust dispersion estimates.

Source data

Source Data Fig. 1

Source Data Fig. 2

Source Data Fig. 3

Source Data Fig. 4

Source Data Extended Data Fig. 1

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