In vitro model of nephrocalcinosis: is apotosis in GDNF silenced HK2 cells the trigger of Ca_2PO_4 mineralization process?

Giovanna Priante[1], Federica Quaggio[1], Lisa Gianesello[1], Ceol Monica[1], Liliana Terrin[1], Claudio Furlan[2], Dorella Del Prete[1], Franca Anglani[1],

[1]Laboratory of Histomorphology and Molecular Biology of the Kidney, Clinical Nephrology, Dept. of Medicine - DIMED, University of Padua, Padua, ITALY; [2]Centre for the ESEM and SEM analyses (CEASC), University of Padua, Padua, ITALY

IDENTIFIER/TOPIC: Renal pathology. Experimental and clinical

INTRODUCTION AND AIMS

The surgical removal of renal cell carcinoma in a MSK patient with a GDNF mutation allowed us to observe that cultured papillary cells spontaneously differentiated into the osteogenic lineage, producing bone protein markers and Ca_2PO_4 deposits.

To investigate the possible relationship between *GDNF* and the observed osteogenic phenomenon, we conducted a study on *GDNF* silenced HK2 cells, confirming its role in the calcification process. Since a close relationship between cell death and pathological calcification has been reported, the aim of this study was to investigate whether apoptosis is involved in the calcification of silenced cells under osteogenic culture *in vitro*.

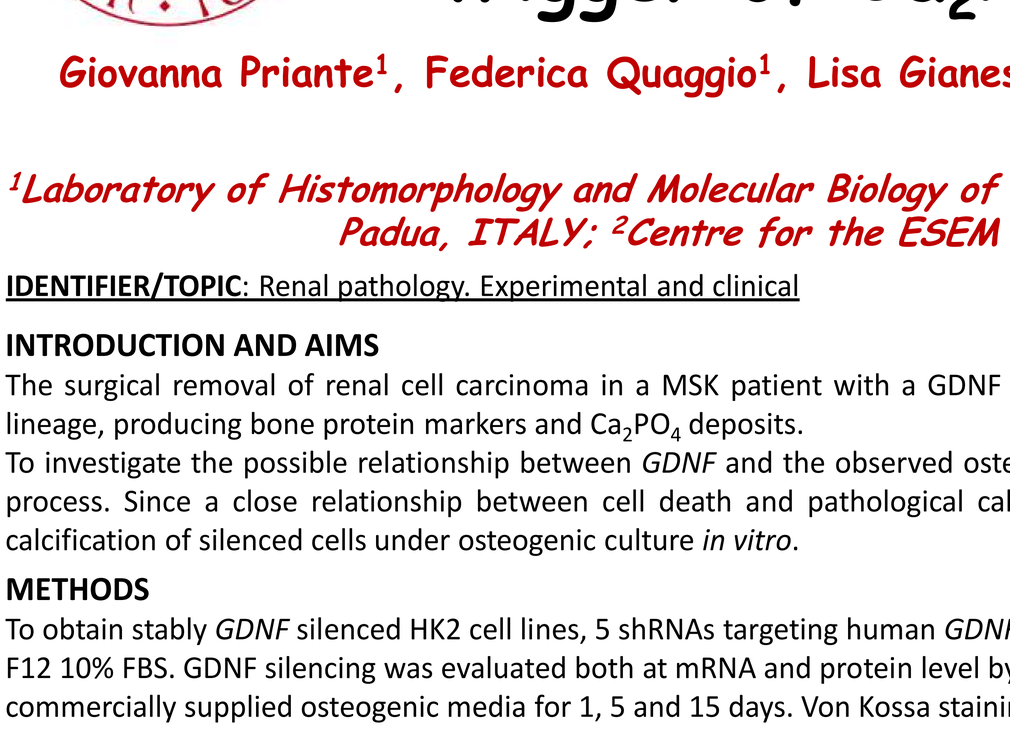
METHODS

To obtain stably *GDNF* silenced HK2 cell lines, 5 shRNAs targeting human *GDNF* were used. As negative control (-) we transfected HK2 cells with an empty vector. Clones were grown in DMEM-F12 10% FBS. GDNF silencing was evaluated both at mRNA and protein level by RT-qPCR and by immunocytochemistry. Efficiently HK2 silenced clones, control (-) and WT cells were cultured in commercially supplied osteogenic media for 1, 5 and 15 days. Von Kossa staining and ESEM were used to detect and analyze crystal deposition. Gene expression analysis was performed by RT-qPCR (ΔΔCt method) to evaluate osteogenic activation (Osteopontin, Osteonectin) and early genes of apoptosis (Bax, Bcl-2).Apoptosis was investigated analyzing: 1) caspase activation by In Cell Western (Caspase 9 as initiator, Caspase 3 as effector and PARP); 2) membrane translocation of phosphotidylserine by Annexin V-FITC staining paired with PI by Cytofluorimetric analysis.

RESULTS

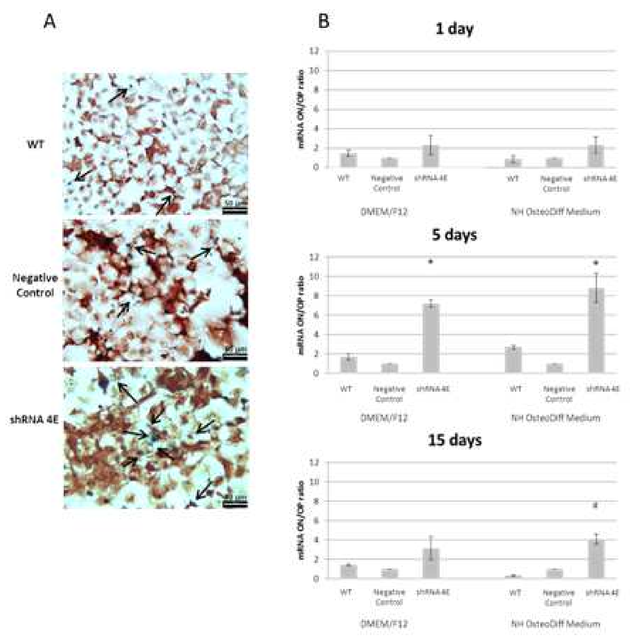
Cell calcification in *GDNF*-silenced HK-2 cells

The presence of Ca_2PO_4 deposition was observed in silenced cells at day 15 with significantly higher levels than controls in osteogenic media. In silenced cells, time-course RT-qPCR experiments showed an increased osteonectin/osteopontin ratio at day 15 in respect to controls. ALP positivity was detected in some of the cells adjacent to the nodules.



Ca_2PO_4 detection in HK-2 cells cultured in either DMEM/F12 with 10% HI-FBS or NH OsteoDiff medium. (**A**) von Kossa staining reveals calcium deposits in some cells and nodules in wild-type (WT), negative control and silenced (shRNA 4E) cells grown in osteogenic medium for 15 days. No calcium deposition is observed in either control or silenced cells cultured under standard conditions (DMEM/F12 supplemented with 10% HI-FBS). (**B**) Quantitative analysis of von Kossa staining performed using morphometric analysis shows a time-dependent increase of deposits that is significant in the silenced cells at 15 days (*p<0.05). (**C**) ESEM images and spectra confirmed that the nodule calcifications in shRNA 4E cells on day 15 consist of Ca_2PO_4. No Ca_2PO_4 deposits were observed in silenced cells cultured under standard conditions. (**D**) Quantitative analysis of calcium and phosphorus levels in WT, negative control and shRNA 4E cells grown in NH OsteoDiff medium for 15 days. ESEM analysis was performed using X-ray fluorescence coupled with energy dispersive spectroscopy (XRF-EDS) (** p<0.05).

Statistically significant differences between the shRNA 4E cells and the WT and negative control cells were evaluated with a non-parametric test (Mann-Whitney U test) using Primer software (McGraw-Hill)

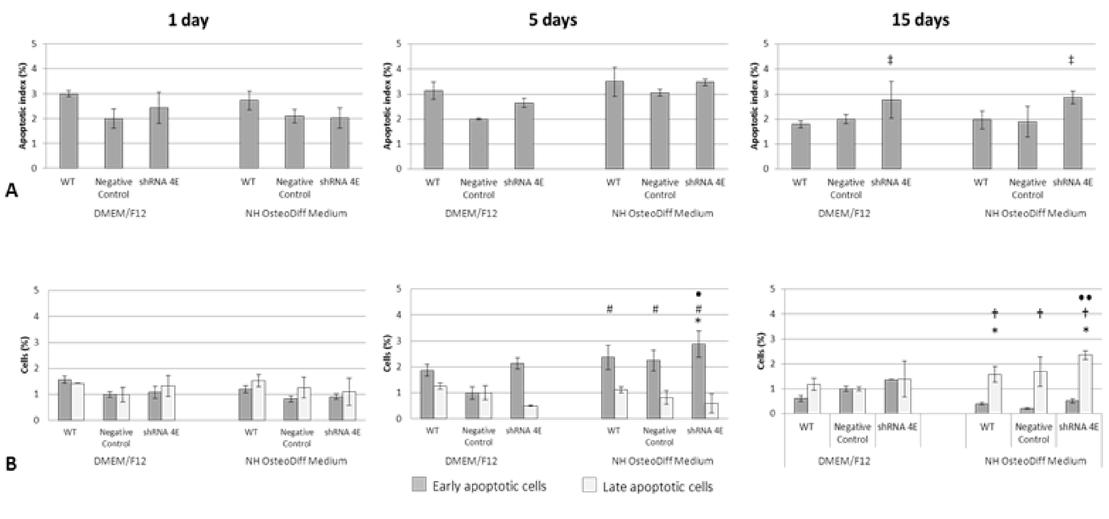


Osteogenic-like process

(**A**) Light microscopy images (magnification 200X) of wild-type (WT), negative control and silenced (shRNA 4E) cells positive for alkaline phosphatase around the nodules after culturing in NH OsteoDiff medium for 15 days. Alkaline phosphatase is indicated in the cells as an intense blue stain (arrows). (**B**) Expression of osteogenesis-related genes as determined by qRT-PCR in wild-type (WT), negative control and silenced (shRNA 4E) cells grown in standard (DMEM/F12 supplemented with 10% HI-FBS) and osteogenic NH OsteoDiff medium. The results are expressed as the ratio of osteonectin (ON) to osteopontin (OP), indicating that the balance between pro- and anti-osteogenic factors in shRNA 4E cells favored an osteogenic process. (* p<0.005; # p<0.05). The results are presented as the mean ± SD of two independent experiments performed in triplicate. Statistically significant differences between the shRNA 4E cells and the wild-type and negative control cells (* p<0.005; # p<0.05) were performed using ANOVA with a between-within design and Bonferroni correction.

Cell death in *GDNF*-silenced HK-2 cells

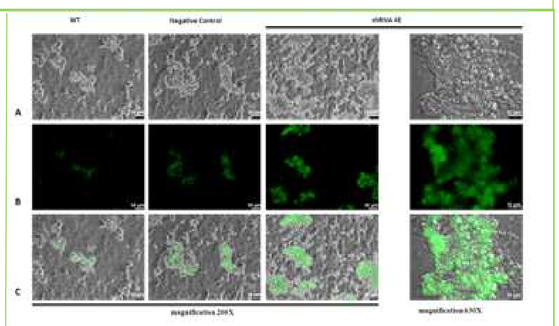
Apoptosis was activated as early at day 5, and lowered at day 15. A transition of the silenced cells in osteogenic medium from early apoptosis to late apoptosis was observed and confirmed by TUNEL assay. Despite these results, we found up-regulation of the antiapoptotic marker Bcl-2 and lower level of caspase 3, 9 and PARP in silenced cells in respect to controls.



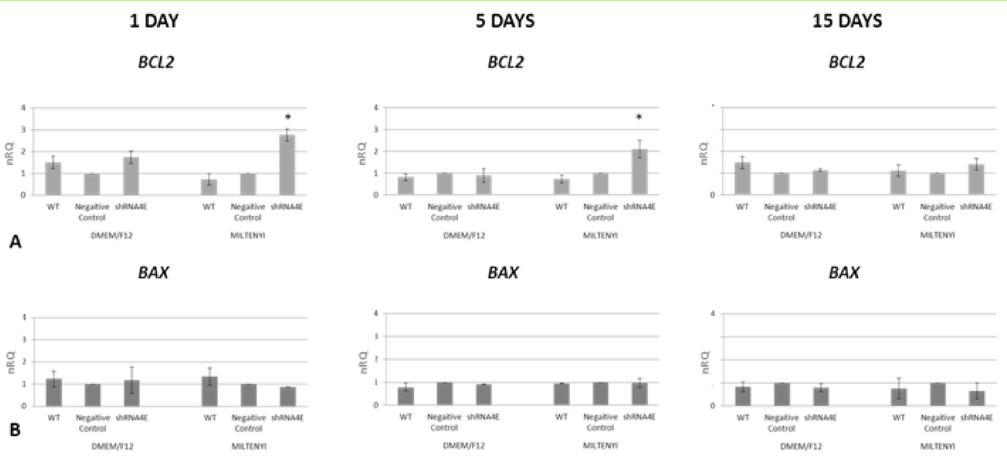
Double staining with annexin V and PI in wild-type (WT), negative control and silenced (shRNA 4E) cells grown in standard (DMEM/F12 10% HI-FBS) and osteogenic (NH OsteoDiff medium) conditions at days 1, 5 and 15. (A) Evaluation of the percentage of apoptotic cells (apoptotic index). (B) Evaluation of the different phases (early and late) of the apoptotic process.

‡ shRNA 4E vs negative control and wild-type, p<0.05; * Early apoptotic cells vs late apoptotic cells, p<0.05; # Early apoptotic cells in NH OsteoDiff medium vs DMEM/F12 medium, p<0.05; † Late apoptotic cells in NH OsteoDiff medium vs DMEM/F12 medium, p<0.05; • Early apoptotic shRNA 4E cells vs control cells (negative control and wild-type) in NH OsteoDiff medium, p<0.05; •• Late apoptotic shRNA 4E cells vs control cells (negative control and wild-type) in NH OsteoDiff medium, p<0.05 .

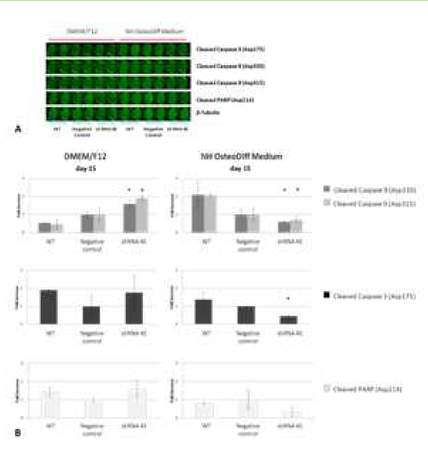
The results are presented as the mean ± SD of two independent experiments performed in triplicate. Statistically significant differences were estimated by using ANOVA with a between-within design and Bonferroni correction



TUNEL-stained of apoptotic nuclei (green staining) in wild-type (WT), negative control and silenced (shRNA 4E) cells grown in NH OsteoDiff medium for 15 days. The images are representative of two independent experiments. A: Bright field; B: FITC; C: Merge.



Expression of apoptosis-related genes in wild-type (WT), negative control and silenced (shRNA 4E) cells grown in standard (DMEM/F12 supplemented with 10% HI-FBS) and NH OsteoDiff media. Changes in the relative *BCL2* (**A**) and *BAX* (**B**) mRNA levels as determined using qRT-PC. * p<0.05. The results are presented as the mean ± SD of two independent experiments performed in triplicate. Statistically significant differences between the shRNA 4E cells and the wild-type and negative control were calculated using ANOVA with a between-within design and Bonferroni correction



Caspase activation in wild-type (WT), negative control and silenced (shRNA 4E) cells grown in normal (DMEM/F12 10% HI-FBS) and NH OsteoDiff media. (**A**) Images acquired at a wavelength of 800 nm. (**B**) Fluorescence quantification at 800 nm normalized to the β-tubulin signal.. *p<0.05. The results are presented as the mean ± SD of two independent experiments performed in triplicate. Statistically significant differences between the shRNA 4E cells and the wild-type and negative control cells were calculated using ANOVA with a between-within design and Bonferroni correction.

CONCLUSIONS: The silencing of *GDNF* gene in HK2 cells induces a biomineralization process similar to that spontaneously occurred in primary papillary cells obtained from a patient with MSK and *GDNF* mutation. GDNF is confirmed as adaptive survival factor whose alteration appears to play a key role in the process of nephrocalcinosis. In our model, cell death seems to be one of the triggering events that give rise to calcium deposits. However, the death process occurs in a programmed way but in complete absence and independently of caspase activation.

54 ERA
73--MP
Renal pathology
Giovanna Priante
DOI: 10.3252/pso.eu.54ERA.2017
ePosters supported by F. Hoffmann- La Roche Ltd.
54th ERA-EDTA CONGRESS Madrid, Spain June 30-July 3 2017
PosterSessionOnline.com