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ETV5 transcription program links BDNF and promotion of EMT at invasive front of endometrial carcinomas

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Myometrial infiltration represents a main clinical determinant of endometrial carcinomas (EC) presenting as aggressive high-grade deeply invasive neoplasms, substantially associated with risk of recurrence and death. The up-regulation of ETV5 transcription factor linked to the promotion of epithelial to mesenchymal transition is considered as a basic mechanism underlying the initial steps of EC invasion. In this work, we aimed to investigate the transcription program of tumor invasion regulated by ETV5. We performed a comparative Chip-on-chip analysis at invasive front and superficial area of human EC. ETV5 specific binding to promoter regions of genes related to cellular migration, adhesion and invasion at deep invasion tumor areas highlighted the relevance of neural networks associated with cellular plasticity. Interestingly, brain-derived neurotrophic factor (BDNF) demonstrated a principal role orchestrating ETV5-mediated epithelial-to-mesenchymal transition in endometrial cancer. Impairment of the BDNF/tropomyosin-related kinase B (TrkB)/extracellular signal-regulated kinase axis in endometrial cancer cell lines reversed the aggressive and invasive phenotype promoted by the up-regulation of ETV5 at the invasive front of EC. Likewise, BDNF directly impacted on the efficiency of ETV5 promoted metastasis in a mice model of endometrial distant dissemination. These results translate the recognized role of BDNF/TrkB on neural plasticity into a relevant cancer metastasis event; suggest common mechanisms shared by neural development and tumor invasion; and offer new therapeutic opportunities specifically directed against disseminated disease in endometrial cancer.

Introduction

Endometrial carcinomas (EC), the most common tumors of the female genital tract, are usually diagnosed at an early stage with uterine-confined disease and an overall favorable prognosis. However, up to 20% of EC present as aggressive neoplasms such as high-grade or deeply invasive lesions, at substantial risk of recurrence and death (1). Tumor invasion defines the frontier between tissue-restricted carcinoma and disseminated tumor cells, with myometrial infiltration, lymph node involvement and lymphovascular space invasion as current clinical parameters defining the probability of recurrent disease. Regarding therapeutic options, radiotherapy and chemotherapy have shown limited efficacy and personalized therapy is still a promise when dissemination and metastasis are present. Therefore, understanding the molecular events related to myometrial infiltration and distant metastasis represents a decisive requirement for the correct management of advanced EC.

A recent example of how comprehensive molecular characterization of endometrial tumors might identify principal triggers of key clinical challenges as tumor invasion and relapse, linked alterations in the PI3K/PTEN/AKT/mTOR pathway with epithelial–mesenchymal transition (EMT) and advanced events in EC progression (2,3). Indeed, main features of EMT provide clues on myometrial invasion in EC (4). Our group has recently described the role of ETV5 transcription factor in the promotion of EMT associated with the initial steps of endometrial tumor invasion, in cooperation with lipoma-preferred partner protein as sensor of extracellular signals (5). In addition, up-regulation of ETV5 in stage IB EC correlated with MMP-2 activity at the invasive front (6). In this work, we aimed to analyze the transcriptional program coordinated by ETV5 specifically at the invasive front of EC. For this, we (i) performed a comparative ChIP-on-chip analysis at paired superficial and deep invasive areas of human EC; (ii) we identified clues on regulation of genes related to neural plasticity; and (iii) we functionally validated, both *in vitro* and *in vivo,* the impact of brain-derived neurotrophic factor (BDNF) on the ETV5-dependent promotion of EMT and invasive abilities in endometrial cancer.

Materials and methods

Patient samples

Fresh-frozen tissue from endometrioid type EC stage IB according to FIGO was obtained from 13 women who underwent surgery at Vall d'Hebron University Hospital (Barcelona, Spain). Informed consent approved by the correspondent ethical committee was signed by all patients. Each sample was evaluated by a pathologist in order to macroscopically dissect the invasive front and the superficial zone of each tumor immediately after surgery. Frozen sections were stained with hematoxylin and eosin (H&E) to evaluate the presence of tumor cells: at the surface, tumor cells predominated representing 80–90% of cells (left upper image in Figure 1B). At the invasive front, tumor cells were admixed with reactive stroma, inflammatory cells and myometrial smooth muscle cells; samples included in the study presenting at least 70% of tumor cells (right upper image in Figure 1B).

Cell lines and cell culture

Human EC cell lines Hec1A and Hec1A stably expressing the ETV5 transcription factor (H-ETV5) were maintained as described (6) (authentication of Hec1A cells has been confirmed at IdentiCell (Denmark) in October 2013 by GenePrint® 10 System allowing co-amplification and three-color detection of nine human loci, including the ASN-0002 loci (TH01, TPOX, vWA, Amelogenin, CSF1PO, D16S539, D7S820, D13S317 and D5S818) as well as D21S11. These loci collectively provide a genetic profile with a random match probability of 1 in 2.92×10^9). To stably silence BDNF expression Hec1A and H-ETV5 cells were infected with shRNA non-mammalian target control (shCtrl) or BDNF-specific shRNA lentiviral particles (shBDNF; NM_001709; shBDNF#2 in Supplementary Table 2, available at *Carcinogenesis* Online) in the presence of polybrene (8 µg/ml; Sigma, St. Louis, MO), and selected with puromycin (1 µg/ml). Efficacy of infection was assessed by quantitative real time PCR (RT-q-PCR) and western blotting. Endometrial cell lines were infected with lentiviruses bearing pLenti CMV V5-LUC Blast (w567-1) (Addgene, Cambridge, MA) to constitutively express the luciferase reporter gene for *in vivo* monitoring of tumor implants, as described previously (7). Infected cells stably expressing luciferase were selected with blasticidine S HCl (3 µg/ml; Invitrogen, Carlsbad, CA).

Chromatin immunoprecipitation, microarray hybridization (ChIP-on-chip) and data analysis

ChIP-on-chip was performed following GeneChip Human Tiling 1.0R Array Set manufacturer's instructions (Affymetrix, Santa Clara, CA). Briefly,

Abbreviations: BDNF, brain-derived neurotrophic factor; ChIP, chromatin immunoprecipitation; EC, endometrial carcinoma; EMT, epithelial-to-mesenchymal transition; IgG, immunoglobulin G; RT-q-PCR, quantitative real-time PCR; TrkB, tropomyosin-related kinase B; ERK, extracellular signal-regulated kinase.

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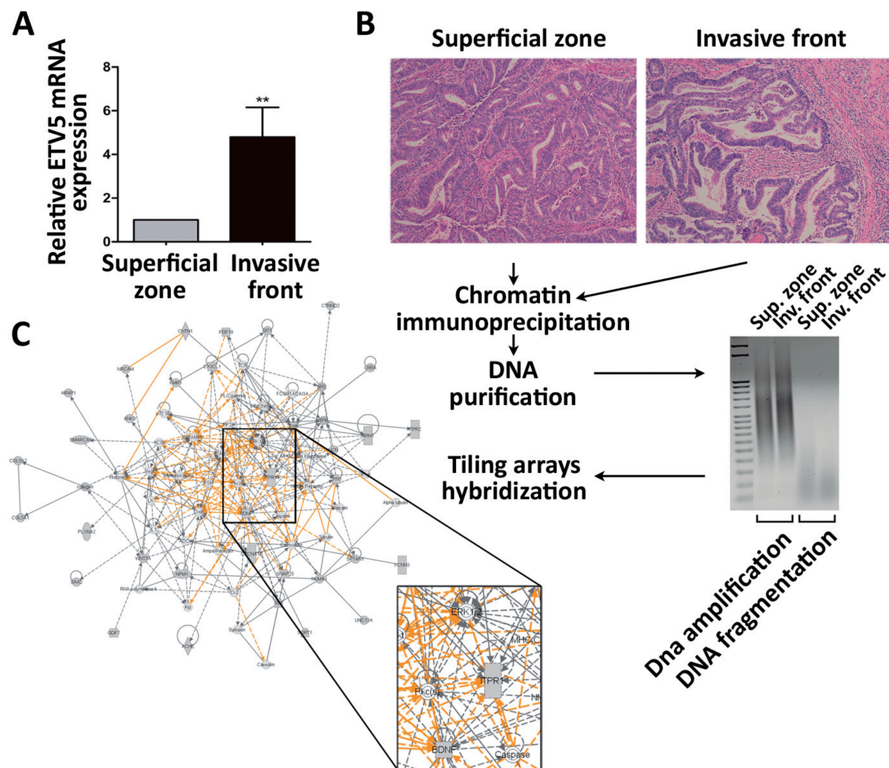

Fig. 1. (**A**) *ETV5* mRNA levels at the superficial zone and invasive front of endometrial carcinomas detected by RT-q-PCR (Wilcoxon test; $**P \le 0.01$). (**B**) Schematic representation of the ChIP-on-chip experimental procedure. (**C**) Ingenuity pathways analysis network of genes regulated by ETV5 at the invasive front and associated with cellular migration, invasion and adhesion highlighted in grey. Magnification shows BDNF located at the core of the network, suggesting a principal role in the orchestration of those cellular functions associated with ETV5 promotion of infiltration in human endometrial carcinomas.

upon chromatin cross-linking in 1% formaldehyde, tissue from paired samples corresponding to the superficial zone and invasive front from six ECs (30 mg) was disaggregated in 600 µl of ChIP lysis buffer (protease inhibitor cocktail, 50 mM Tris–HCl pH 8.0, 10 mM ethylenediaminetetraacetic acid, 1% sodium dodecyl sulfate). After sonication (Branson sonifier 150; Biogen), chromatin fragments (200–1000 bp) were pre-cleared with 100 µl of Protein G Agarose/Salmon Sperm DNA beads (Millipore, CA). Immunocomplexes of chromatin fragments and ETV5 antibodies (10 µg; Santa Cruz Biotechnologies, CA) or irrelevant Immunoglobulin G (IgG) as negative control (10 µg; Santa Cruz Biotechnologies), were recovered with Protein G beads (60 µl) and de-cross-linked (5M NaCl at 65°C overnight). Resulting DNA was purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany), labeled with GeneChip WT Double-Stranded DNA Terminal Labeling Kit (Affymetrix) and hybridized to GeneChip Human Tiling 1.0R Arrays (Affymetrix) for 16 h at 45°C and 60 r.p.m., according to supplier's instructions. GeneChips were scanned with the Affymetrix GeneChip scanner 3000 TG Plus and data from. CEL files (*Affymetrix GeneChip Operating Software*, GCOS, v1.2) were imported into the Partek Genomic Suite Software, pre-normalized using the Robust Multichip Averaging algorithm (Partek, MO) and converted to $\log_2$. Mean signal from each probe corresponding to the superficial tumor zone was subtracted to that of the corresponding invasive area. Promoter regions with positive signals representing preferential ETV5 occupation sites at the invasive front were selected. Statistical parameters were set at $P \le 0.005$ (paired *t*-test). By using Ingenuity Pathway Analysis 2.0 software (Ingenuity Systems), genes related with tumor invasion, cellular migration and metastasis were selected to identify functional pathways and gene interaction networks characterizing those promoter genes significantly regulated by ETV5 at the invasive front of EC.

BDNF promoter ChIP

Immunoprecipitated DNA was analyzed by PCR using previously designed primers for *BDNF* promoter (Forward: 5′-GTGACCACCCAGGTGTAGAAT-3′; Reverse: 5′-GCA GGAATGTGTGACAGGAAC-3′; annealing temperature 57°C, 40 cycles). Equal volumes of PCR products were separated on 2% acrylamide gels. Data were normalized to input by densitometry with Image J software.

RNA extraction and quantitative real-time PCR (RT-q-PCR)

Total RNA from the invasive front and superficial area of frozen tumor samples ($n = 13$) was extracted using the MasterPure Complete DNA and RNA Purification Kit (Epicentre Biotechnologies, Madison, WI), according to manufacturer's protocol. Alternatively, total RNA was isolated from different cell lines using High Pure RNA Isolation Kit (Roche, Applied Science, Indianapolis, IN) according to the manufacturer. cDNA synthesis was performed with MuLV Reverse Transcriptase kit (Applied Biosystems, Foster City, CA) following manufacturer's protocol, and RT-q-PCR was performed using TaqMan assays (Supplementary Table 3, available at *Carcinogenesis* Online; Applied Biosystems) in an ABI 7500 Real-Time PCR System. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal normalization control. The results were represented as the fold change in gene expression relative to *GAPDH* gene expression ($2^{-\Delta\Delta Ct}$).

Western blot and immunofluorescence

Protein levels from invasive and superficial tumor areas and from cell extracts were assessed by western blot or immunofluorescence as described (5), with primary antibodies at indicated dilutions (Supplementary Table 4, available at *Carcinogenesis* Online) and total extracellular signal-regulated kinase (ERK), tubulin and actin as internal controls. Protein signal was detected using Immobilon Western Blotting Kit (Millipore, Billerica, MA). BDNF stimulation was performed under serum starved conditions for 24 h and further incubation with recombinant human BDNF (250 ng/ml; Peprotech) for indicated times. Cells were harvested in the presence of protein phosphatase inhibitors.

Migration and invasion assays

Migration and invasion assays were performed as described previously (5). Moreover, cells were treated or not with the ERK inhibitor, PD98059 (10 µM; Calbiochem, Darmstadt, Germany), or tropomyosin-related kinase B (TrkB) inhibitor, K252a (Calbiochem), with or without 250 ng/ml recombinan human BDNF (Peprotech) for 24 h and 5 days, respectively.

In vivo *assay and bioluminescent imaging*

Five-week-old female athymic Nude-Foxn1nu mice were purchased from Harlan Laboratories (Indianapolis, IN), housed and maintained under specific pathogen-free conditions and manipulated in accordance with institutional guidelines

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approved by the correspondent committee for animal care. Mice were divided in three groups with Hec1A–shCtrl, H-ETV5–shCtrl or H-ETV5–shBDNF stably expressing luciferase cells (5×10^5 cells in 100 µl of sterile phosphate-buffered saline) being inoculated into animals by intracardiac injection under 2% isoflurane/air anesthesia. A successful intracardiac injection was indicated on day 0 by images showing systemic bioluminescence distributed throughout the animal. Five animals per group, with evidence of a satisfactory injection, continued in the experiment. Three weeks after cells injection and before killing, IVIS system (Xenogen, Caliper Life Sciences, PerkinElmer, Waltham, MA) coupled to Living Imaging software 4.2 (Xenogen) were used to detect tumor lesions by bioluminescent imaging. For non-invasive bioluminescence tumor imaging, luciferin (Firefly Luciferin, Caliper Life Science, Hopkinton, MA) was used as the substrate for the luciferase expressing tumor cells and injected intraperitoneally at a concentration of 150 mg/kg in phosphate-buffered saline.

Statistical analysis

Statistical analyses were conducted using SPSS (Chicago, version 15.00 for Windows) and GraphPad Prism 4.00 software (GraphPad Softwares, San Diego, CA). Wilcoxon signed test was used to determine the differences in relative gene expression between paired superficial zone and invasive front of EC. For other analysis Mann–Whitney and Kruskal–Wallis (followed Dunn's post-test) non-parametric tests were used to determine the differences between conditions. Statistical significance was set at $P \leq 0.05$.

Results

ETV5-transcription program at invasion front of EC

Paired samples of deep invasive front and superficial areas from EC (n = 13) were macroscopically dissected with a minimal of 70–80% tumor epithelial component, respectively (see Materials and methods). Total RNA was extracted and differential relative expression levels of *ETV5* transcription factor at the areas of myometrial infiltration were confirmed by RT-q-PCR (n = 13; P = 0.0017; Figure 1A). We then explored the differential transcriptional program that the specific up-regulation of ETV5 might be triggering at the invasive front of EC. For this, we performed an ETV5-driven ChIP-on-chip comparative analysis at paired tumor areas (deep invasive versus superficial) of EC (n = 6). Briefly, fresh tumor tissue from the invasive front and superficial area of EC was used for ChIP with antibodies directed against ETV5 transcription factor. As a control or readout of technical efficiency, differential promoter occupation of ETV5 at the deep invasive versus corresponding superficial areas was confirmed by evaluation of the known ETV5 target cyclooxygenase-2 (*COX2*) promoter gene (8); ETV5-immunoprecipitated DNA demonstrated an enrichment of *COX2* promoter region (n = 10; Supplementary Figure 1A, available at *Carcinogenesis* Online). Purified DNA was further processed for amplification and fragmentation following manufacturer's instructions for hybridization onto Affymetrix promoter arrays (Human Tilling Arrays, Affymetrix) (Figure 1B). Data mining with Partek analysis software resulted in a list of 1523 differentially occupied promoter regions between invasive front and superficial areas of EC ($P \leq 0.005$; GEO accession no. GSE57870).

Further filtering with Ingenuity Pathway Analysis software to comprehensively analyze those gene pathways and networks that might be related to myometrial infiltration (cellular migration, adhesion or invasion), resulted in a list of 104 ETV5-occupied promoter's gene eventually responsible for ETV5-dependent promotion of tumor invasion in EC (Supplementary Table 1, available at *Carcinogenesis* Online). Main biological functions associated with ETV5-occupied promoter regions at the invasive front pointed to cell-to-cell signaling interaction, organ development, cellular movement, and connective tissue disorders. More interestingly, development and function of nervous system highlighted as the principal network characterizing an ETV5-regulated transcription program of endometrial tumor invasion (Figure 1C). This neural network, orchestrated by Pkc signaling pathway in cooperation with Akt and ERK1/2, identified *BDNF* at the core suggesting a principal role in the ETV5-transcription program at the invasive front in EC (magnification in Figure 1C). The described role of ETV5 and neurotrophic factors mediating some features of neuronal plasticity, prompted us to further investigate the implication of BDNF in ETV5-dependent endometrial tumor invasion.

ETV5 up-regulates BDNF at the invasive front of EC and triggers ERK1/2 signaling pathway

The differential occupation of *BDNF* promoter region by ETV5 transcription factor at the invasive front of EC was confirmed by ChIP in paired human samples (Figure 2A). Specific immunoprecipitation of *BDNF* promoter by ETV5 antibodies compared with non-specific IgGs was increased at the deep invasive area of EC compared with their corresponding superficial areas (n = 6; histogram in Figure 2A). Concordantly, the expression levels of *BDNF* at the invasive area were significantly higher compared with those at superficial areas of EC (n = 13; P = 0.0215; Figure 2B). This specific up-regulation was linked to an enhanced expression of NTRK2 (TrkB) receptor (Figure 2B) and the activation of ERK1/2 signaling pathway (Figure 2C), as TrkB is known to be coupled to the activation of the RAS (rat sarcoma viral oncogene homolog family)/ERK signaling pathways during synaptic plasticity (9). Significant relative expression of *FYN* and *NRCAM* genes at the invasive front of EC reinforced the neural network characterizing ETV5-dependent transcription program during myometrial infiltration (Supplementary Figure 1B, available at *Carcinogenesis* Online).

The specific occupation of the *BDNF* promoter region by ETV5 was further confirmed in the endometrial cancer cell line Hec1A and its ETV5 over-expressing cell line counterpart H-ETV5 (5). As shown in Figure 2D, ETV5 antibodies were able to immunoprecipitate higher levels of *BDNF* promoter region in ETV5-overexpressing cells compared with the basal levels found in the parental cell line expressing basal levels of ETV5. Similar results were obtained with the Ishikawa endometrial cancer cell line and its ETV5-overexpressing variant (Supplementary Figure 2A, available at *Carcinogenesis* Online). Also concordantly, increased RNA levels of *ETV5* resulted in enhanced expression of *BDNF* in H-ETV5 cell line (Figure 2E). At the protein level, up-regulation of ETV5 led to an enhanced cytoplasmic BDNF expression as evidenced by fluorescent microscopy (Figure 2F), and of both the pro- and the mature protein forms shown by western blot (Figure 2G). Finally, increased ETV5 transcription factor levels leading to an enhanced BDNF expression resulted in the activation of ERK1/2 signaling in the endometrial cancer cell line Hec1A (Figure 2G). Likewise, addition of BDNF to Hec1A resulted in the activation of ERK pathway (Figure 2H), suggesting a feedback mechanism of ETV5-dependent modulation of ERK pathway. Similar results were obtained in the Ishikawa cell line with up-regulated expression of ETV5 (Supplementary Figure 2B and C, available at *Carcinogenesis* Online). All these data link the up-regulation of ETV5 at the invasive front of EC during the initial steps of myometrial infiltration with the activation of the BDNF–TrkB–ERK1/2 axis, a crucial signaling pathway related to axonal guidance and plasticity in neural development.

BDNF mediates ETV5-dependent EMT in EC cell lines

As mentioned, ETV5 has a direct role on EMT resulting in the acquisition of migratory and invasive capabilities in endometrial cell lines (5). We thus investigated whether BDNF was intervening in ETV5-mediated plasticity; for this, we evaluated *in vitro* and *in vivo* the impact of BDNF silencing in ETV5 over-expressing cell lines (Supplementary Figure 3A, available at *Carcinogenesis* Online). We first confirmed the reversion of the plasticity phenotype promoted by the up-regulation of ETV5 in the Hec1A endometrial cancer cell line. The acquisition of morphological and mesenchymal biomarkers like increased Vimentin or diminished E-cadherin concomitant to the up-regulation of ETV5 and BDNF in H-ETV5 cells, was partially restored both at the RNA and protein levels by silencing of BDNF (Figure 3A). The reversion from a mesenchymal to an epithelial phenotype was also evidenced by restoration of EMT-related transcriptions factors upon silencing of BDNF in the ETV5-overexpressing Hec1A cells (Supplementary Figure 3B, available at *Carcinogenesis* Online). No impact of BDNF silencing could be observed in the parental Hec1A cells with basal levels of ETV5 (data not shown). Similar results were obtained in the Ishikawa endometrial cancer cell line (Supplementary Figure 4, available at *Carcinogenesis* Online).

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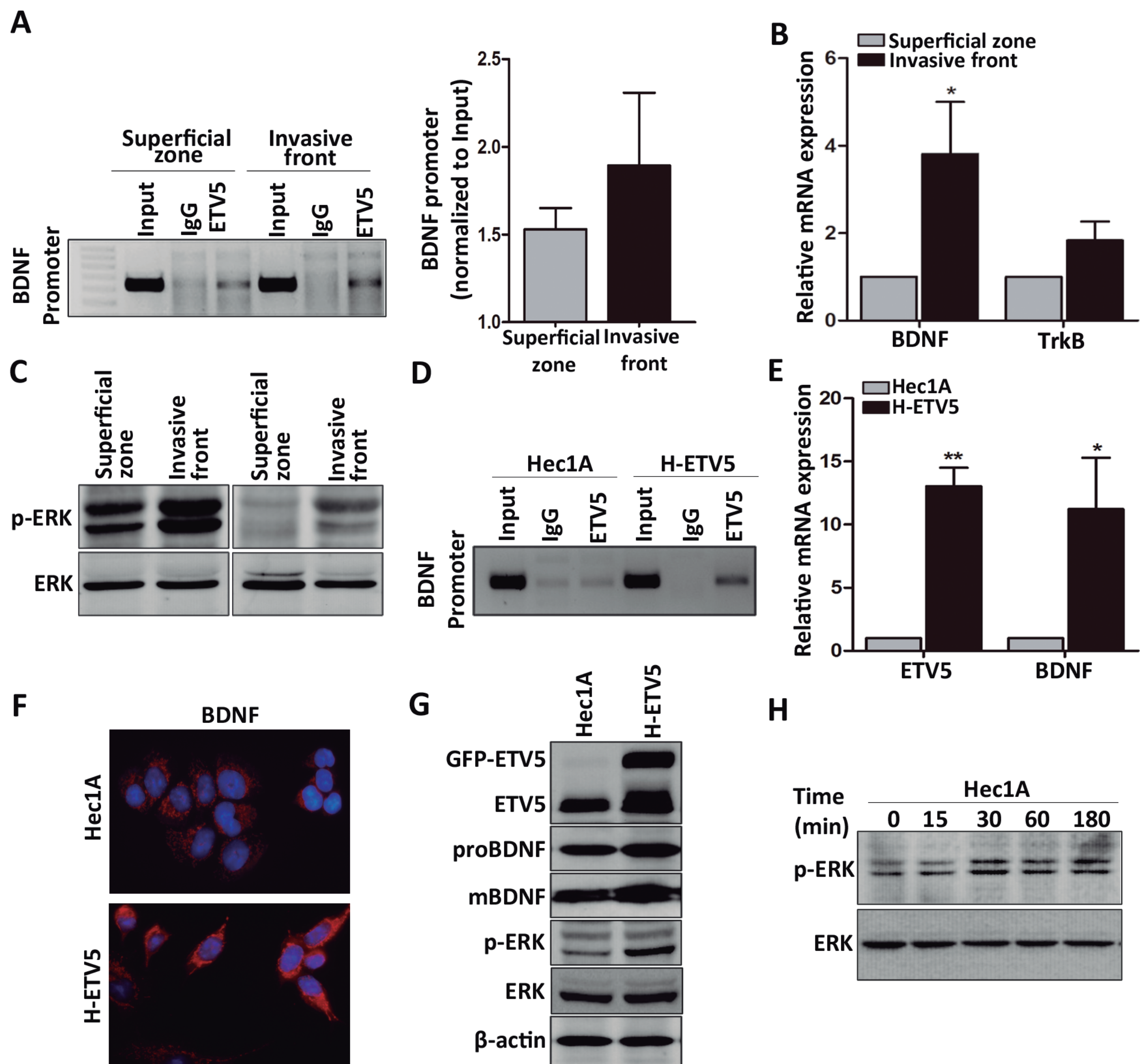

Fig. 2. (**A**) Representative PCR analysis of the *BDNF* promoter region in chromatin immunoprecipitated with anti-ETV5 antibody (ETV5) at the superficial zone and invasive front. Irrelevant IgG are shown as negative control (IgG) (left panel). Optical densities of *BDNF* promoter-enriched chromatin immunoprecipitated with anti-ETV5 antibody in the superficial zone and invasive front (Wilcoxon test; $*P \leq 0.05$) (histogram in right panel). (**B**) *BDNF* and *NTRK2* mRNA expression levels analyzed by RT-q-PCR in the superficial zone and invasive front (Wilcoxon test; $*P \leq 0.05$). (**C**) Representative western blot images of p-ERK in paired samples including superficial zone and invasive front. Total ERK was used as loading control. (**D**) Validation of ETV5 binding to *BDNF* promoter region in chromatin immunoprecipitated with ETV5 antibody (ETV5) in Hec1A and H-ETV5 cells. Irrelevant IgG are shown as negative control (IgG). (**E**) Analysis of *ETV5* and *BDNF* mRNA levels by RT-q-PCR in Hec1A and H-ETV5 cells (Mann–Whitney test; $*P \leq 0.05$, $**P \leq 0.01$). (**F**) Immunofluorescence staining for BDNF in Hec1A and H-ETV5 cell lines. DAPI staining was used to detect nuclei. (**G**) Western blot analysis with antibodies against ETV5, BDNF and p-ERK in Hec1A and H-ETV5 cell lines. Total ERK and β-actin were used as a loading control (mBDNF, mature BDNF). (**H**) Western blot analysis showing changes in ERK phosphorylation in Hec1A and H-ETV5 cells after stimulation with human recombinant BDNF for indicated times. Total ERK was used as a loading control.

Interestingly, silencing of BDNF also reversed the activation of ERK signaling pathway resulting from the ETV5/BDNF axis in the endometrial cancer cell lines (Figure 3A).

Consistently, the migratory abilities promoted by the up-regulation of ETV5 were reversed by silencing of BDNF in H-ETV5 endometrial cell line (Figure 4A). Moreover, inhibition of ERK1/2 signaling by specific inhibitor (10 µM PD98059) significantly reduced the ETV5–BDNF-mediated Hec1A migration even in the presence of human recombinant BDNF (Figure 4A), further supporting the impact of BDNF–ERK1/2 axis in ETV5 mediated myometrial infiltration. Regarding tumor invasion, *in vitro* inverted invasion assay also evidenced that BDNF silencing reverted the invasive properties developed by the up-regulation of ETV5 in the Hec1A cells (Figure 4B). Consistently, specific inhibition of TrkB (100 nM K252a) resulted in a complete abrogation of the migratory properties exhibited by Hec1A cells upon activation of the ETV5–BDNF axis (Figure 4A). All these results reinforced the mediation of BDNF neurotrophic factor in the ETV5-dependent acquisition of a plasticity phenotype in EC leading to the promotion of migration and invasion during myometrial infiltration.

BDNF influences the metastatic efficiency of ETV5-overexpressing cells in vivo

We finally evaluated the impact of the reversion of the aggressive plasticity phenotype resulting from the over-expression of the transcription factor ETV5, in an *in vivo* mice model of tumor dissemination. For this, we compared the pattern of metastasis upon intra-cardiac injection of stably transfected luciferase reporter gene Hec1A cells,

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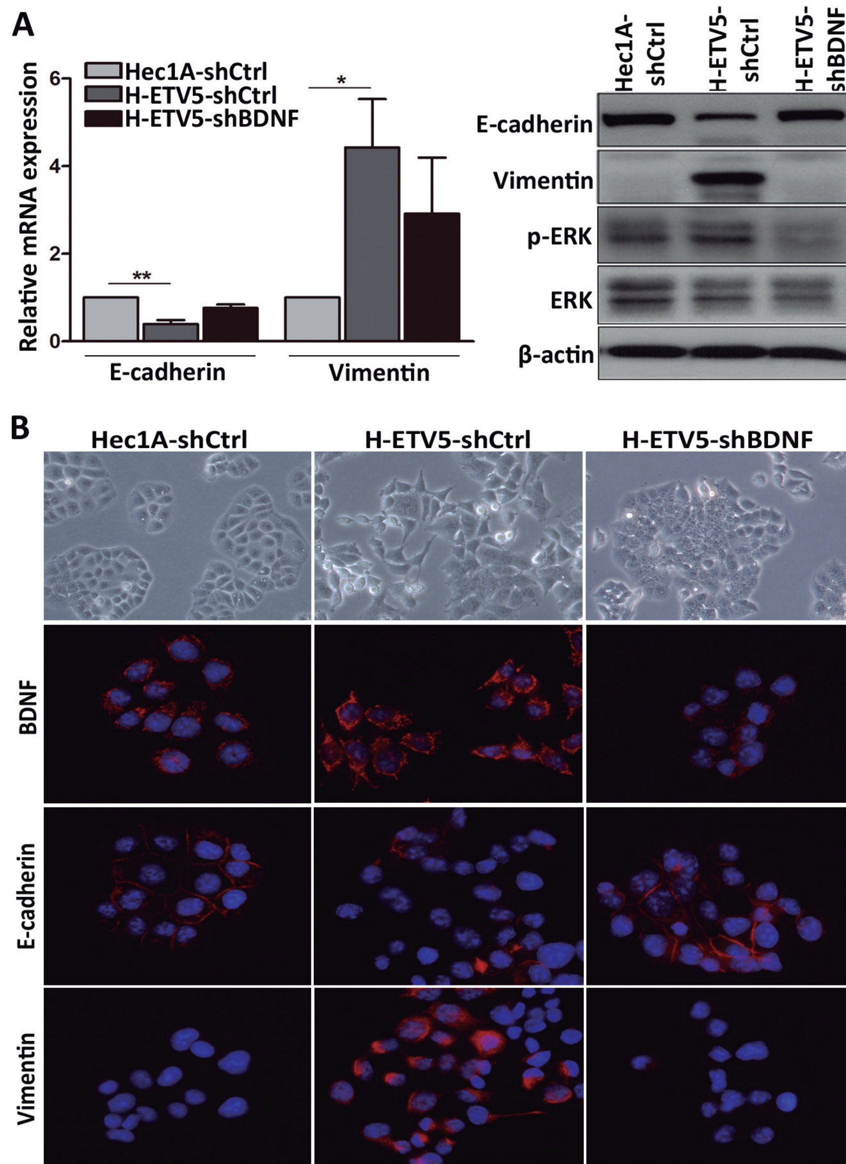

Fig. 3. (**A**) E-cadherin and Vimentin levels in Hec1A–shCtrl, H-ETV5–shCtrl and H-ETV5–shBDNF cells analyzed by RT-q-PCR (histogram in left panel) (Kruskal–Wallis test; $*P \leq 0.05$, $**P \leq 0.01$) and western blot (right panel). Total ERK and β-actin were used as a loading control (right panel). (**B**) Phase contrast microscopy images (upper panels) and immunofluorescence staining for BDNF, E-cadherin and Vimentin (lower panels) in Hec1A–shCtrl, H-ETV5–shCtrl and H-ETV5–shBDNF cells. DAPI staining was used to detect the nuclei.

over-expressing or not ETV5 and in the presence or not of BDNF. Two representative animals per group are shown in Figure 5, with parental Hec1A–shCtrl cells-injected mice presenting metastasis implants mainly at bones, ovaries and adrenal glands 3 weeks after intra-cardiac injection (n = 5; Figure 5A and B). Up-regulation of ETV5 resulted in massive systemic dissemination with implants at pancreas, gonadal fat pad and intestine in addition to bones, ovaries and adrenal glands (n = 5; Figure 5A and B). Remarkably, silencing of neurotrophic factor BDNF in ETV5 over-expressing Hec1A cells resulted in a reversion of the metastatic pattern (n = 5; Figure 5A and B). Quantification of organ-related bioluminescence signal demonstrated the restoration to the less dramatic pattern of metastasis dissemination generated by Hec1A endometrial cancer cells upon reversion of the ETV5-promoted plasticity phenotype through BDNF silencing (Figure 5B). These results further translated into a clinically relevant *in vivo* model the impact of the plasticity phenotype associated with the up-regulation of ETV5 during myometrial invasion, systemic dissemination and homing at sites of metastasis. In addition, these *in vivo* data also reinforced the interplay of BDNF in the promotion and modulation of this aggressive phenotype, providing with evidences that the known role of the BDNF–TrkB–ERK1/2 axis on neuronal plasticity and organogenesis might be mediating pro-metastatic events triggered by the up-regulation of ETV5 at least in endometrial cancer.

Discussion

In this work we have addressed the transcription program underlying ETV5-promotion of myometrial infiltration specifically at the invasive front of EC. Notably, we have identified a principal role for the ETV5-dependent activation of the BDNF–TrkB–ERK1/2 axis, relevant in neuronal plasticity. To this regard, PEA3 transcription factor family members ETV4 and ETV5 have been found mediating retrograde signaling and axonal growth in response to nerve growth factor (10). Likewise, ETV5 has been described as a critical mediator of the glial cell line-derived neurotrophic factor signaling, acting as an upstream inducer of several genes essential in spermatogonial stem cells fate regulation (11). BDNF is a

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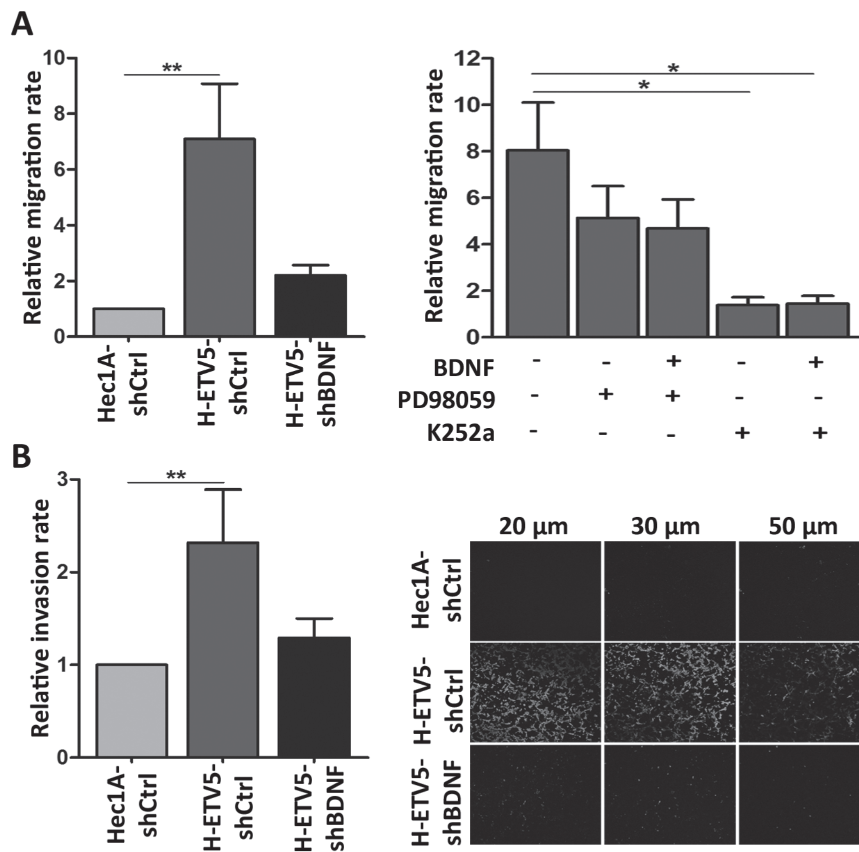

Fig. 4. (**A**) Transwell migration assay with Hec1A–shCtrl, H-ETV5–shCtrl and H-ETV5–shBDNF cells quantified as relative fluorescence compared with Hec1A–shCtrl cells (histogram in left panel) (Kruskal–Wallis test; $**P \le 0.01$). H-ETV5–shCtrl cell migration assays in the presence or absence of BDNF with or without PD98059 and K252a treatment (histogram in right panel) (Kruskal–Wallis test; $*P \le 0.05$). (**B**) *GAPDH* mRNA levels of cells invaded into Matrigel were employed to quantify the relative invasion rate compared with Hec1A–shCtrl cells (histogram in left panel) (Kruskal–Wallis test; $**P \le 0.01$). Representative images of confocal microscopy sections with invasive cells stained with calcein acetoxymethyl ester (right panels).

highly conserved neurotrophin in gene structure and function during vertebrate evolution, serving an important role during brain development and in synaptic plasticity. It participates in the formation of appropriate synaptic connections in the brain, particularly on axon guidance (12). Of note, polymorphisms in ETV5 and BDNF have been associated with obesity, maybe through a role in neurite outgrowth affecting the development of the energy balance circuitry (13,14). Trk receptors are single transmembrane catalytic receptors with intracellular tyrosine kinase activity, coupled to the Ras/ERK, Cdc42/Rac/RhoG, MAPK, PI3-K and phosphoinositide-specific phospholipase C (PLC) gamma signaling pathways (15). TrkB has highest affinity for BDNF and is involved in neuronal plasticity, long-term potentiation and apoptosis of central nervous system neurons. BDNF/TrkB axis has in fact been related with invasion and metastasis in several types of cancer (16–19).

We previously demonstrated that ETV5 cooperates with lipoma-preferred partner protein as a sensor of extracellular signals and promotes EMT in EC. ETV5 modulates Zeb1 expression and E-cadherin repression leading to a complete reorganization of cell–cell and cell–substrate contacts in endometrial cancer (5). Here, we introduced a level of ETV5-dependent EMT modulation through BDNF and its known role on plasticity. In line with our results, studies with HNSCC (head and neck squamous cell carcinoma) cell lines revealed that stimulation with BDNF promoted the migration and invasion of HNSCC cells, and both transient and stable suppression of TrkB resulted in significant abrogation of constitutive and ligand-mediated migration and invasion. Furthermore, enforced overexpression of TrkB results in altered expression of molecular mediators of EMT, including down-regulation of E-cadherin and up-regulation of Twist (20). TrkB-induced EMT is also related to anoikis resistance and metastasis (21). More interestingly, recent evidences suggested a direct role of TrkB in endometrial cancer, with increased levels correlated with lymph node metastasis and lymphovascular space involvement (22). TrkB-mediated EMT and promotion of the migratory and invasive capacity of endometrial tumor cells reinforced our results linking ETV5 and BDNF during the initial events of EC invasion. Likewise, TrkB has been involved in a regulatory loop with miR-204-5p during endometrial tumorigenesis, suggesting restoration of miR-204-5p expression as a potential new therapeutic target in EC (23). Also in line with recent evidences in EC invasion, expression of ERK1/2 has been described to be heterogeneous and, noteworthy, p-ERK1/2 expression increased at the myoinvasive front compared with the corresponding superficial tumor area of EC (24). The *in vivo* data further reinforced the interplay of BDNF in the promotion and modulation of an invasive tumor cell phenotype, and the impact that these aggressive cells might ensure in metastasis efficiency.

In summary, we have performed a comparative ChIP-on-chip analysis at the invasive front of human EC, to identify candidate genes involved in ETV5-dependent promotion of myometrial infiltration. Bioinformatics highlighted BDNF neurotrophic factor as a main component of the gene network that characterized ETV5 transcription at the invasive front in EC. *In vitro* and *in vivo* studies demonstrated the role of BDNF in the modulation of EMT induced by ETV5 and the acquisition of cell migratory/invasive capabilities, and the consequent impact on the efficiency of metastasis. To our knowledge, this is the first time that ETV5-dependent transcription program of invasion provided with new evidences that the known role of the BDNF–TrkB–ERK1/2 axis on neuronal plasticity and organogenesis might be mediating pro-metastatic events at least in endometrial cancer. The presence of tyrosine kinase inhibitors with potent activity

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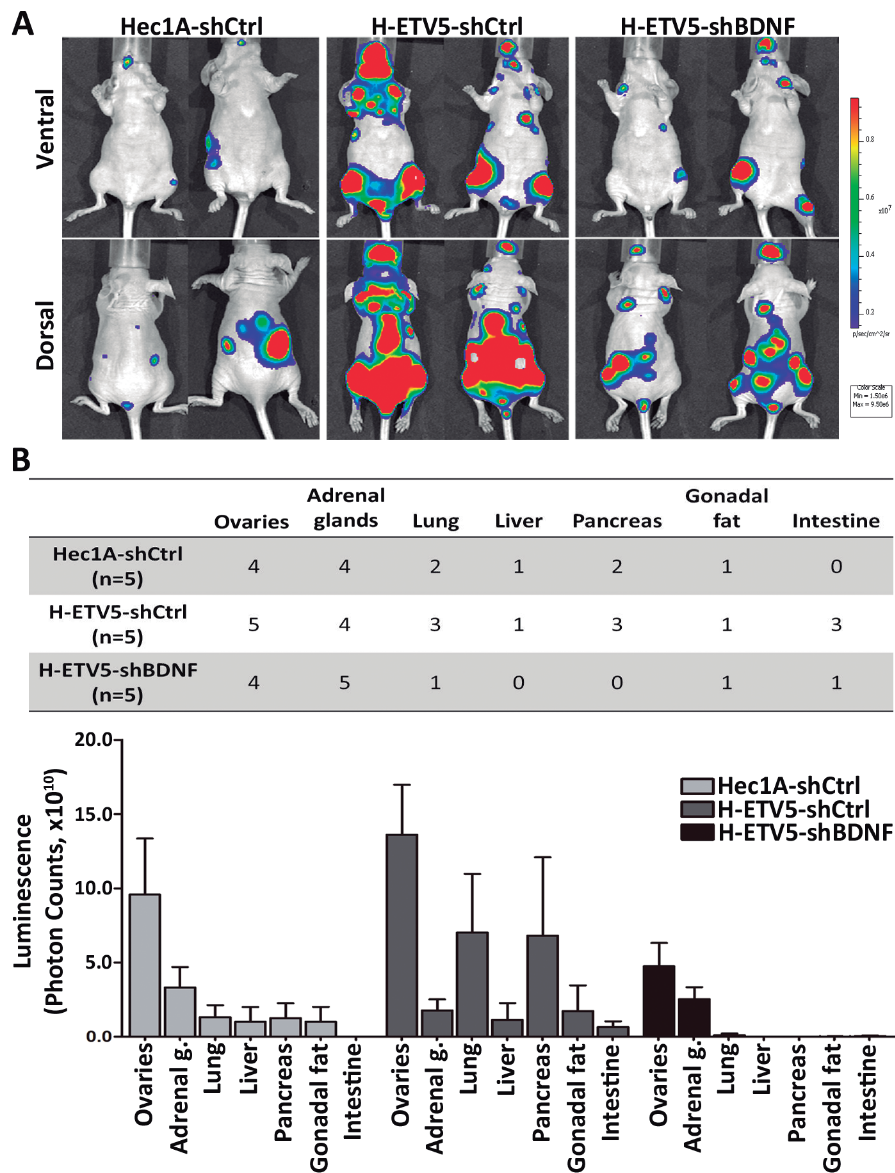

	Ovaries	Adrenal glands	Lung	Liver	Pancreas	Gonadal fat	Intestine
Hec1A-shCtrl (n=5)	4	4	2	1	2	1	0
H-ETV5-shCtrl (n=5)	5	4	3	1	3	1	3
H-ETV5-shBDNF (n=5)	4	5	1	0	0	1	1

Fig. 5. (**A**) *In vivo* bioluminescence representative images of the pattern of metastasis upon intracardiac injection of Hec1A–shCtrl (left panels), H-ETV5–shCtrl (middle panels) and H-ETV5–shBDNF (right panels) cells at ventral (upper panels) and dorsal (lower panels) positions. (**B**) Tumor cell dissemination, homing and metastasis evaluated by affected tissues (table) and luminescence quantification of metastasis in mice injected with Hec1A–shCtrl, H-ETV5–shCtrl and H-ETV5–shBDNF cells (histogram; adrenal g., adrenal glands).

like lestaurtinib in early phase clinical trials (25,26), represents a new therapeutic expectative for the treatment of high-grade EC.

Supplementary material

Supplementary Table S1–S4 and Figures S1–S4 can be found at http://carcin.oxfordjournals.org/

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