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Short Research Paper

Cathelicidin LL-37 Promotes or Inhibits Cancer Cell Stemness Depending on the Tumor Origin

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Abstract

Antimicrobial peptides play critical protective roles in a range of human diseases, including cancer. Multiple studies have demonstrated functions—such as proliferation, angiogenesis, apoptosis and immunomodulation—of these peptides in crucial cancer pathways. We investigated the role of the antimicrobial peptide LL-37 on stemness in breast cancer (SKBR3) and melanoma cells (A375). PCR array analysis of differential gene expression in SKBR3 and A375 cancer cell lines downregulated for LL-37 expression by siRNA revealed downregulation of genes related to stemness, including telomerase reverse transcriptase, forkhead box D3 and undifferentiated embryonic cell transcription factor 1, remarkably in breast cancer cells. Furthermore, SKBR3 cells knocked down for LL-37 expression showed a decreased production of oncospheres in comparison with negative controls, while A375 cells exhibited increased production. Taken collectively, our findings indicate a role for LL-37 in cancer cell stemness depending on the cell type.

Key words: LL-37, cancer, stemness, pluripotency, self-renewal

Introduction

Antimicrobial peptides play crucial roles in critical molecular pathways in cancer, such as cell proliferation, epithelial cell migration, angiogenesis promotion, induction of apoptosis and immunomodulation (1).

The effects of the antimicrobial peptide LL-37 in cancer remain unclear. While LL-37 acts as a positive regulator of ovarian, breast, melanoma and lung cancer progression, it also suppresses colorectal and gastric cancer cell growth (2), indicating that its effects are tumor-specific.

Recently, the concept of clonal tumor evolution has been challenged by the observation of cancer stem cells (CSCs) in a variety of tumors. CSCs possess increased invasive and metastatic capabilities and render tumors more resistant to several microenvironmental stresses, including the action of several anti-cancer drugs (3).

Here we investigated the effects of LL-37 on stemness in both breast cancer and melanoma cells. We performed array analysis to examine the expression of 84 genes related to DNA damage in wild-type and LL-37-knockdown cancer cells.

Material and Methods

The study protocol was approved by the

Hospital das Clinicas Ethical Committee, protocol 034/14.

Cell culture.

Immortalized human breast cancer cells (SKBR-3) and skin malignant melanoma cells (A375) were used in this study. Cells were maintained according to the guidelines of the ATCC (American Type Culture Collection).

Real-time PCR.

RNA was extracted from cultured cells using TRIZOL® protocol. RNA was quantified using NanoVeu (GE Heathcare) systems and RT-PCR was performed using the StepOne SuperScript® III (Applied Biosystems) protocol as provided by the manufacturer. Beta-2 microglobulin (B2M) gene was used as an internal control; primers were as follows: GAT GAG TAT GCC TGC CGT TGC, and CAA TCC AAA TGC GGC ATC T. The reactions were performed in a StepOne™ system (Applied Biosystems) at 50°C for 10 min, 95°C for 5 min and then 95°C for 15 s followed by 60°C for 30 s, and 72°C for 30 s for 40 cycles. Quantification was performed by $2^{-\Delta\Delta CT}$ method.

LL-37 gene silencing.

Cells were plated at 2.5×10^5 cells per well in a 6-well plate overnight. LL-37 Silencer Selected Pre-designed short interfering RNA (siRNA) or negative scramble siRNA (Ambion®) (10 nM each) was combined with 5 µL of Lipofectamine™ RNAiMAX reagent for 20 min. Opti-MEM® I Reduced Serum Medium (Invitrogen) was added to a final volume of 2.5 mL per well after cells were rinsed with PBS. After 24 h (SKBR3) or 48 h (A375), experimental assays were performed.

PCR array.

Total RNA was converted into cDNA using the RT² First Strand Kit (SABiosciences, Frederick, USA) and cDNA was then combined with the RT² SYBR Green qPCR Master Mix (SABiosciences). Each sample was added to 24 Human DNA Damage PCR arrays (Qiagen, USA) according to the StepOne equipment protocol (Applied Biosystems). PCR-array data analysis was performed at the manufacturer's website (http://www.sabiosciences.com/pcrarraydataanalysis.php).

Sphere-forming assays.

The assay was performed as previously reported (4, 5). Briefly, after siRNA treatment, cells were detached and a single cell suspension was obtained after passing cells through 25 G needles. Cells (1.0×10^5) were plated in their respective cell culture media containing B27 supplement and rEGF (100 ng/ml; Sigma Aldrich, Poole, UK; E-9644). After 5 days, the number of spheres that were greater than 50 µm in diameter were counted and sphere forming efficiency (%) was determined.

Statistical analysis.

Results were analyzed suing Mann-Whitney test and are shown as mean ± standard deviation. A p-value < 0.05 was considered significant.

Results

LL-37 production upregulates several pathways related to stemness.

We next analyzed differential gene expression in cancer cell lines downregulated for LL-37 expression by siRNA. Knockdown efficiency of LL-37 expression was more than 90%, as evaluated by qPCR (data not shown). PCR arrays showed a downregulation of several genes related to stemness, especially in SKBR3, for LL-37 compared with control cells (**Tables 1 and 2**).

LL-37 knockdown cells show decreased production of oncospheres in breast cancer cells and increased production in melanoma cells.

We next analyzed the production of cancer-derived extracellular vesicles, oncospheres, as a hallmark of stemness in cancer cell lines depleted for LL-37 expression. Our results showed that SKBR3 cells knocked down for LL-37 expression produced a decreased number of oncospheres compared with negative controls (**Figure 1A**), while A375 produced an increased number of oncospheres compared with negative controls (**Figure 1B**).

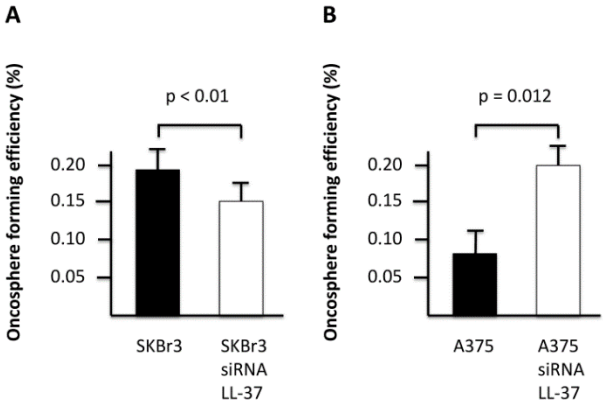

Figure 1. Oncosphere-forming efficiency in SKBR3 (A) and A375 (B) cell lines depleted for LL-37 expression by siRNA.

Table 1. Genes upregulated in SKBR3 breast cancer cells compared with SKBR3 cells transfected with LL-37 siRNA treatment. Genes related to stemness are in bold.

Symbol	Gene Name	Fold Change	p-value
KAT2A	K(lysine) acetyltransferase 2A	1.6337	0.008484
COL2A1	Collagen, type II, alpha 1	1.8903	0.012853
GDF3	Growth differentiation factor 3	1.8402	0.014975
HNF4A	Hepatocyte nuclear factor 4, alpha	1.9064	0.016682
TERT	**Telomerase reverse transcriptase**	**2.2468**	**0.018056**
HAND1	Heart and neural crest derivatives expressed 1	2.1076	0.02039
HSPA9	Heat shock 70kDa protein 9 (mortalin)	3.7997	0.020459
SOX15	SRY (sex determining region Y)-box 15	2.1014	0.02309
NAT1	N-acetyltransferase 1 (arylamine N-acetyltransferase)	26.925	0.026202
ALPL	Alkaline phosphatase, liver/bone/kidney	1.838	0.026346
PARD6A	Par-6 partitioning defective 6 homolog alpha (C. elegans)	14.2326	0.026805
OLIG2	Oligodendrocyte lineage transcription factor 2	1.7598	0.031648
FOXD3	**Forkhead box D3**	**2.6502**	**0.03305**
MYBL2	V-myb myeloblastosis viral oncogene homolog (avian)-like 2	1.6863	0.035958
LIN28A	Lin-28 homolog A (C. elegans)	1.8108	0.036787
TP53	Tumor protein p53	1.9757	0.040021
FGF2	Fibroblast growth factor 2 (basic)	2.0355	0.044985
NES	**Nestin**	**1.8498**	**0.047692**
UTF1	**Undifferentiated embryonic cell transcription factor 1**	**5.2139**	**0.048209**

Discussion

Self-renewal and pluripotency are the key characteristics of stem cells. Here, our findings suggest that LL-37 regulates stemness. Cancer stem cells (CSCs) are a considerable clinical problem, since they are highly resistant to radiation and chemotherapy (6). The mechanism of resistance is not fully understood, but enhanced DNA repair capacities and low intracellular reactive oxidative species concentrations are implicated (7). CSCs also proliferate more slowly than non-stem carcinoma cells, circulate in the bloodstream (8) and can lead to tumor metastasis and relapse. Definitive markers of CSCs do not exist, but our results demonstrated many interesting results in SKBR3 and A375 wild-type cells, when compared with LL-37 knockdown cells. Nestin, for example, is an important marker of CSCs, regulates proliferation, migration and invasion of cancer cells (9) correlating to a worse prognosis (10).

Telomerase reverse transcriptase, forkhead box D3 (FOXD3) and undifferentiated embryonic cell transcription factor 1 (UTF1) are other genes related to stemness, so we hypothesized that LL-37 should be important to maintain stem cell identity in breast cancer cells (**Table 1**). Upregulation of telomerase is a prerequisite for cellular immortalization and has been associated with stemness in various human cancers (11). FOXD3 induces cancer progression by epithelial-mesenchymal transition (12) and UTF1 increases stem cells reprogramming to pluripotency (13).

Table 2. Genes upregulated in A375 melanoma cells compared with A375 cells transfected with LL-37 siRNA treatment. Genes related to stemness are in bold.

Symbol	Gene Name	Fold Change	p-value
LIN28A	Lin-28 homolog A (C. elegans)	1.5252	0
TCF3	Transcription factor 3 (E2A immunoglobulin enhancer binding factors E12/E47)	2.9868	0
RUNX2	**Runt-related transcription factor 2**	**1.7998**	**0.000007**
CCNA2	Cyclin A2	1.3786	0.000011
FOXA2	Forkhead box A2	1.5997	0.000018
CD34	CD34 molecule	1.3355	0.000025
BGLAP	Bone gamma-carboxyglutamate (gla) protein	1.5147	0.000044
CDH2	**Cadherin 2, type 1, N-cadherin (neuronal)**	**1.6844**	**0.000044**
FABP7	Fatty acid binding protein 7, brain	1.6157	0.000056
CCNE1	Cyclin E1	1.472	0.000063
OLIG2	Oligodendrocyte lineage transcription factor 2	1.5713	0.000086
NCAM1	Neural cell adhesion molecule 1	1.2164	0.00013
REST	RE1-silencing transcription factor	1.3996	0.000231
TUBB3	Tubulin, beta 3	1.4953	0.000248
CDC42	Cell division cycle 42 (GTP binding protein, 25kDa)	1.5889	0.00025
KRT15	Keratin 15	1.469	0.000278
GATA2	GATA binding protein 2	1.5547	0.000372
ALPL	Alkaline phosphatase, liver/bone/kidney	1.4816	0.000379
GDF3	Growth differentiation factor 3	1.7102	0.000422
TBX3	T-box 3	1.5775	0.000669
AICDA	Activation-induced cytidine deaminase	1.3863	0.001057
RUNX1	**Runt-related transcription factor 1**	**1.2238**	**0.001949**
KLF4	Kruppel-like factor 4 (gut)	1.1139	0.00216
FGF2	Fibroblast growth factor 2 (basic)	1.4219	0.002267
NAT1	N-acetyltransferase 1 (arylamine N-acetyltransferase)	1.3888	0.002528
LEFTY2	Left-right determination factor 2	1.3489	0.003161
MYCN	V-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	1.1672	0.003271
HAND1	Heart and neural crest derivatives expressed 1	1.2117	0.003824
ZFP42	Zinc finger protein 42 homolog (mouse)	1.2421	0.004222
HSPA9	Heat shock 70kDa protein 9 (mortalin)	1.4029	0.005582
FGFR1	Fibroblast growth factor receptor 1	1.3626	0.005687
FGF4	Fibroblast growth factor 4	1.1789	0.006383
NODAL	**Nodal homolog (mouse)**	**1.6944**	**0.0064**
ALDH1A1	**Aldehyde dehydrogenase 1 family, member A1**	**1.2544**	**0.007374**
KAT2A	K(lysine) acetyltransferase 2A	1.2006	0.007585
PAX6	Paired box 6	1.1578	0.008689
DNMT3B	DNA (cytosine-5-)-methyltransferase 3 beta	1.2005	0.011056
HNF4A	Hepatocyte nuclear factor 4, alpha	1.4146	0.011275
CDK1	Cyclin-dependent kinase 1	1.1471	0.012381
COL1A1	Collagen, type I, alpha 1	1.1761	0.015877
LEFTY1	Left-right determination factor 1	1.226	0.015976
DPPA3	Developmental pluripotency associated 2	1.3138	0.024156
NR5A2	Nuclear receptor subfamily 5, group A, member 2	1.1412	0.026799
EP300	E1A binding protein p300	1.3847	0.029084
KAT7	K(lysine) acetyltransferase 7	1.967	0.029885
PARD6A	Par-6 partitioning defective 6 homolog alpha (C. elegans)	1.2703	0.033506
POU5F1	POU class 5 homeobox 1	1.1131	0.042862
ESRRB	Estrogen-related receptor beta	1.2021	0.044154

The results were not so evident in A375 melanoma cells, but the presence of Runt-related transcription factors 1 and 2 (14-16), Cadherin 2 (17, 18), Nodal homolog (mouse) (19) and Aldehyde dehydrogenase 1 (family member A1) pointed to the same direction (20) (**Table 2**). However, the production of oncospheres in A375 cells put in evidence that LL-37 has opposing effects on cancer cell stemness depending on the cell type.

Conclusion

Emerging evidence highlights the role of antimicrobial peptides in non-infectious diseases, such as cancer. The mechanisms triggered by antimicrobial peptides are broad and lead to unexpected cell responses. Here, we show that the antimicrobial peptide LL-37 is implicated in cancer stemness. Further research needs to be directed to better clarify this phenomenon and the role of other antimicrobial peptides in this scenario.

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Competing Interests

The authors have declared that no competing interest exists.

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