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F-BOX PROTEIN FBXL5 NUCLEAR RETENTION BY SPECIFIC INHIBITORS OF NUCLEAR EXPORT INDUCES SNAIL UBIQUITINATION LEADING TO REVERSAL OF EMT

Irfana Muqbil[1], Amro Aboukameel[1], Yosef Landesman[2], Michael Kauffman[2], Sharon Shacham[2], Ramzi M. Mohammad[1], Asfar S. Azmi[1].

[1]Wayne State University, Detroit, MI; [2]Karyopharm Therapeutics, Newton, MA

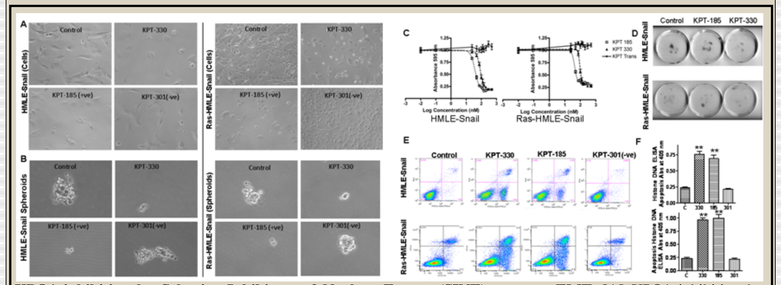
ABSTRACT

FBXL5 is an F-box protein and one of four subunits constituting the ubiquitin protein E3 ligase complex (SKP1-cullin-F-box). It has been shown to promote snail nuclear ubiquitination therefore down regulating snail induced epithelial-to-mesenchymal transition (EMT). However, cancer associated up regulation of the nuclear exporter Crm1/Exportin 1 (XPO1) expression results in nuclear exclusion of FBXL5 leading to snail stability and EMT. Here we demonstrate that XPO1 inhibition by Selective Inhibitors of Nuclear Export (SINE) results in nuclear retention of FBXL5, leading to nuclear degradation of SNAIL and to the reversal of EMT in HMLE-SNAIL models.

METHODS

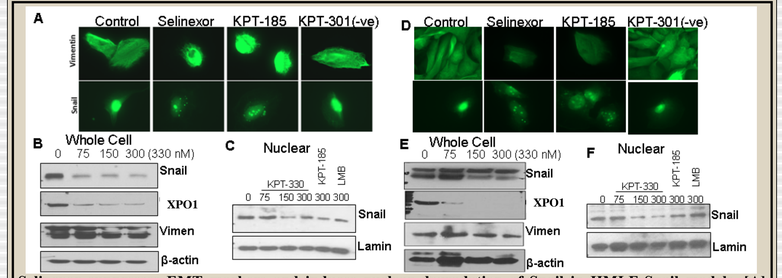
Reagents: HMLE-SNAIL cell pairs were provided by Dr. Robert Weinberg from the Whitehead Institute. Cell growth inhibition, apoptosis, protein expression, localization and ubiquitination were evaluated using MTT, Annexin V FITC, Histone ELISA, colonogenic immunofluorescence, western blotting and Co-IPs respectively. For systems analysis microarray expression profiling RNA from quadruplet samples was performed at 24 hrs. Xenograft were developed by injecting 1x10^6 Ras-HMLE-snail cells bilaterally at subcutaneous site in ICR SCID mice.

RESULTS

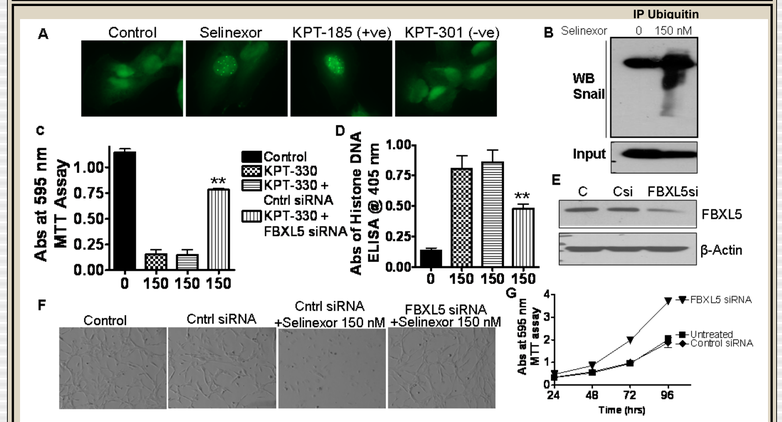


XPO1 inhibition by Selective Inhibitors of Nuclear Export (SINE) reverses EMT. [A] XPO1 inhibition by Selinexor/KPT-330 or KPT-185 (24 hrs) reverses EMT in Snail transduced Human Mammary Epithelial cells. **[B]** SINE suppress spheroid formation (treated at a concentration of 150 nM once a week for two weeks); **[C]** inhibit growth (MTT 72 hrs) IC_{50s} <100 nM; **[D]** suppress clonogenicity **[E]** induce apoptosis (Annexin V FITC assay at 72 hrs and **[F]** Histone DNA ELISA apoptosis analysis at 72 hrs.

RESULTS

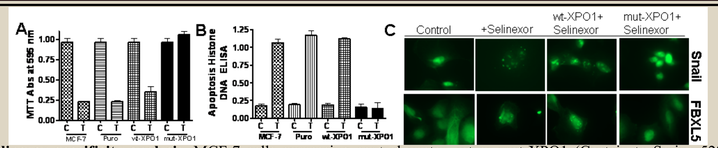


Selinexor suppresses EMT markers and induces nuclear degradation of Snail in HMLE-Snail models. [A] HMLE-Snail Cells [exposed to 150 nM of either KPT-330/Selinexor; KPT-185 or KPT-301 –ve control) for 24 hrs]. (100 X) confocal images (GFP staining) showing reduction in cytosolic Vimentin and nuclear degradation of Snail (speckled staining). Western blotting of whole cell lysates **[B]** and nuclear lysates **[C]** under similar treatment conditions. **[D, E & F]** ras-HMLE-Snail under similar conditions. LMB was used as +ve control at 100 nM.



Role of FBXL5 in Snail turnover and consequent reversal of EMT. [A] FBXL5 confocal images (100X) of HMLE-Snail cells exposed to SINE for 24 hrs **[B]** IP of nuclear lysates (from Selinexor exposed cells 24hrs) using ubiquitin antibody followed by Western blotting with snail. **[C&D]** MTT and Apoptosis at 72 hrs post KPT-330/ Selinexor treatment in the absence or presence of siRNA for FBXL5 (10 nM). **[E]** Western blot showing FBXL5 siRNA silencing **[F]**. siRNA treatment abrogates Selinexor mediated reversal of EMT and **[G]** FBXL5 siRNA treated cells show enhanced proliferation indicating its role in regulating snail expression.

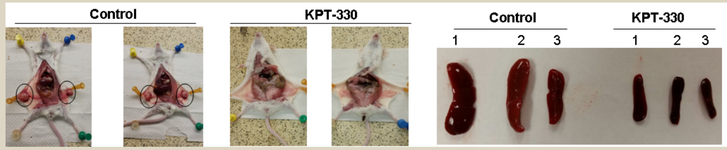
RESULTS



Selinexor specificity analysis. MCF-7 cells expressing control vector, wt or mut-XPO1 (Cystein to Serine 528 mutation) were exposed to 100 nM of Selinexor. **[A]** MTT assay and **[B]** apoptosis by Histone DNA ELISA. Note: Selinexor is ineffective in mut-XPO1 harboring cells (black bars). **[C]** Confocal images showing lack of snail degradation or FBXL5 nuclear localization in mut-XPO1 cells.

Change	Gene	Location	Function
2.381	FBOX2	Cytoplasm	Enzyme
1.498	FBXO33	Unknown	Other
1.347	FBXL17	Unknown	Other
-1.554	FBXO37	Unknown	Transcription

Systems analysis of SINE activity in HMLE-SNAIL cells. Table showing selected F-BOX gene expression changes ($p<0.001$). [**Center**] Pathway analysis reveals down-regulation of Snail network and [**Right**] DAVID enrichment analysis showing enhancement in transcription of cytosolic proteins.



Selinexor completely inhibits HMLE-Snail xenograft. Mice (n=6) with bilateral tumors were exposed to 15 mg/kg Selinexor orally once a day for three weeks. Control mice show enlarged spleen and treated with normal spleen.

CONCLUSIONS

Inhibition of the nuclear exporter XPO1 by Selinexor reverses EMT

EMT reversal results from the nuclear retention of the F-BOX protein FBXL5 and the nuclear degradation of SNAIL

Selinexor leads to complete tumor inhibition (tumor free at 120 days) in ras transformed HMLE-snail derived sub-cutaneous xenografts

This study demonstrates a novel strategy of targeting EMT at the nuclear pore using Selinexor