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Triptolide from TCM abolishes NF-κB-signaling, EMT and Stem-like Features in a Hypoxic Microenvironment of Pancreatic Cancer

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Introduction: Pancreatic cancer is characterized by a pronounced hypoxic tumor-microenvironment and the level of hypoxia is considered as an independent factor of poor prognosis. It has been identified that the induction of cancer stem cells (CSCs) characteristics, migratory properties and epithelial-to-mesenchymal transition (EMT)-like signaling in pancreatic cancer cells upon hypoxia. Hypoxia-induced NF-κB signaling is an important mediator of CSC-like cells by induction of EMT signaling. Triptolide from traditional chinese medicine (TCM) is a potent inhibitor of transcriptional activation of NF-κB.Targeting of NF-κB by specific inhibition of c-Rel or Triptolide could be beneficial for strategies aiming to eliminate CSC-like cells in the hypoxic microenvironment of pancreatic cancer.
Aims: To investigate the effect of hypoxia on pancreatic CSCs and to further elucidate the effect and molecular mechanism of Triptolide anti-cancer action under hypoxic microenvironment.
Methods: MIA PaCa-2, AsPC-1 and BxPC-3 pancreatic carcinoma cell lines were used as representatives of CSC^{high}, CSC^{high}, and CSC^{low} cell lines, respectively. The ability of hypoxia and Triptolide to target hypoxia-induced CSCs was evaluated by spheroid, colony formation assays, measuring ALDH1 activity in vitro. The effect of Tiptolid on the CSC/EMT-related and proteins expression evaluated by Western blot analysis upon hypoxia. To elucidate the mechanism of Triptolid on hypoxia-induced EMT and CSC related features NF-κB signaling were investigated.

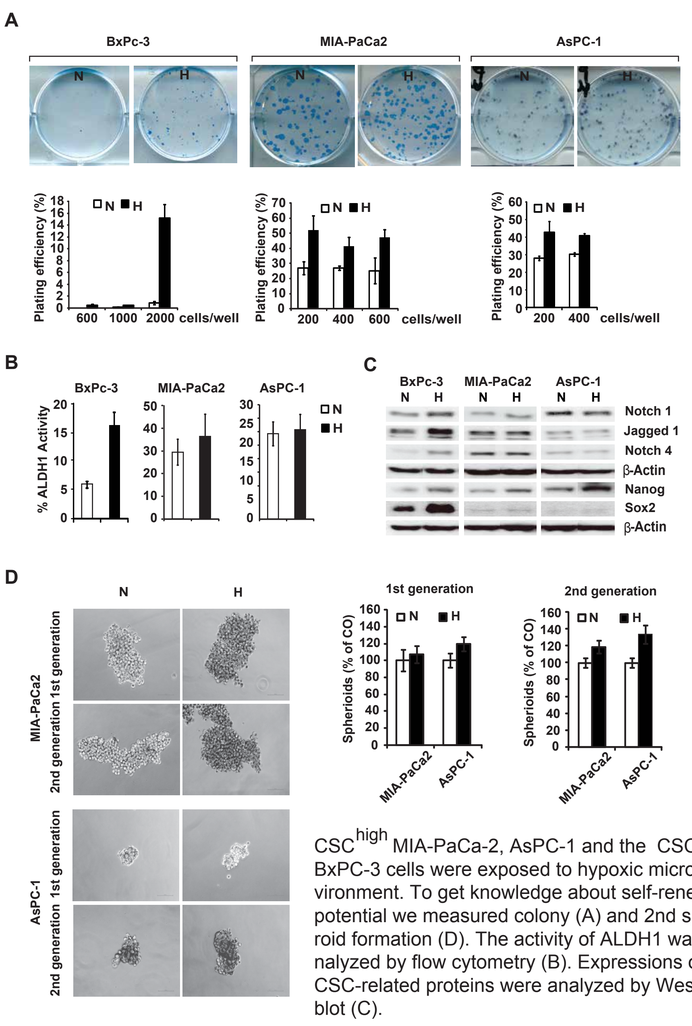
1 Characterization of established human pancreatic cancer cell lines

	CSC^{high}		CSC^{low}	References
	MIA-PaCa2	AsPC-1	BxPc-3	
ATCC No.	CRL-1420	CRL-1682	CRL-1687	ATCC
Source	Primary tumor	Ascites	Primary tumor	ATCC
Degree of tumor differentiation	Poor	Moderate-poor	Well	ATCC
Histology of primary tumor	PDAC, G3	PDAC, G2	PDAC, G2	[1]
In vitro morphology	spheroidal + attached	loosely attached single cells	densely attached cell formations	present study
Colony-forming capacity	+++	+++	+	[2], present study
Spheroid-forming capacity	+++	+	-	[2], UOD
ALDH activity	+++	++	-	[2], UOD
Tumorigenic in mice	+++	+++	+	[2], UOD
Histology of xenografts	G3	G2/G3	G2/G3	[1]
$CD44^{+}/CD24^{-}$	+++	+++	+	[2], UOD
E-Cadherin Protein	-	++	+++	[3], UOD
Vimentin Protein	+++	+++	+	present study
p53	MT	MT	MT	[1]
K-ras	MT	MT	WT	[1]

ATCC: American Tissue Culture Collection;
PDAC; Pancreatic Ductal Adenocarcinoma;
UOD: unpublished own data;NE: not examined;
-: none; +: weak; ++: median; +++: strong.

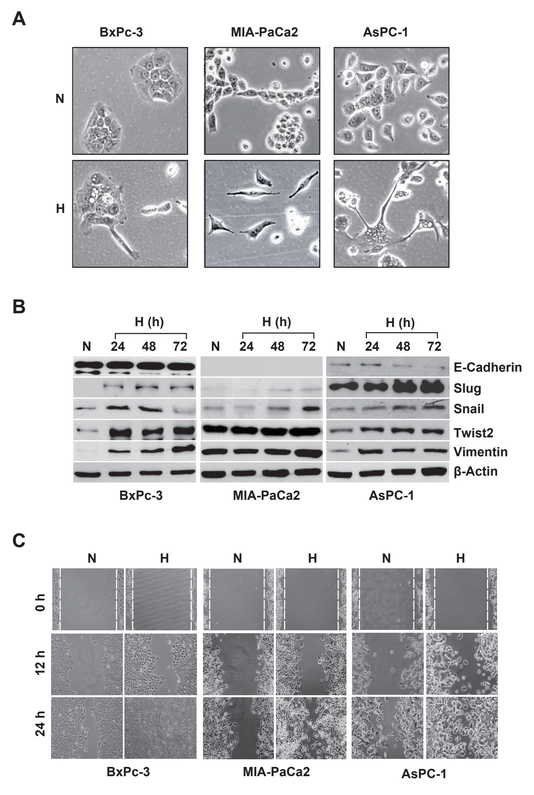
References:
1. Gukovskaya AS, Pandol SJ. Cell death pathways in pancreatitis and pancreatic cancer. Pancreatology 2004; 4: 567-86.
2. Brown JM, Giaccia AJ. The unique physiology of solid tumors: opportunities (and problems) for cancer therapy. Cancer Res 1998; 58: 1408-16.
3. Chia SK, Wykoff CC, Watson PH, Han C, Leek RD, Pastorek J, Gatter KC, Ratcliffe P, Harris AL. Prognostic significance of a novel hypoxia-regulated marker, carbonic anhydrase IX, in invasive breast carcinoma. J Clin Oncol 2001; 19: 3660-8.

2 Hypoxia increases stemness characteristics of pancreatic cancer cells



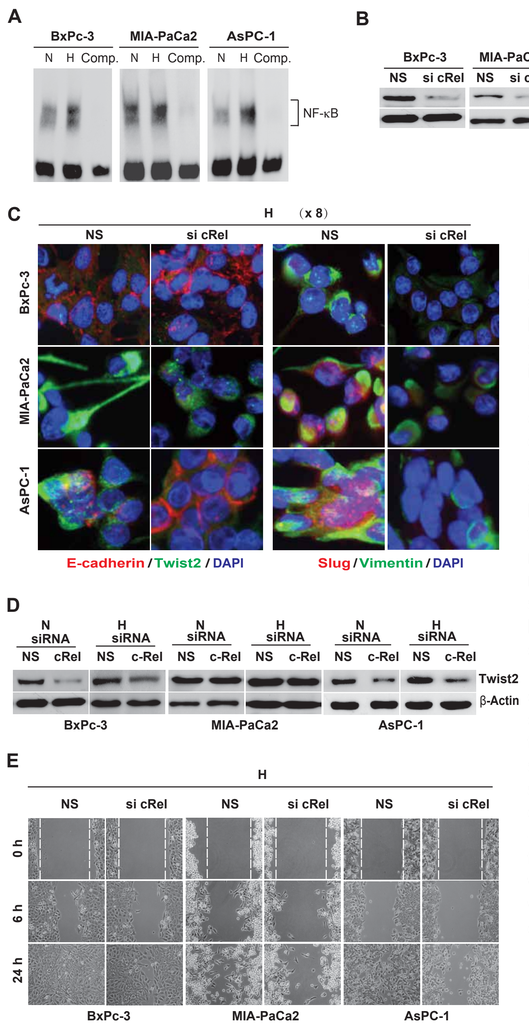
CSC^{high} MIA-PaCa-2, AsPC-1 and the CSC^{low} BxPC-3 cells were exposed to hypoxic microenvironment. To get knowledge about self-renewal potential we measured colony (A) and 2nd spheroid formation (D). The activity of ALDH1 was analyzed by flow cytometry (B). Expressions of CSC-related proteins were analyzed by Western blot (C).

3 Hypoxia induces morphological changes, EMT features and migration



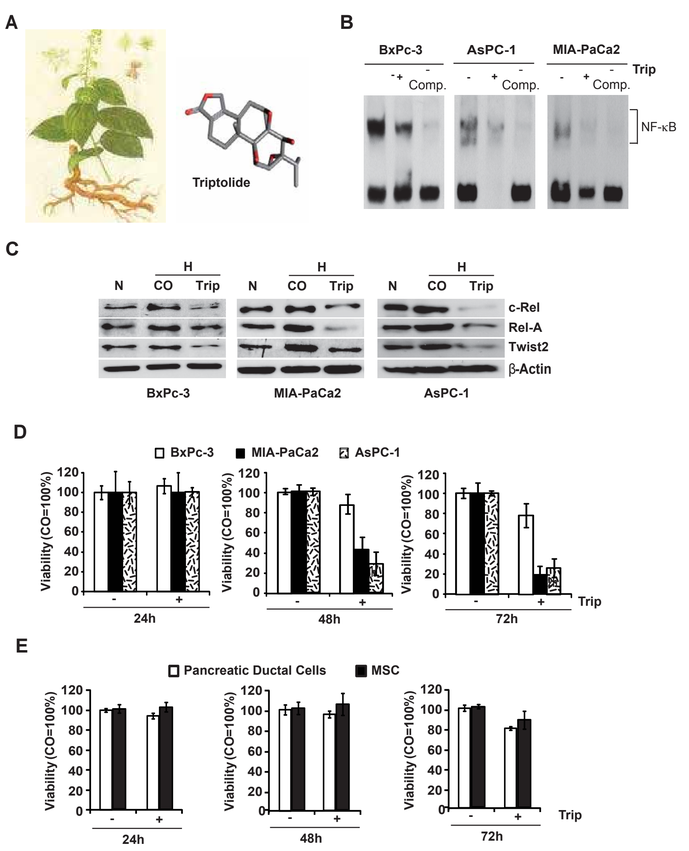
The above changes were associated with a switch in morphology from an epithelial to a more fibroblastoid/mesenchymal phenotype (A) and protein expression typical for EMT, namely down-regulation of E-cadherin and up-regulation of Vimentin, Slug, Snail and T wist2 (B). Corresponding with the aggressive phenotype of MIA-PaCa2 these cells had high basal expression of vimentin and Twist2 and did not show pronounced further up-regulation by hypoxia. Correspondlingly, MIA-PaCa2 had no E-Cadherin expression, while expression of this adhesion protein was high in BxPc-3 cells according to its attached growth in cell aggregates. The migratory potential was increased after hypoxia as evident from faster closure of wounded regions 12 and 24 h after scratching and compared to cells cultured under normoxic conditions (C).

4 c-Rel siRNA inhibits EMT-related features and migration potential



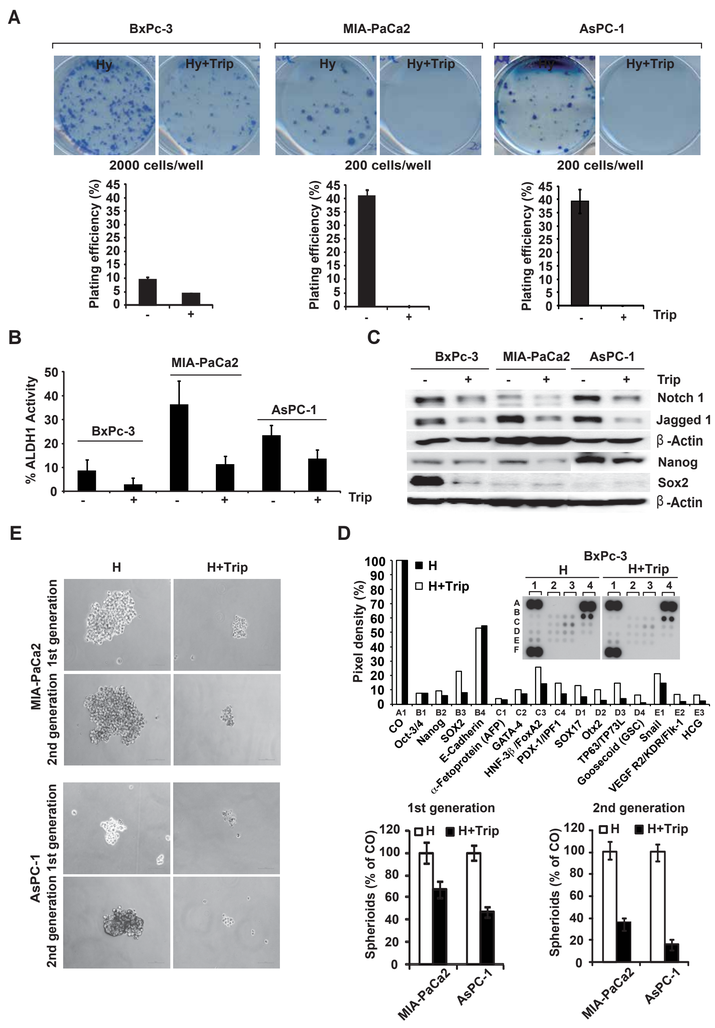
Nuclear cell extracts were prepared 24 h after hypoxia and DNA binding was analyzed by EMSA for NF-κB signaling and it is involved in induction of hypoxia (A). Incubation of cells with specific c-Rel siRNA strongly downregulated c-Rel expression in all three cell lines as examined by Western blot analysis and compared to cells transfected with nonsense siRNA (B). Downregulation of cRel was associated with reversion of hypoxia-induced EMT-related protein expression as examined by immunohistochemistry in double stained cells (C). Compared to cells transfected with nonsense siRNA, the specific c-Rel siRNA led to downregulation of EMT-related protein Twist -2 (D). Finally, faster migration under hypoxic condition was totally inhibited in cells with downregulated c-Rel expression (E).

5 Triptolide inhibits hypoxia-induced EMT-related protein and NF-κB signaling



The molecular structure of the NF-κB inhibiting diterpenoid Triptolide (A) used in TCM as anti-inflammatory and immunosuppressive treatment. Triptolide inhibited the expression of hypoxia-induced NF-κB signaling (B). Triptolide also downregulated expression of the NF-κB subunits c-Rel and Rel-A, and of the EMT-related protein Twist2 within 24 h of treatment as examined by Western blot analysis (C).Pancreatic cancer cell lines (D), normal pancreatic ductal cell and MSC (E) were treated with Triptolid after 24 h, 48 h and 72 h, cell viability was measured by MTT assay.

6 Triptolide abolishes hypoxia-induced CSC-like features



Triptolid abolished hypoxia-induced colony (A), 2nd spheroid (E), the activity of ALDH1 (B) and CSC-related proteins (C,D).

Conclusions: Hypoxic environment increased migratory capacities of pancreatic cancer cells with elevated stem cell characteristics and EMT features by induced NF-κB activity. In additiom, traditional chinese medicine-Triptolide treatment inhibited hypoxia-induced NF-κB activity. Furthermore, Triptolide abolished EMT and CSC-like features. Our data suggested that NF-κB signaling is in volved in the process of hypoxia and EMT. Above results support that Triptolide from TCM may be a good candidate for the treatment of pancreatic cancer enriched in CSCs.

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