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ORIGINAL ARTICLE

Impact of silencing eEF2K expression on the malignant properties of chordoma

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Abstract

Background Eukaryotic elongation factor 2 kinase (eukaryotic elongation factor 2 kinase, eEF2K) is a calcium calmodulin dependent protein kinase that keeps the highest energy consuming cellular process of protein synthesis under check through negative regulation. eEF2K pauses global protein synthesis rates at the translational elongation step by phosphorylating its only kown substrate elongation factor 2 (eEF2), a unique translocase activity in ekaryotic cells enabling the polypeptide chain elongation. Therefore, eEF2K is thought to preserve cellular energy pools particularly upon acute development of cellular stress conditions such as nutrient deprivation, hypoxia, or infections. Recently, high expression of this enzyme has been associated with poor prognosis in an array of solid tumor types. Therefore, in a growing number of studies tremendous effort is being directed to the development of treatment methods aiming to suppress eEF2K as a novel therapeutic approach in the fight against cancer.

Methods In our study, we aimed to investigate the changes in the tumorigenicity of chordoma cells in presence of gene silencing for eEF2K. Taking a transient gene silencing approach using siRNA particles, eEF2K gene expression was suppressed in chordoma cells.

Results Silencing eEF2K expression was associated with a slight increase in cellular proliferation and a decrease in death rates. Furthermore, no alteration in the sensitivity of chordoma cells to chemotherapy was detected in response to the decrease in eEF2K expression which intriguingly promoted suppression of cell migratory and invasion related properties.

Conclusion Our findings indicate that the loss of eEF2K expression in chordoma cell lines results in the reduction of metastatic capacity.

Keywords Chordoma · eEF2K · Migration · siRNAs

Abbreviations

eEF2	Eukaryotic Elongation Factor 2
eEF2K	Eukaryotic Elongation Factor 2 Kinase
siRNAs	Silencing RNAs
PI3K	Phosphoinositide 3-kinase
mTOR	Mammalian target of rapamycin-rapamycin in mammals
MAPK	Mitogen-activated protein kinase
AMPK	Adenosine monophosphate-activated protein kinase
STR	Short tandem repeats
TRAIL	TNF-related apoptosis-inducing ligand
TNF	Tumor necrosis factor
ESCC	Esophageal squamous cell carcinoma
EGFR	Epidermal Growth Factor Receptor
RPMI	Roswell Park Memorial Institute
PBK	Protein Kinase B
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

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MTS	3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
FITC	Fluorescein isothiocyanate
PI	Propium Iodide
NC	Negative Control
PKM2	Pyruvate kinase isozymes M1/M2
c-My	C-myelocytomatosis
Stat3	Signal transducer and activator of transcription 3
EMT	Epithelial Mesenchymal Transition
Twist1	Twist Basic Helix-Loop-Helix Transcription Factor 1

Introduction

Chordoma is a slowly developing, soft tissue type of cancer that typically originates from the notochordal remnants in the embryonic period and tends to localize at the base of the skull and around the sacrum in the axial skeletal system [1]. Chordoma was first reported by Virchow in 1857 as a clivus tumor [2]. According to SEER data, the rate of diagosis reported is 1 in 10^6 in the USA, and it mostly occurs between the ages of 40–70 and its incidence under the age of 20 is 1% [3]. Although basis for gender-based predisposition is not well understood, chordoma is seen approximately twice more in women than in men, [4–7]. One of the pathophysiological features of chordomas is the overexpression of the gene called *T* (*BRACHYURY*, mouse homolog), which is used as a biomarker for chordoma diagnosis [8]. Chordoma is highly resistant to traditional therapies like chemotherapy and relatively more responsive to radiotherapy which is -therefore- preferred as a follow up treatment option particularly ensuing radical surgeries [9–11]. Therefore, alternative treatment methods are needed because of the inadequacy of existing chemotherapy options. For that matter, understanding the molecular mechanisms driving chordoma carcinogenesis is extremely important for the discovery and development of novel targeted molecules of high efficacy. To study the molecular pathogenesis of the disease there have been several cell lines established either from primary or recurred tumors collected from patients. MUG-Chor1 and UM-Chor1 chordoma cell lines, both of which were the selected model systems for this study, are examples of such in vitro models. MUG-Chor1 originates from sacrococcygeal recurrent tumor tissue, while UM-Chor1 was established from clivial primary tumor tissue [12–14].

In an attempt to uncover a novel point of intervention for the treatment of chordoma tumors we undertook understanding the therapeutic potential of silencing of eEF2K which recently has emerged as a suitable molecular target in several solid tumors types such as breast, pancreas, lung, and brain cancer [15].

Protein synthesis is an essential cellular process for cell vialbility and is accomplished by the action of a humbling machinery comprised of several factors acting in a highly sophisticated and meticulously orchestrated fashion [16]. Furthermore, synthesis of nascent proteins dedicated to different functions to sustain cell viability claims 30–50% of cellular energy [15]. In that respect, eEF2K acts as a reversible break of this multi-member machinery to sustain metabolic energy until stress conditions are reverted back to normal [17]. eEF2K executes its break function to bring polypeptide chain elongation to a halt by phosphorylating its only known substrate, eEF2 (Eukaryotic Elongation Factor 2), which possesses a unique translocase activity in eukaryotic cells allowing the progression of ribosome on an mRNA being translated [18, 19]. In response to acute stress, the activity of eEF2K toward eEF2 increases so that the interaction of the phosphorylated eEF2 with the ribosome is abrogated, and thereby, protein synthesis becomes paused until the inhibitory phosphates are removed to allow its re-activation and re-access to the ribosome when normalcy in the growth conditions are re-attained [20].

Majority of the cancer cells in tumor bed have a higher division capacity as well as metabolic activity compared to normal cells which forms the basis for the severity of the living conditions in their microenviroment that is deficient in oxygen, nutrients and energy and is highly acidic. Initially, the association between high eEF2K expression in cancer cells and worse degree of malignancy appeared to be contradicting the prediction that a high expression of a negative regulator for protein synthesis does not reconcile with meeting the demand for the high protein synthesis rates. However, from the perspective of cytoprotective impact of eEF2K activity through energy preservation under cellular stress conditions, it becomes explicable why cancer cells of certain tissue types favors elevation of eEF2K levels in their deprived microenvironment where they have endure. In addition, in order provide high protein synthesis rates explotation of other regulators of protein translation machinery is a frequently encountered event in cancer cells where there is a strong propensity to increase the expression of those genes important for cancer development while decreasing the expression of others that prevent cancer formation [21]. Several lines of evidence obtained in different cancer models suggest that aberrant proliferation, angiogenesis, metastasis, pro-tumorigenic immune response and cancer energetics meets such pathological needs through aberrant protein translation [16].

Regulation of eEF2K function is highly complex under the control of several mitogenic signaling pathways, including PI3K (Phosphoinositide 3-kinase), mTOR (mammalian target of rapamycin-rapamycin in mammals)

and MAPK (mitogen-activated protein kinase) the activity of which blocks eEF2K activity to allow protein synthesis in accordance with proliferation inducing stimuli [22]. In contrast, AMPK, the major energy sensor signaling activates eEF2K through direct phosphorylation. In several types of cancer, de-regulation of these pathways has been shown to be pivotal events driving the oncogenesis and determining the degree of malignancy whereby more aggressive phenotypes are more frequently associated with malfunction of these pathways executing key roles in cell division, differentiation, metabolism and motility. Strikingly, depending on the nutrient and energy status of the cell, signaling through these decision making pathways for the cell fate converge on eEF2K whose outcome activity directs protein synthesis accordingly [23].

Previous studies indicate that, silencing of eEF2K protein in glioma cells is shown to trigger Tumor Necrosis Factor (TNF)-related apoptosis-inducing ligand (TRAIL) dependent death mechanism [24] and reduces the proliferation of breast cancer cells by increasing sensitivity to chemotherapeutics such as doxorubicin [25]. Likewise, knockdown of higher expression of eEF2K compared to normal in esophageal squamous cell carcinoma (ESCC) slowed down migration and proliferation rate [34]. So far there has been no such study showing the association of high expression of eEF2K and bad prognosis in chordoma and whether eEF2K has any effect on the drug resistance [34].

Recent reports highlighted Afatinib, an Epidermal Growth Factor Receptor (EGFR) inhibitor, as a promising agent that inhibits the proliferation of chordoma cells via decreasing Akt/PKB pathway (negative regulator of eEF2K activity) and lowering the expression of T (BRACHYURY, mouse homolog) [26] suggesting that the antiproliferative effects of afatinib could be associated with re-activation of eEF2K as seen in the colon carcinoma in vitro and in vivo models [26–29]. This is contrasting to the data obtained from patient tumors and overall survival in TNBC, pancreatic, lung carcinoma and glioblastoma in the TGCA database as well as the experimental data from the in vitro and in vivo models of these cancer types where reduction in eEF2K expression results in decreased tumorigenicity [25].

To uncover the contributory role of eEF2K to the oncogenic processes in chordoma cells, we took a gene silencing approach where lowering eEF2K protein levels promoted an increase in cellular proliferation. Based on this observation and the commonly known fact that the failure of conventional chemotherapeutics in the treatment of chordoma that target DNA synthesis can be attributed to the slow proliferation kinetics of these cells, we hypothesized whether increased proliferation of chordoma cells in a eEF2K-silenced background could potentiate the standard chemotherapeutics.

In other words, it is worthwhile to address whether knock down of eEFK2 in combination with conventional therapeutics would result in improved efficacy of standard of care agents that elicit cell death through massive DNA damage.

Materials and methods

Cell culture

Chordoma cell lines, MUG-Chor1 and UM-Chor1 [30], were kindly gifted from the Chordoma Foundation and they were routinely checked for mycoplasma contamination and STR analysis. The cell lines were grown in glatin coated flasks with culture medium containing IMDM (Gibco cat no: 31980–022, ThermoScientific, USA), RPMI (11,875–093, ThermoSecientific, USA), 10% FBS and 1% Penicillin–Streptomycin-Anphoteracin (PSA).

Silencing of eEF2K expression via siRNAs)

Chordoma cells were transfected either with eEF2K-specific siRNA (4,392,420 Thermo Scientific) or negative control siRNA (AM4611 Thermo Scientific) using the liposomal-based carrier "Lipofectamine RNAi Max Transfection Reagent" (13,778,100, Thermo Scientific) according to the manufacturer's instructions. Briefly, cells were grown in 6-well plates prior to the transfection and siRNAs –diluted in Opti-MEM (31,985,062, ThermoScientific, USA) with no serum and mixed with 3 µL Lipofectamine™ RNAiMAX- were introduced into the cells with a final concentration of 50 nM. Following a 36 h incubation with the siRNA coctails, cells were returned to fresh medium to be harvested at 72 h post-transfection.

Gene expression analysis and protein quantification

The silencing of eEF2K expression was investigated both at the mRNA and protein level on the 3rd day following siRNA transfection. GAPDH was used as an internal control. Total RNA was isolated using Trizol reagent (15,596,018, ThermoScietific, USA) according to the manfacturer's protocol. Complementary DNA was synthesized from 1000 ng RNA with the "High-Capacity cDNA Reverse Transcription Kit": (4,368,814, Thermo Fisher) in accordance with the manufacturer's protocol. Taqman primer probes targeting eEF2K (Hs00179434_m1,) and GAPDH (Hs02786624_g1), and Universal Taqman Master Mix (4,440,038, Applied Biosystem) were purchased from Thermo Fisher to run the realtime PCR according to the manufacturer's protocol. GAPDH was used as an internal control to normalize the results. As for the analysis $2^{-\Delta\Delta Ct}$ method was used to find difference in fold change.

Proteins were isolated from chordoma cell lines, which were transfected with siRNAs, by using a RIPA buffer solution (9806, CST, USA). Briefly, upon transfection, cell pellets were lysed with RIPA buffer containing protease and phosphatase inhibitor cocktail (78,440, Thermo Fisher) on ice, and centrifuged for 30 min at 14,000 *g*. Protein concentration was determined by Pierce™ BCA Protein Assay Kit" (23,225, Thermo Fisher, USA). Proteins were loaded onto polyacrylamide gels, transferred onto the nitrocellulose membrane, blocked with skim milk, incubated with eEEF2K (ab45168, Abcam, USA) and GAPDH (5174, Cell Signaling Technology) antibodies (diluted in 1:1000), and finally visualized with a chemiluminescent solution containing hydrogen peroxide in the Bio Rad Chemidoc imaging system. Band lengths were normalized to GAPDH by the software and protein amounts were determined.

Proliferation assay

The effects of eEF2K silencing on the viability of chordoma cells were investigated on the 3rd day with a 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), viability assay (G3581, Promega, USA) which is based on the breakdown of tetrazolium salts into formazan crystals through the mitochondrial activity. Briefly, two days after the transfection, cells were seeded onto the four 96-well plates (CLS6509, Corning, USA) at a density of 5×10^3/well and treated with 10% MTS reagent and incubated in regular culture conditions for 1 h in dark. Absorbance values of each well were determined by a plate reader (ELx800, Biotek Instruments, USA) at wavelength 490 nm. Results were compared with negative controls.

Apoptosis assay

Early apoptotic cells were detected using the FITC Annexin V Apoptosis Detection Kit I (556,547, BD Biosciences, USA) according to manufacturer's protocol. Briefly, cells were labeled with Propium Iodide (PI) and annexin V, which is an early apoptotic marker, and the resultant intensity of fluorescence was measured by using the BD Facs Calibur instrument [31].

Cell cycle analysis

Upon inhibition of eEF2K in cells with siRNA, the cell cycle profile in cells was determined using the BD Facs Calibur device using a previously established protocol [32]. This analysis was performed 3 days after siRNA transfection. The cells were harvested and fixed in cold in 70% ethanol at least 2 h prior to the analysis. The cells were then incubated with a solution containing 0.3 mg/ml RNAse A and 5 µg/ml PI and incubated at 37 °C for 30 min. The cell cycle analysis was performed by measuring the fluorescence intensity of DNA using the BD Facs Calibur device.

Invasion and migration assays

Chordoma cell lines transfected with siRNA were trypsinized and seeded at 2×10^5 cells by dissolving in 200 µl serum-free chordoma medium into the transwells, which was then placed in wells of a-24 well plate containing 1300 µL of regular chordoma medium and kept at 37 °C for 24 h. At the end of the incubation, cells were processed for the staining protocol applied in the invasion assay (described below). For the invasion assay, cells were seeded at 2×10^5 cells in matrigel-coated transwells (354,480, Corning, USA) according to the manufacturer's protocol. After 24 h of incubation, the cells were fixed with 3.7% formaldehyde, permeabilized with 100% methanol, and stained with 0.1% Crystal Violet dye. The cells stained with the dye on the reverse side of the transwells were considered as the migratory and invasive cells, and compared to the unstained counterparts for the analysis.

Combinatorial treatment of eEF2K siRNA with chemotherapeutic agents

After the transfection with siRNAs, MUG-Chor1 and UM-Chor1 cells were treated for 72 h with conventional chemo agents such as Etoposide and Cisplatin (each at 10 µM as was used previously [33] to which they have resistance [34, 35]. The viability of the cells was determined by the "CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS)" according to the manufacturer's protocol described before. The combined treatment of cells silenced with eEF2K and the chemotherapeutics were compared to the negative counterparts. The percentages of the viability were determined the MTS assay.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 5 (GraphPad Software, La Jolla, CA) and comparisons were made using unpaired t-test, post-test, and post hoc test where applicable two-way analysis of variance (ANOVA) and Bonferroni's test. A P value of <0.05 was considered statistically significant.

Results

Transient silencing of the *eEF2K* in cells and the its confirmation

To verify that the endogenous levels of eEF2K in the UM-Chor1 and MUG-Chor1 chordoma cell lines are reduced with the gene silencing methodology used in the study, both total RNA and protein samples were prepared. Changes in gene expression both at the mRNA and protein levels were analyzed with qPCR and Western Blot Analysis. Interestingly, although MUG-Chor1 cells appeared to have higher remainder eEF2K mRNA compared to UM-Chor1 cell line under post-eEF2K-siRNA treatment conditions, the magnitude of reduction in eEF2K protein levels appear to be comparable in both MUG-Chor1 and UM-Chor1 cells. When MUG-Chor1 and UM-Chor1 cells were transiently transfected with siRNA against eEF2K, a significant decrease both in gene expression (Fig. 1A, B) and protein levels of eEF2K (Fig. 1C) was observed, which was accompanied with a reduction in its endogenous phosphorlyation target of eEF2 as indicated by the decrease in the signal from phospho-T56-eEF2 antibody (Supplemental figure). Differences in eEF2K amounts are calculated by their normalization to the GAPDH levels in each sample.

The effects of decrease in eEF2K levels on cell proliferation and cell cycle

To address the impact of reduced eEF2K expression on cell proliferation and distribution of cell cycle populations of chordoma cell lines, we used both an MTS-based assay and cell cycle analysis. The diminished levels of eEF2K did not significantly affect the cellular division rate in both cell lines (Fig. 2A, B). However, silencing of eEF2K, caused a mild increase in the number of cells in S and G2/M phases in MUG-Chor1 cells (Fig. 2C). On the other hand, there was not a substantial alteration in the phases of the cell cycle in UM-Chor1 cells upon eEF2K silencing (Fig. 2D).

The impacts of silencing eEF2K on cellular death

Next, we addressed whether silencing of eEF2K had any impact on cell death. To measure apoptosis Annexin V staining was performed on cells treated with either control or eEF2K siRNA. The data indicate that –albeit with minor differences- the silencing of eEF2K resulted in differential apoptotic or necrotic responses in MUG-Chor1 (Fig. 3A) and UM-Chor1 cells (Fig. 3B). For example, UMChor1 cells show a slightly higher percentage of living cells in both groups (control and sieEF2K) than the same conditions in MUG-Chor1 cells (Fig. 3A and B) and lower percentage of late and early apoptotic populations. The lower number of viable cells in the MUG-Chor1 cells is accompanied with slightly higher percentages of sub-apoptotic and necrotic populations. Although reasons for this difference is not investigated in-depth in this study, the mutational burden and variation in the genomic background between the two cell lines that are harvested from tumors found in different locations of the body could account for the discrepancies seen in death profiles.

The changes in the migration and invasion capacity of UM-Chor1 and MUG-Chor1 cells upon eEF2K silencing

To measure the potential changes in the migration and invasiveness of the two cell lines, control siRNA or eEF2K-siRNA treated cells were seeded in transwell plates and the number of cells that migrate across were determined through absobance readings in a Gimsa Stain-based protocol. Our findings indicate that the reduction of eEF2K expression was associated with a decrease in the number of cells that migrated across the transwell. For example,

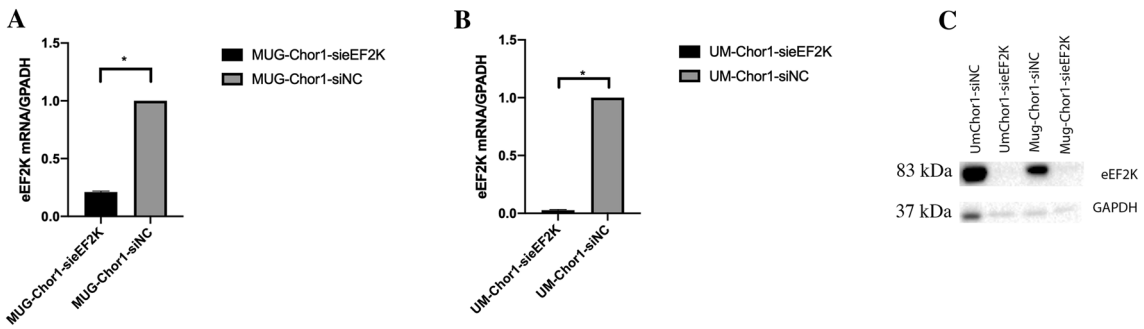

Fig. 1 The confirmation of knock-down by gene expression and western blot analyses

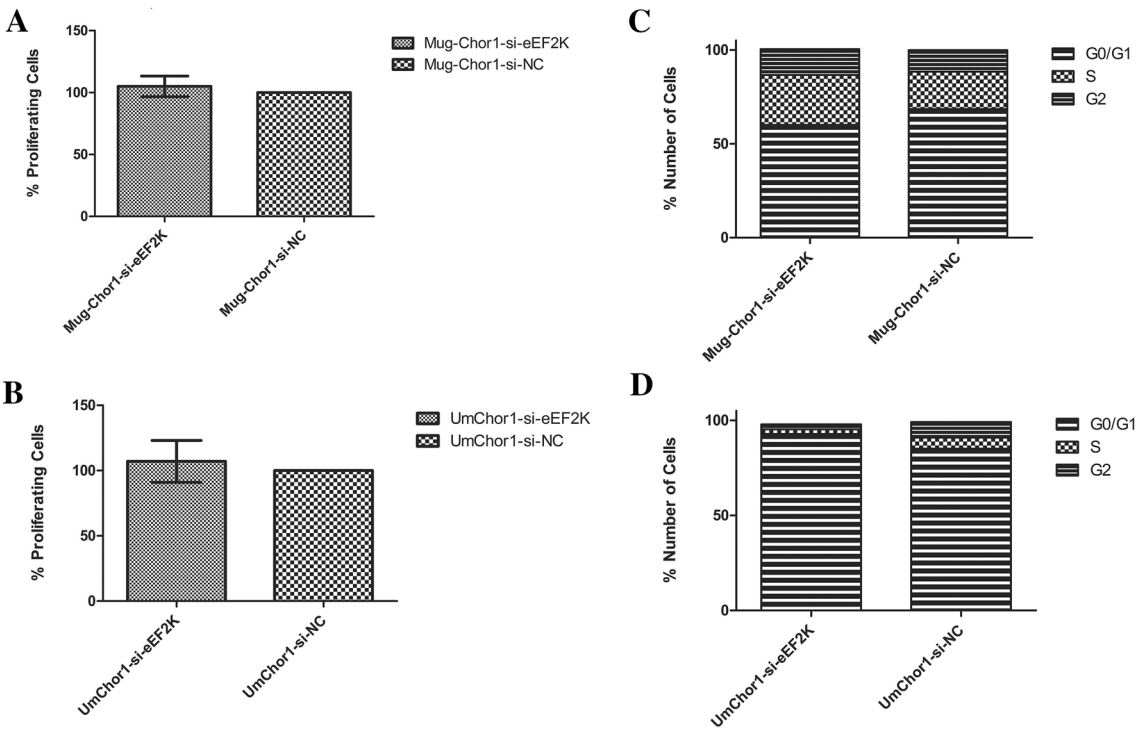

Fig. 2 The changes in the proliferation status of chordoma cells upon silencing. **A** MUG-Chor1 cells **B** UM-Chor1 cells. The changes in the cell cycle profile of chordoma cells upon silencing. **C** MUG-Chor1 cells and **D** UM-Chor1 cells

when normalized to the control cells (no eEF2K silencing) only 60% of both MUG-Chor1 (Fig. 4A) and UM-Chor1 (Fig. 4B) cells were able to migrate through the transwells. Similar results were also obtained in the invasion assay, where control or eEF2K-siRNA treated cells were allowed to migrate across a matrigel layer in same transwell setting. Accordingly, around 70% of MUG-Chor1 and 50% of UM-Chor1 (Fig. 4C and D) cells invaded the matrigel coated transmembrane in presence of eEF2K suppression.

Investigation of the changes in drug-resistance in response to eEF2K silencing

Transient administration of eEF2K siRNAs in both cell lines, chemosensitized these cells to both Etoposide and Cisplatin partially. However, the resultant reduction in cell viability obtained via the combination treatment did not exceed 50%. MUG-Chor1 cells that are silenced for eEF2K were treated with 10 μM Cisplatin and Etoposide. Nonetheless, lower percentage of cells were viable in groups co-treated with Cisplatin compared to those co-treated with Etoposide suggesting a more significant therapeutic outcome in the Cisplatin and eEF2K siRNA dual treatment (Fig. 5A). In contrast, response of UM-Chor1 to the identical treatment schemes indicated a higher percentage of viability when compared to the MUG-Chor1 cells (Fig. 5B). All together, the combination of eEF2K silencing and chemotherapeutics caused the viability to stay above 50%; therefore, this therapy regime is not considerably detrimental on both cells.

Discussion

The small interference-RNAs (si-RNAs) based gene silencing has become a promising tool in developing new methods in regard to elucidating the molecular mechanisms that underlie many diseases including cancer [36]. si-RNAs are essential tools in decipfering the function of particular genes as it promotes silencing by blocking translation of mRNAs into proteins [37]. In addition to being used as a tool for understanding the gene function, in recent years siRNAs have emerged as novel drug cancdidates due to their ability to silence expression of those genes involved in oncogenesis. In our study, physiological consequences of eEF2K silencing on malignant properties of chordoma cells were investigated via transient transfection. To our knowledge, this is the first study that addresses the link between chordoma malignancy and downregulation of eEF2K expression which has been reported to exert pro-tumorigenic effects both in vitro and in vivo models of breast, ovarian, lung, glioma, medulloblastoma, hepatocellular carcinoma, pancreatic cancer and prostate cancer [38]. Suppression of eEF2K expression in

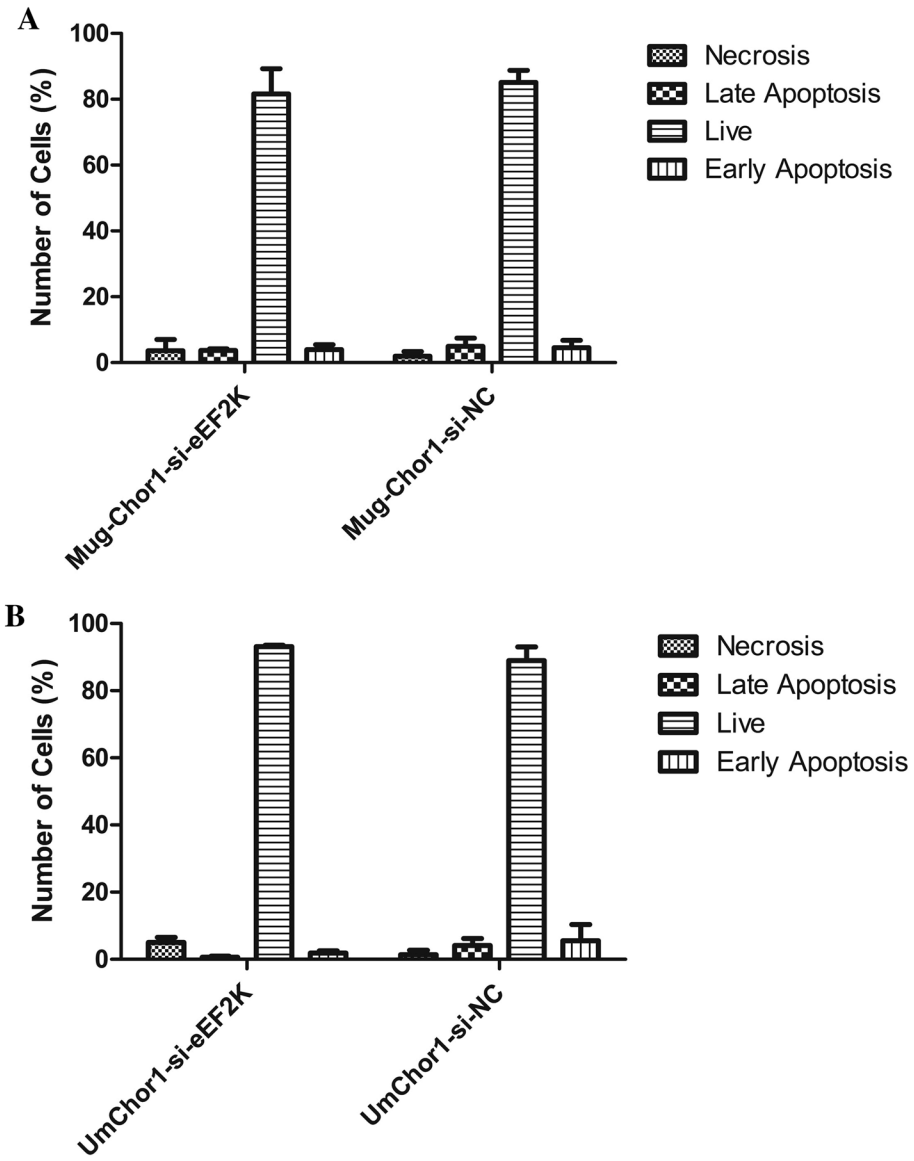

Fig. 3 The cell death profile of cells via Annexin V analysis after the silencing with si-eEF2K. **A** MUG-Chor1 cells **B** UM-Chor1 cells

the slow-growing chordoma cell line model rather hindered migratory and invasiveness in terms of malignant characteristics, while having a mior effect on the proliferative properties.

eEF2K is an atypical Ca2 + /calmodulin-linked protein kinase and negatively regulates mRNA translation by phosphorylating the eEF2 protein that bocks its transocase activity [39]. Various stress stimuli such as nutrient depletion, hypoxia, energy crisis, and viral infections force the cells to adapt their new environment trigger eEF2K catalytic activity toward eEF2 to turn off translation which claims more 90% of the energy dedicated to protein synthesis [40]. It is known that the levels of eEF2K is increased in various rapidly dividing tumor cells and attenuation of eEF2K expression through gene silencing methodologies potentiatiates cancer cell death induced by chemotherapeutics via various cellular death mechanisms [25]. eE2FK is overexpressed in many metastatic cancer types and has been associated with poor prognosis [17, 41]. A recent study highlighted the importance of eEF2K as a metastatic as well as prognostic biomarker in people diagnosed with gastric cancer [42]. A similar study done by Xie et al. showed that si-RNA-mediated eEF2K silencing in human lung cancer cells suppressed the tumor growth and metastasis [43]. Silencing eEF2K reduced the number of proliferating esophageal squamous cell carcinoma cells [34]. In the same study Zhu and colleagues also showed that as a result of suppression of eEF2K in cells, there was a decrease in the number of cells in the S and M phases [44]. In contrast, our findings demonstrated that the proliferation rate of chordoma cells did not decrease upon the suppression of eEF2K, which -in fact- resulted in a mild increase in cell division. The cell cycle profiles for both lines

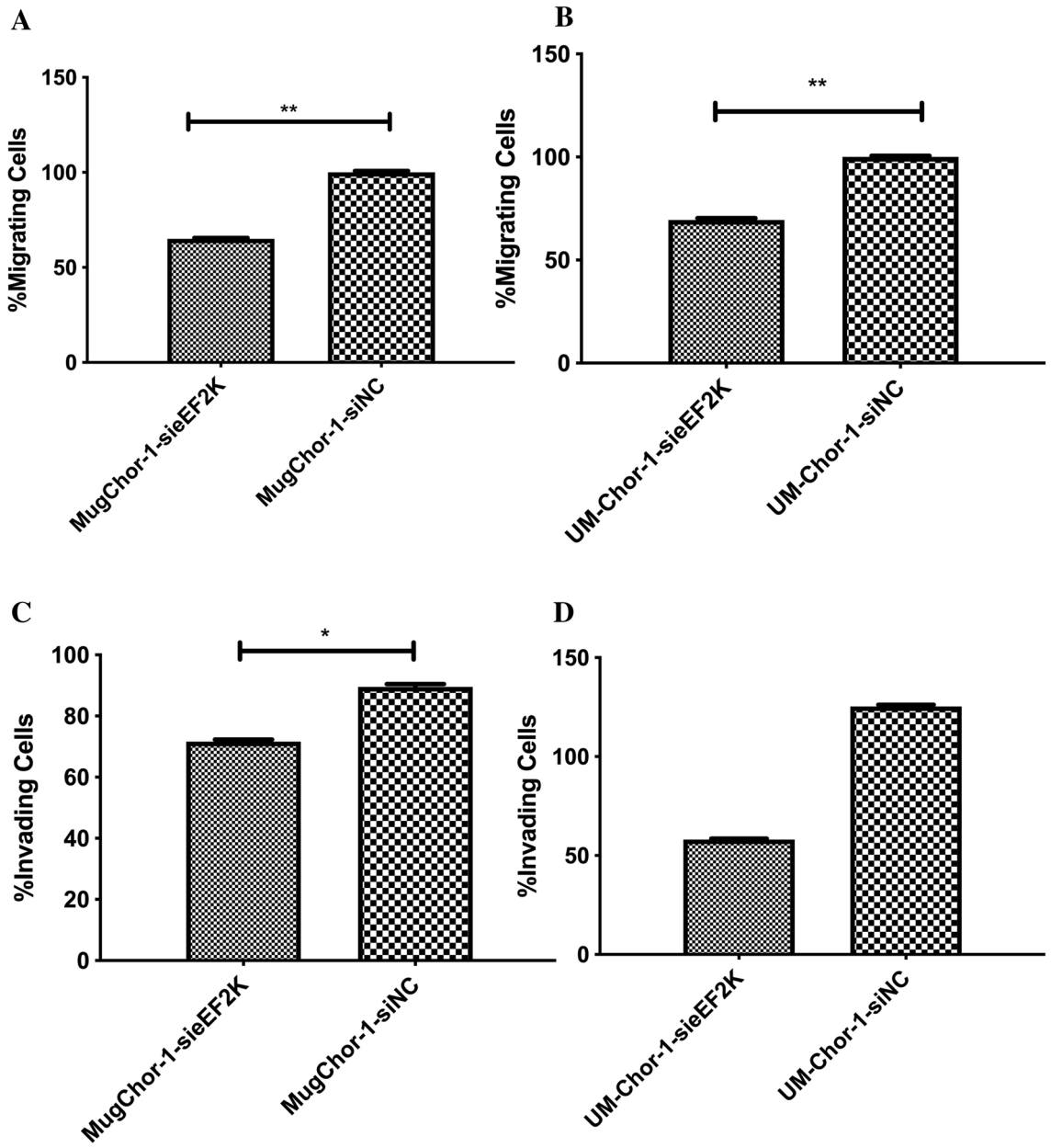

Fig. 4 The numbers of migratory cells upon si-eEF2K silencing on chordoma cells **A** MUG-Chor1 cells **B** UM-Chor1 cells. The number of invasive cells upon si-eEF2K silencing on chordoma cells **C** MUG-Chor1 cells and **D** UM-Chor1 cells

were found to be similar except for a slight increase in S and G2/M phases in MUG-Chor1 cells.

As opposed to previous findings which suggest the inhibition of eEF2K expression in reducing the proliferation rate [45], other studies provide evidence that unleashing eEF2K activity via removal of the inhibitory upstream signaling exerts antitumorigenic effects including breast [46], intestinal, colorectal and lung cancer models [27, 47]. Particularly, translation-independent antitumorigenic effects of eEF2K attenuation detected in the non-small cell lung cancer model are interesting in the sense that an inhibitory phosphorylation of PKM2 directly by eEF2K ultimately results in suppression of glycolysis concomitant with STAT3 dependent transcription and decrease in c-Myc expression [48]. In contrast, high eEF2K expression is associated with poor patient survial and a more efficient siRNA-based silencing eEF2K in a panel of lung carcinoma cell lines resulted in a drastic suppression of colony formation, invasion and tumor volume in an earlier study [49]. Hence, it is worthwhile to understand whether differences in the gene silencing efficiency could account for the dual contrasting roles eEF2K in cancer.

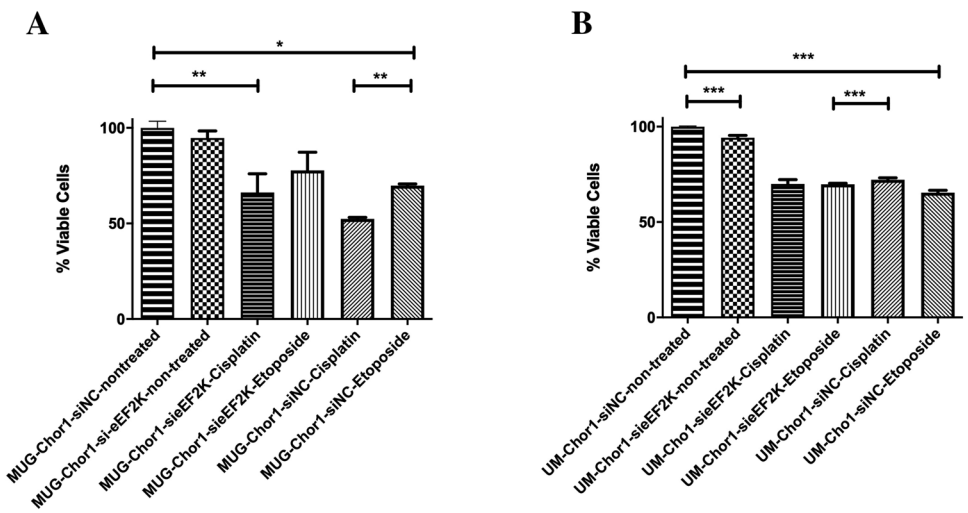

Fig. 5 The chemosensitivity assay shows the number of viable cells synergistically upon silencing with si-eEF2K and drugs (Etoposide and Cisplatin). **A** MUG-Chor1 cells **B** UM-Chor1 cells

The role of eEF2K in protecting cells with more proliferative capacity including stem cells was demonstrated in a study done by Liao et al. [50]. The authors claimed that in the absence of eEF2K expression in knock-out mice were protected against low dose ionizing radiation. In the same study, they also showed the reduction in death of stem cells of the bone marrow in the eEF2K knock out mice. The authors further explained that at lower doses of ionizing radiation (IR), the absence of eEF2K allows cells escape from (G2/M) checkpoint in the cell cycle accounting for the increased survival phenotype of the animals; however, at higher doses of IR eEF2K deficiency of the knock-out cells increases their susceptibility to undergo higher degree of mitotic catastrophe. This would explain why at least one type of cells are found arrested in S and G2/M phases of the cell cycle.

Therapeutics that target eEF2K are continued to be developed and become of great interest in the treatment of triple negative breast as therapeutic effect of eEF2K knock down was demonstrated both in cancer cell lines [51] as well as in in vivo models [52]. Silencing eEF2K increases the sensitivity against lapatinib in human nasopharyngeal carcinoma cells [53], and further sensitizes human glioma cells to agents such as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and temozolomide [24]. Combined therapy of an eEF2K inhibitor –mitoxantrone- and mTOR targeting increased the efficiency of treatment method against breast cancer [54]. mTOR is highly expressed in chordoma cells and is a well known biomarker [55]. Targeting mTOR is not only promising for other cancer types, but also for chordoma [56]. Peng et al. showed that targeting mTOR sensitized the cells to Cisplatin [57]. Zhang et al. showed that silencing of eEF2K increased the sensitivity of hepatocellular carcinoma cells to Cisplatin [58]. In our study, MUG-Chor1 cells responded to Cisplatin better than they did to Etoposide. The cell lines used in this study originate from different parts of the spine and therefore show varying characteristics in their response as well as drug resistance to chemotherapy. The decrease in eEF2K levels might have increased the rate of proliferation and sensitizing the cells to chemotherapy considerably. This showed us that drug resistance in chordoma cells partially attenuated when silenced with eEF2K siRNAs. As suggested by our group that there is a population of cancer stem cells in chordoma [59] and hence they might be deprived of the protecting role of eEF2K due to the silencing [50]. Therefore, the partial death observed in the chordoma cells upon treatment with chemotherapeutics in presence of silencing eEF2K could be due to losing its protective role.

According to our findings, downregulation of eEF2K reduced the metastatic capacity of chordoma cells. A recent study suggests that targeting eEF2K could be a promising therapeutical approach in hindering melanoma progression [60].

Other studies show that there is an association between mTOR expression and migration [61]. Considering that both mTOR and eEF2K are highly expressed in chordoma and the findings of Guan and colleagues in the breast carcinoma model, it would be of interest to test whether inhibition of eEF2K could help potentiating the targeting mTOR pathway in the treatment of this cancer type in a future study. Nonetheless, based on the findings of the current work, we can conclude that chordoma cells may have lost their migratory capacity significantly due to the silencing of eEF2K.

Angiogenesis is the process in which newly formed tumor cells, become invasive and migrate into the other parts of the body. It is found that eEF2K induces an increase in the VEGF expression and, thereby, ultimately contributes to angiogenesis. A chain of events occur upon inhibition of eEF2K including a decrease in PI3K/Akt/STAT3 pathway, which is highly expressed in chordoma [11]. This might explain the reduced migratory capacity

of chordoma cell when eEF2K is silenced. Ashour et al. showed that silencing of eEF2K in pancreatic cancer cells suppressed EMT and reduced metastasis and invasion [45]. Previously we have addressed genes that regulate the invasiveness of chordoma cells and reported that TWIST -a prominent marker of Epithelial Mesenchymal Transition (EMT)- also underlies invasiveness of chordoma [62]. In a similar study, Xie et al. delineated the molecular switches that control the metastatic capabilities of breast and lung cancer cells through suppression of eEF2K suggesting EMT-modulatory roles of eEF2K loss [43]. Therefore, we investigated the impact of eEF2K silencing on the metastasis and invasion capacities of the chordoma cells in the current study. Similar to the results in the lung [38] and breast cancer study [25], our data also point out that upon eEF2K suppression, a significant reduction in metastasis and invasion capabilities both MUG-Chor1 and UM-Chor1 cells is observed.

Chordoma is a rare type of bone tumor and yet classiffied as often presenting with malignant properties, including recurrence and ability for metastasis. Chordoma is also highly resistant to conventional drug-based treatment methods and therefore requires new strategies that can improves the clinical outcome. Proliferation of the cells are largely depend on the proper protein synthesis, but under suboptimal conditions, proliferation needs to be blocked until the environmental conditions go back to normal. eEF2K is an enzyme that negatively regulates the protein synthesis by phosphorylating eEF2 and causes an inhibition. When cells are exposed to energy depletion eEF2K becomes activated and trigger a reduction in overall translational rates. This mechanism is also adapted by the tumor cells as they are faced with various stress conditions including nutrient deprivation, high acidity and low oxygen levels in their poorly vascularized microenviroment, which account for these harsh living conditions as a consequence of exponential growth in the tumor mass. Chordoma cells express high eEF2K levels that might explain the slow proliferating nature of the cells. By inhibiting eEF2K we were able to force the cells to become more sensitive Cisplatin and Etoposide. Upon eEF2K depletion the chordoma cells significantly lost the metastatic and invasive capacities, which holds a promise for future preclinical studies and clinical trials.

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Declarations

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