Keratin 8 and 18 knockdown increases cisplatin-induced apoptosis and invasive potential through claudin-1/PI3K/NFkB up-regulation in epithelial carcinoma cells

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Keratins are epithelial-specific intermediate filament (IF) proteins, which are expressed in a tissue- and differentiation state-specific manner. As part of the cytoskeleton, keratins are important for the mechanical stability and integrity of epithelial cells and tissues. Moreover, a number of keratins are involved in intracellular signalling pathways which regulate response to injuries and non-mechanical stresses [1, 2], cell growth [3-5], cell death [6-9] and cancer progression [10-12]. Keratins 8 and 18 (K8/18) are typically co-expressed as the primary keratin pair in simple epithelial cells and their expression are maintained during malignant transformation, hence their use as diagnostic marker in tumor pathology. However, in recent years different studies have shown that IF should not be considered only as markers proteins but also as regulators of cancer cell signaling and that they might play an active role in malignant transformation. In the present study, we addressed the question as to whether K8/18 expression affects tumor fate and behaviour.

Our results show that K8/18 stable knockdown using shRNA increases cisplatin sensitivity in different epithelial cancer cell lines. Indeed, western blot analysis of caspases activation and flow cytometry analysis of Annexin V/PI staining show that K8/18 knockdown sensitizes cells to cisplatin-induced apoptosis. Increased FasL expression and FasR membrane targeting suggest that apoptosis is enhanced via the death receptor pathway.

Moreover, using *in vitro* wound healing and transwell invasion assays, we observed that K8/18-deficient cancer cells display an increased cellular motility and invasion. Interestingly, we observed that these cells present higher PIP3 levels in the plasma membrane as determined by flow cytometry analysis. Consequently, the K8/18-shRNA clones show PI3K/Akt/NFkB pathways hyperactivation and increased MMP-9 expression. Furthermore, these processes are shown to be partially regulated by the tight junction's protein claudin-1, which is highly increased in K8/18-shRNA clones.

To our knowledge, these results represent the first indication that K8/18 can influence the phenotype of epithelial cancer cells. Knockdown of K8/18 increases cisplatin sensitivity and invasive potential of epithelial cancer cell lines through the regulation of different cell signaling pathways, involving claudin-1-dependent PI3K activation and NFkB transcription activity. Several studies demonstrate that over-expression of claudin-1 protein is associated with increased invasiveness and metastatic behavior [13-15], partly through the up-regulation of MMPs [16-18]. Moreover, NFkB is known to regulate MMP-9 expression in some carcinomas [19-22]. Our results demonstrate that K8/18 constitute a signaling platform capable of modulating cell invasion/survival-dependent signal transduction in tumor cells. K8/18 knockdown induces deregulation of the expression of junctional proteins that seems to be key steps in invasion. Moreover, our results suggest that K8/18 could also play a key role in the location of cell death receptors to the plasma membrane so that the cisplatin-induced apoptosis is improved. These results support the hypothesis that keratins 8 and 18 play an active role in cancer progression.

Model cell lines— We used two epithelial cancer cell lines: endometrial carcinoma KLE cells (expressing constitutively-activated Akt isoforms) and hepatocellular carcinoma HepG2 cells.
Transfections with shRNAs— Stable transfection of the cells was carried out with keratin 8, keratin 18 or scrambled negative control (NC) shRNAs (SABiosciences) using FuGene 6 reagent. G418 was applied to isolating resistant clones.
Targeting of claudin1 by siRNA— Cells were transfected for 24h with 100nM claudin1 or scrambled negative control (NC) siRNA (Ambion) using *Trans*IT-TKO reagent (Mirus).
qRT-PCR— Quantitative real-time PCR was performed with Mx3000P (Stratagene) in duplicates from at least three independent experiments. RNA 18s and β-actin was used as reference genes.
Invasion and wound-healing assay— Invasive properties were measured using 2mg/mL of Matrigel-coated Transwell inserts. Invasive cells that had adhered to the porous insert were fixed in methanol and nuclear staining was performed with Hoechst dye. To evaluate cell motility, cells were grown to near confluency and a wound was created with the blunt end of a yellow tip. Each experiment was performed in duplicates and repeated three times.
Cell fractionation— Separation and preparation of cytoplasmic, membrane and nuclear extracts from cells were done with the Subcellular Protein Fractionation Kit (Thermo Scientific).
Apoptosis analysis by flow cytometry— Cells were dual stained with propidium iodide and Alexa Fluor 488-annexin V using Dead Cell apoptosis kit (Life Technologies) according to the manufacturer's protocol. Stained cells were analyzed by FC 500 MPL system (Beckman Coulter).
PIP3 levels analysis by flow cytometry— Cells were fixed in 2% paraformaldehyde, blocked with 10% normal goat serum in PBS, and incubated 1h at 4°C with mouse anti-PIP3 monoclonal antibody (Echelon). Primary antibody was detected with Alexa488-conjugated donkey anti-mouse antibody (Molecular Probes). Samples were acquired on a FC 500 MPL system (Beckman Coulter).
Luciferase Reporter Assay— Cells were transfected with NFkB-Luc reporter plasmid and TK-hRLuc, followed by dual luciferase assay (Promega). Each experiment was repeated three times.

Keratin 8 and 18 knockdown increases cisplatin-induced apoptosis in epithelial cancer cells.

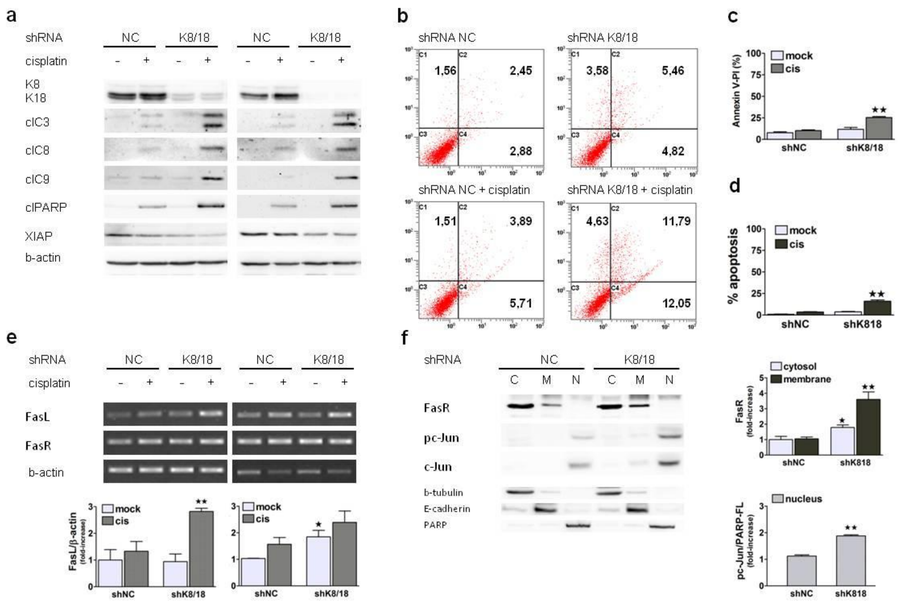

Figure 1. a) Western blot analysis of keratin 8, keratin 18, cleaved caspase 3, 8 and 9, cleaved PARP and XIAP in KLE (left) and HepG2 (right) shRNA clones treated with 10μM cisplatin for 24h. b-c) Flow cytometry analysis of Annexin V (*x* axis)-PI (*y* axis) levels in KLE shRNA clones treated with 10μM cisplatin for 24h. d) Apoptotic level determined by Hoechst nuclear staining in KLE shRNA clones treated with 10μM cisplatin for 24h. e) qPCR analysis of Fas ligand and Fas receptor in KLE (left) and HepG2 (right) shRNA clones treated with 10μM cisplatin for 24h. f) Western blot analysis of Fas receptor, phosphorylated c-Jun and c-Jun in subcellular fractions (C, cytosol; M, membrane; N, nucleus) of KLE shRNA clones treated with 10μM cisplatin for 24h. ★ $p < 0.05$; ★★ $p < 0.01$

Claudin1 regulates motility of K8/18-deficient cancer cells.

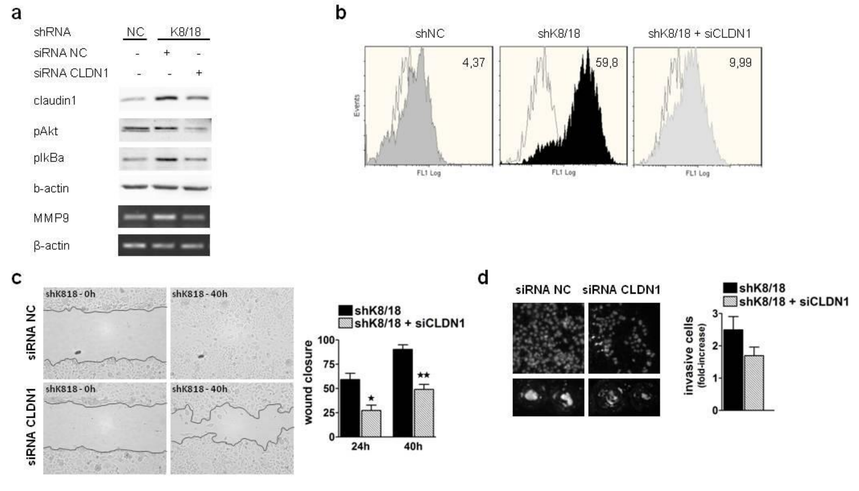

Figure 4. a) Protein levels of claudin1, phosphorylated Akt, phosphorylated IkBa and mRNA level of MMP9 in KLE shRNA clones transfected with negative control or claudin1 siRNA. b) Percentage of PIP3 levels in KLE shRNA clones transfected with negative control or claudin1 siRNA. c) Wound-healing assay with KLE shRNA clones transfected with negative control or claudin1 siRNA. d) Invasion assay through matrigel with KLE transfected with negative control or claudin1 siRNA. ★ $p < 0.05$; ★★ $p < 0.01$

Keratin 8 and 18 knockdown increases cancer cell motility and invasion.

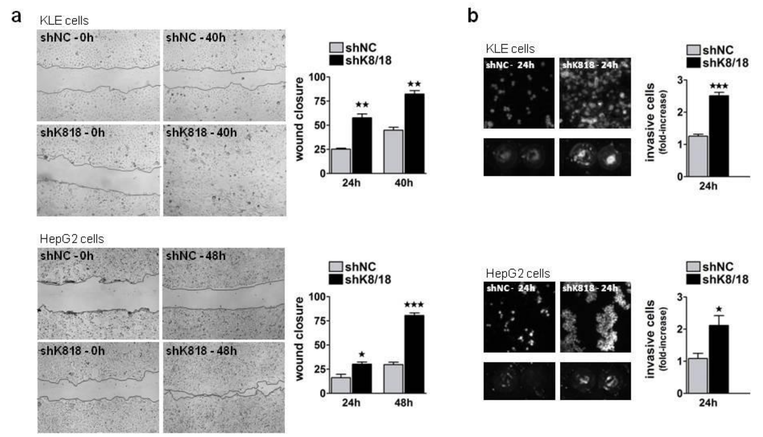

Figure 2. a) Wound-healing assay with KLE and HepG2 shRNA clones. b) Invasion assay through matrigel with KLE and HepG2 shRNA clones. ★ $p < 0.05$; ★★ $p < 0.01$; ★★★ $p < 0.001$

Keratin 8 and 18 knockdown improves Akt phosphorylation and NFkB transcriptional activity in epithelial cancer cells.

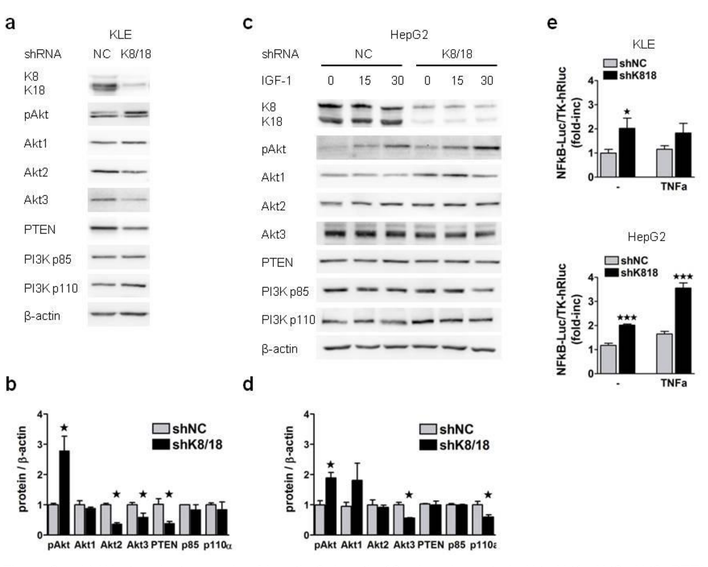

Figure 3. a-b) Western blot analysis of keratin 8, keratin 18, phosphorylated Akt, Akt1, Akt2, Akt3, PTEN, PI3Kp85 and PI3Kp110a in KLE shRNA clones. c-d) Western blot analysis of keratin 8, keratin 18, phosphorylated Akt, Akt1, Akt2, Akt3, PTEN, PI3Kp85 and PI3Kp110a in HepG2 shRNA clones treated with 20nM IGF-1 for 0, 15min and 30min. e) Dual luciferase reporter assay for NFkB transcription (firefly luciferase) and constitutive internal control (renilla luciferase) in KLE and HepG2 shRNA clones. TNFa treatment was used as a positive control for NFkB transcription. ★ $p < 0.05$; ★★★ $p < 0.001$

References

1.Ku NO, Omary MB *J Cell Biol* 2006; 2.Zatloukal K et al. *Am J Pathol* 2000; 3.Kim S et al. *Nature* 2006; 4.Galarneau L et al. *Exp Cell Res* 2007; 5.Ku NO et al. *PNAS* 2002; 6.Inada H et al. *J Cell Biol* 2001; 7.Gilbert S et al. *J Cell Biol* 2001; 8.Caulin C et al. *J Cell Biol* 2000; 9.Ku NO et al. *Hepatology* 2003; 10.Long HA et al. *J Cell Sci* 2006; 11.Buhler H et al. *Mol Cancer Res* 2005; 12.Schaller G et al. *Clin Cancer Res* 1996; 13.Dhawan P et al. *JCI* 2005; 14.Dos Reis PP et al. *Cancer* 2008; 15.Myal Y et al. *J Biomed Biotechnol* 2010; 16.Leotlela PD et al. *Oncogene* 2007; 17.Miyamori H et al. *JBC* 2001; 18.Oku N et al. *Cancer research* 2006; 19.Yeh CB et al. *PloS one* 2012; 20.Moon MC et al. *Can J Physiol Pharmacol* 2008; 21.Dell'Agli M et al. *Bioorg Med Chem Lett* 2009; 22.Min C et al. *J Cell Biochem* 2008.