2-Methoxyestradiol-mediated induction of Frzb contributes to cell death and autophagy in MG63 osteosarcoma cells

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Short Title: 2-ME-mediated induction of FrzB

Key words: 2-Methoxyestradiol, estrogen metabolite, Frzb, sFRP3, Wnt, autophagy, Osteosarcoma.

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†This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/jcb.25809

Received 29 July 2016; Revised 22 November 2016; Accepted 22 November 2016
Journal of Cellular Biochemistry
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DOI 10.1002/jcb.25809
Abstract

Osteosarcoma is a bone tumor that mainly affects children and adolescents. Although its pathogenesis is still not fully understood, activation of Wnt signaling has been implicated in the development and metastasis of osteosarcoma. In this report, we have investigated the effect of the anti-tumor compound, 2-methoxyestradiol (2-ME) on Wnt antagonist frizzled-related protein b (Frzb), also known as secreted frizzled-related protein (sFRP)3 in human osteosarcoma (MG63) cells. Our results show that 2-ME treatment induces Frzb gene promoter activity, and increases Frzb mRNA and protein levels in osteosarcoma cells. In addition, 2-ME treatment regulates downstream Wnt signaling, increasing the cytoplasmic levels of β-catenin, and blocking β-catenin-mediated Wnt activation in osteosarcoma cells. 2-ME-mediated induction of Frzb protein expression is specific to osteosarcoma cells, as it does not affect Frzb expression in normal primary human osteoblasts. Furthermore, 2-ME-induced apoptosis and autophagy are blocked in osteosarcoma cells transfected with Frzb siRNAs. Taken together, these studies demonstrate that Frzb protein plays an important role in 2-ME-mediated anti-tumor mechanisms in osteosarcoma cells. This article is protected by copyright. All rights reserved
Introduction

Osteosarcoma is the most common primary bone tumor which mostly originates in the growing ends of bones and primarily affects children and adolescents [Arndt et al., 2012]. About 30% of patients diagnosed with osteosarcoma will develop metastatic diseases. Although a combination of surgery and chemotherapy has improved the survival rate many patients fail to achieve long-term disease free survival [Arndt et al., 2012; Hayden and Hoang, 2006; Kansara and Thomas, 2007; O’Reilly et al., 1996]. Part of the difficulty in treating osteosarcoma involves the ability of osteosarcoma cells to develop genetic modifications that enable resistance to therapeutic interventions [Chou and Gorlick, 2006; Wagner et al., 2011].

The Wnt signaling pathways play key roles in cell proliferation, survival, stem cell self-renewal and differentiation, and maintaining the homeostasis of many tissues [Clevers, 2006; Logan and Nusse, 2004]. Abnormalities in the Wnt signaling pathways have been widely implicated in tumorigenesis, including the development of osteosarcoma [Fujie et al., 2001; Jessen, 2009; MacDonald et al., 2009; Morin et al., 1997; Polakis, 2007; Woo et al., 2001]. Several natural and synthetic Wnt antagonists have been investigated for their anti-tumor therapeutic effects [Chen et al., 2009; Kim et al., 2016; Martins-Neves et al., 2016; Saraswati et al., 2010; Suzuki et al., 2004]. Secreted Frizzled-Related Proteins (sFRPs) are natural antagonists that directly bind to Wnt ligands and inhibit canonical and non-canonical Wnt pathways. The sFRP3, also known as frizzled-related protein b (Frzb) acts as a tumor suppressor in osteosarcoma and other cancers [Guo et al., 2008b; Mandal et al., 2007; Zi et al., 2005].

2-Methoxyestradiol is a metabolite of mammalian estrogen 17β-estradiol but it differs from the parent compound and acts as an anti-tumor agent in many cell types. 2-ME has been implicated in the regulation of many signaling pathways [LaVallee et al., 2003; Mukhopadhyay and
Roth, 1997; Schumacher et al., 1999; Seegers et al., 1997; Wimbauer et al., 2012]. 2-ME has been shown to induce cell death and autophagy in osteosarcoma cells, but not in normal osteoblasts [Maran et al., 2008; Shogren et al., 2007; Wimbauer et al., 2012; Yang et al., 2013]. Since the Wnt antagonist Frzb protein has been shown to have a tumor suppressive role in osteosarcoma [Mandal et al., 2007], in this report, we have investigated whether 2-ME-mediated anti-tumor effects in osteosarcoma cells involve the Frzb protein.

Materials and Methods

Cell culture

The human osteosarcoma cells (MG63, KHOS and 143B) [Maran et al., 2008; Maran et al., 2002; Shogren et al., 2007; Wimbauer et al., 2012] and primary human osteoblasts (HOB) were maintained in DMEM/F12 medium with 10% FBS. HOB cells were established as previously described [Maran et al., 2002]. 17β-Estradiol (E₂), 2-methoxyestradiol (2-ME), 4-hydroxyestradiol (4-OHE) and 16α-hydroxyestradiol (16-OHE) were purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions of the metabolites at their respective concentrations were made in 95% ethanol.

Reporter assay

Luciferase constructs containing the Frzb promoter used in this study were constructed by subcloning a 5935bp human Frzb promoter fragment into the KpnI (5’) and BglII (3’) sites in the plasmid pGL3-basic vector (Promega, Madison, WI). MG63 osteosarcoma cells plated in 6-well plates (1.5 X 10⁶ cells/well) were transfected at 60% confluence using the transfection reagent Lipofectamine, as described in the manufacturer’s protocol (Invitrogen, Carlsbad, CA). All
transfections performed had a control plasmid containing Renilla Luciferase (Promega, Madison, WI), which allowed normalization of luciferase units measured with an illuminometer. To monitor TCF-dependent transcription, reporter constructs containing TCF binding site (TOP flash) or mutant binding site (FOP flash) were transfected using FuGene HD reagent as described (Promega). The luciferase activity was calculated by determining the ratio of TOP flash/FOP flash, and after normalization to renilla luciferase units.

RNA analysis

Osteosarcoma cells were treated with vehicle (Veh) and 2-ME (10 μM) for 24hrs and then were used for RNA isolation. Total cellular RNA was extracted and isolated using trizol reagent (Invitrogen), and the mRNA levels of sFRP3, Wnt and the control actin were analyzed by the quantitative PCR as described [Wimbauer et al., 2012]. The following primer sequences were designed as described previously for Frzb [Qu et al., 2008], and actin [Wimbauer et al., 2012] genes and obtained from Integrated DNA Technologies, Inc (Coralville, IA):

Frzb: forward 5’ gag gag ctg cca gtg tac gac 3’ and reverse 5’ gaa aat cag ctc cgt ccg c 3’
Actin: forward 5’ tgcctcaggc 3’ and reverse 5’ gctgtgctatccctgtac 3’.

Protein Analysis

For western analysis, the cytoplasmic extracts were prepared from vehicle and 2-ME-treated cells as described [Shogren et al., 2007], and analyzed by western blot hybridization using anti-β-catenin, anti-LC3-I/II (Cell Signaling Technology, Danvers, MA), anti-sFRP3 (Santa Cruz Biotechnology, Dallas, TX) and anti-actin antibodies (Sigma, St.Louis, MO). Co-immunoprecipitation studies were carried out as described [Kennedy et al., 2005]. Briefly, nuclear
extracts (300 μg protein) from vehicle and 2-M-treated cells were precipitated with anti-TCF4 antibody (Cell Signaling Technology) or non-immune IgG controls (Dako, Carpineria, CA). The immunoprecipitates were incubated with protein G sepeharose for 60 min at 4°C and analyzed by western blot hybridization using anti-β-catenin (Cell Signaling Technology) antibodies. Quantitation of the corresponding bands was carried out using densitometry and analyzed using Quantity One software (Bio-Rad, Hercules, CA).

siRNA-mediated inhibition of gene expression

Osteosarcoma cells (5 × 10⁴) in 24-well plates were transfected with Frzb duplex siRNA [5’gga ucg acu cgg uaa aaaa 3’ and 5’uuuu uua ccg agu cga ucc 3’] and control non-targeting siRNA [5’ uguuuuaaugucagcuaa 3’] following the manufacturer's protocol (Dharmacon, Lafayette, CO). Twenty-four hours after transfection, cells were treated with vehicle control and 2-ME (5 μM). At the end of 48 hrs of treatment, cells were processed for apoptosis assay, autophagosome formation and western blot hybridization.

Hoechst Assay

The apoptosis assay to detect chromatin condensation was performed using Hoechst staining as described earlier [Shogren et al., 2007]. Briefly, Twenty-four hours after siRNA transfection, cells were treated with Veh and 2-ME (5 μM). At the end of 48hrs of treatment, cells were fixed, stained with Hoechst and quantitated as described [Shogren et al., 2007].

Transmission Electron Microscopy (TEM)
Autophagosome formation was investigated by TEM as described [Yang et al., 2013]. Briefly, osteosarcoma cells were grown on aclar and treated with Vehicle (Veh) and 2-ME (5 μM) for 48 hours. After treatment, the cells were fixed using Trump’s fixative and processed for TEM at Mayo Clinic’s electron microscopy core facility.

Statistical Analysis

StatView version 5.0.1 (SAS Institute Inc.) was used for statistical analysis of the data and all of the values are expressed as mean +/- standard error of mean. Statistical analysis was accomplished by using oneway analysis of variance (ANOVA). When the overall ANOVA F-test was found to be significant, pair-wise comparisons were performed using Tukey0s studentized range test in order to maintain the overall experiment-wise error rate. P<0.01 was considered statistically significant.

Results

2-ME induces FrzB expression in MG63 osteosarcoma cells

To determine whether 2-ME regulates the Wnt antagonist, Frzb, we have studied the effect of 2-ME on Frzb gene activity and expression in osteosarcoma cells. Compared to vehicle (Veh) control, 2-ME treatment stimulated the Frzb-driven luciferase activity by 9-fold over 48 hours in MG63 osteosarcoma cells (Fig.1A). Real time PCR analysis of total RNA from vehicle and 2-ME-treated osteosarcoma cells show that 2-ME treatment increased Frzb mRNA levels by 2-fold (Fig.1B). Western blot hybridization using cell extracts from vehicle and 2-ME-treated MG63 cells show that 2-ME treatment did not affect actin levels but induced Frzb protein levels by 4-fold compared to the vehicle control (Fig. 1C).
2-ME-mediated induction of Frzb expression is specific to osteosarcoma cells

To validate the specificity of 2-ME-mediated induction of Frzb, we have studied the effect of 2-ME in two additional osteosarcoma cells (KHOS and 143B) and in normal human osteoblast (HOB) cells. Our results show that 2-ME induces Frzb protein levels in KHOS and 143B osteosarcoma cells (Fig.2A). In contrast, 2-ME did not affect Frzb expression in normal human osteoblast cells, HOB1 and HOB2 (Fig.2B), which are resistant to the anti-proliferative effects of 2-ME [Maran et al., 2008; Maran et al., 2002]. The levels of control protein actin were not influenced by either vehicle or 2-ME treatment in osteosarcoma and HOB cells (Fig.2).

To further establish the specificity of 2-ME in regulating Frzb expression, we evaluated the effects of various estrogenic compounds on MG63 cells. Our results showed that the anti-tumorigenic metabolite, 2-ME, induced Frzb expression but the tumorigenic metabolites, 4- and 16α-hydroxyestradiol and the parent estrogen, 17β-estradiol did not have any effect on Frzb expression (Fig.3). None of these estrogenic compounds had any effect on the control actin expression (Fig.3).

2-ME treatment regulates β-catenin levels and blocks Wnt signaling in osteosarcoma cells

To further investigate whether 2-ME-treatment regulates Wnt pathway, we have determined the levels of β-catenin in Veh and 2-ME-treated osteosarcoma cells by using western blot analysis. We found that 2-ME treatment increases the cytoplasmic levels and decreases the nuclear levels of the Wnt canonical pathway protein β-catenin (Fig.4A &4B). The results show that the control cytoplasmic actin and nuclear lamin B are not affected by 2-ME treatment (Fig.4A & 4B). Additionally, the influence of 2-ME on the association of β-catenin and transcription factor 4
(TCF4) was examined by co-immunoprecipitation studies. Our analysis using nuclear extracts from Veh and 2-ME-treated cells demonstrate a decreased association of β-catenin and TCF-4 protein in the presence of 2-ME treatment (Fig.4C).

In order to determine whether the induction of Frzb gene expression in the presence of 2-ME leads to a decreased Wnt signaling, we studied the effect of vehicle and 2-ME on β-catenin-independent TCF luciferase reporter gene activity. MG63 cells were transiently-transfected with the luciferase reporter plasmids containing either (TOP flash) or mutant (FOP flash) TCF binding sites and renilla luciferase. Our results showed that 2-ME decreased β-catenin-mediated TCF signaling (Fig.5). 2-ME blocked LiCl-mediated induction of TCF- luciferase by 1.8 and 6-fold following the co-treatment and pre-treatment of LiCl, respectively (Fig.5).

**siRNA-mediated inhibition of Frzb reverses 2-ME-mediated effects on osteosarcoma cells**

To further establish the role of Frzb in 2-ME actions, 2-ME-mediated regulation of β-catenin and osteosarcoma cell death were investigated in MG63 cells transfected with siRNAs directed against Frzb (Fig.6). Down-regulation of Frzb expression through siRNA transfection blocked the 2-ME-mediated decreases in nuclear β-catenin levels. Whereas in control transfected with non-specific siRNAs, the nuclear β-catenin protein levels decreased by 50% following 2-ME treatment (Fig.6A & 6B). Also, the inhibition of Frzb expression decreased the rate of apoptosis in 2-ME-treated osteosarcoma cells compared to the non-specific siRNA (NS siRNA)-transfected controls (Fig.6C). Our results show that 2-ME induced apoptosis by 99% and 62% in the presence of NS siRNA and Frzb siRNA transfection, respectively (Fig.6A). Westernblot analysis shows that transfection of siRNA directed against Frzb affects 2-ME-
mediated increase in Frzb protein level, whereas the 2-ME actions are not affected in cells transfected with NS siRNAs (Fig.6D). The results further confirm that siRNA transfection did not affect control actin levels (Fig.6D).

Previous report shows that 2-ME induces autophagy in osteosarcoma cells. To determine whether Frzb upregulation influences the 2-ME-mediated induction of autophagy, we have investigated the effect of 2-ME on autophagosome formation (Fig.7) and the autophagy marker, microtubule-associated protein 1 light chain 3 (LC3) protein levels (Fig.8) in MG63 osteosarcoma cells after transfection with siRNAs directed against Frzb. Compared to vehicle controls 2-ME treatment induces autophagosome formation in NS SiRNA-transfected osteosarcoma cells (Fig.7c) but does not induce autophagosome formation in cells that have been transfected with Frzb siRNAs (Fig.7d). Further, the results show that downregulation of Frzb expression changes the expression of LC3 and the conversion of LC3-I to LC3-II protein (Fig.8A). Quantitative analysis of western blots reveal that the ratio of LC3II/I in NS siRNA- and Frzb siRNA transfected-cells are 2.4 and 1.2, respectively (Fig.8B).

Discussion

Wnt signaling plays an important role in osteogenesis, and potentially osteogenic dysregulation leading to tumorigenesis. We have previously shown that the Wnt pathway is associated with osteosarcoma, and demonstrated that Frzb is downregulated in osteosarcoma patients [Mandal et al., 2007]. In this report, we show that Frzb is upregulated in osteosarcoma cells by the anti-tumor compound, 2-ME which has been reported to kill specifically osteosarcoma cells but not normal osteoblasts [Maran et al., 2008; Maran et al., 2002]. 2-ME treatment leads to an increase in Frzb gene activity and expression levels in osteosarcoma cells, but has no effect on
Frzb expression in normal osteoblasts. Also, the cell killing effects of 2-ME are inhibited by silencing the Frzb gene in osteosarcoma cells. Furthermore, 2-ME-mediated induction of autophagosomes and autophagic flux is blocked in the absence of Frzb expression. Thus, our results suggest that 2-ME induces anti-tumor effects in osteosarcoma cells through inducing Frzb protein and by blocking the Wnt activation in osteosarcoma cells.

The Wnt signaling cascade has been implicated in the pathogenesis of several malignant tumors [Hayden and Hoang, 2006; Logan and Nusse, 2004; MacDonald et al., 2009; Mitsui et al., 2014; Morin et al., 1997; Polakis, 2007]. In particular, the Wnt signaling has been demonstrated to regulate osteosarcoma cell growth and invasiveness [Goldstein et al., 2016; Guo et al., 2008a; Lin et al., 2014; Wagner et al., 2011]. Additionally, 2-ME has been shown to have anti-tumor effects in several cancers both in vitro and in vivo [Cicek et al., 2007; Fotsis et al., 1994; Kambhampati et al., 2013; LaVallee et al., 2003; Muh et al., 2014; Mukhopadhyay and Roth, 1997; Schumacher et al., 1999; Seegers et al., 1997; Shogren et al., 2007]. Our investigation provides evidence for an association between the upregulation of Frzb expression and the anti-tumor effects of 2-ME in osteosarcoma cells.

The Wnt antagonist Frzb belongs to a family of five secreted glycoproteins which share similar sequences with the frizzled receptor extracellular domain. Frzb antagonizes Wnt signaling by binding to Wnt ligands, competitively inhibiting the ligand and receptor interactions. The Frzb molecule has been shown to act as a tumor suppressor, and decrease the growth and invasiveness of prostate cancer and fibrosarcoma cells [Guo et al., 2008b; Zi et al., 2005]. Our current results add to an earlier finding that Frzb expression is downregulated in osteosarcoma patients [Mandal et al., 2007] and reveal that the anti-tumor effects of 2-ME are mediated through the protein Frzb. Our data point out that Frzb upregulation is specific to the anti-tumor effects of
2-ME, as tumorigenic metabolites 4- and 16α-hydroxyestradiol, and the parent estrogen do not have any effect on Frzb expression. 2-ME treatment induced Frzb levels in many osteosarcoma cells that were examined. In addition, Frzb gene expression is not induced in 2-ME-treated normal osteoblasts, which are known to be resistant to the anti-proliferative effects of 2-ME.

The β-catenin protein functions as an intracellular signal transducer in the Wnt canonical pathway. Mutations and increases in nuclear level and activity of β-catenin have been reported in malignant cells. Previous studies have demonstrated small molecule-mediated and targeted inhibition of β-catenin [Amado et al., 2014; Gang et al., 2014; Park and Choi, 2010]. Our studies demonstrate that 2-ME treatment in osteosarcoma cells results in an increase in the cytoplasmic levels and a decrease in nuclear levels of β-catenin. 2-ME treatment decreased TCF-dependent transcriptional activity and blocked TCF-luciferase expression by Lithium chloride, a known activator of Wnt signaling. These results thus reveal that 2-ME treatment regulates downstream effects, in addition to targeting Frzb, in osteosarcoma cells.

Our results further confirm that Frzb is directly involved in 2-ME-mediated anti-tumor effects, since inhibition of Frzb expression through siRNAs partially reverses the 2-ME-mediated decrease in nuclear levels of β-catenin and induction of apoptosis in osteosarcoma cells. Thus, these studies suggest that Frzb is necessary for 2-ME-mediated anti-tumor actions in osteosarcoma cells. Further work is necessary to determine whether Frzb is sufficient or additional mechanism is involved.

The current results show that 2-ME-mediated induction of autophagosome formation and autophagic flux requires Frzb expression as silencing of Frzb blocks 2-ME-mediated effects on autophagy in osteosarcoma cells. Autophagy is a homeostatic mechanism through which cellular proteins and organelles are enveloped in autophagosomes and subjected to lysosomal
degradation and recycling. We have previously demonstrated that 2-ME induces autophagy in osteosarcoma cells but not in normal osteoblasts [Yang et al., 2013]. Autophagy in cancer has been widely investigated and shown to have a dual role by contributing to either cell survival or as a step to cell death. Hence, autophagy and apoptosis could be under the control of similar cellular signals and cross-regulate each other. Autophagy reduces the capacity of cells to undergo apoptosis, and triggering of apoptosis is coupled to the suppression of autophagy. 2-ME-induced autophagy has been shown to follow cell death in other cancer cells [Chen et al., 2008; Lorin et al., 2009; Yang et al., 2013]. Previous studies show that damage-regulated autophagy modulator (DRAM) [Lorin et al., 2009] and RNA-dependent protein kinase (PKR) [Yang et al., 2013] are involved in the induction of autophagy and cell death. It remains to be established whether Frzb-dependent autophagy contributes to cell death in the presence of 2-ME treatment. Current findings show that Frzb protein is involved in the stimulation of 2-ME-mediated apoptosis and autophagy. Further work is necessary to delineate the mechanism of action of Frzb in 2-ME-induced autophagy in osteosarcoma cells, and establish the role of autophagy in the regulation of osteosarcoma.

Acknowledgment

This work was supported by funding from Riviera Foundation and the Mayo Clinic. DB was supported by Mayo Clinic’s Center for Clinical and Translational Science Master program.

Disclosure: The authors report no conflict of interest.
References


Figure legends

**Fig.1. 2-ME induces Frzb expression in MG63 osteosarcoma cells.** A) Frzb-Luciferase reporter gene activity: MG63 cells transiently transfected with Frzb-luciferase plasmid constructs were treated Vehicle control (Veh) and 2-ME (10µM) for 48 hrs and the luciferase activity was measured. B) mRNA levels: MG63 cells were treated with Veh and 2-ME (10µM) for 24hrs and the total RNA isolated from the cells was analyzed by real time PCR. C) Protein analysis: Cytoplasmic extracts prepared from MG63 cells treated with Veh and 2-ME (10µM) for 24hrs and analyzed by western blot hybridization using anti-sFRP3 and anti-actin (Santa Cruz Biotechnology) antibodies. *P< 0.01 vs Veh.

**Fig.2. 2-ME induced Frzb expression and is associated with anti-tumor effects.** Cytoplasmic extracts from cells treated with Veh and 2-ME (10µM) for 24hrs were analyzed by western blot. A) KHOS and 143B osteosarcoma cells; B) Primary human osteoblasts [HOB 1 & 2].

**Fig.3. Effect of various estrogenic compounds in MG63 cells.** Cells were treated for 24 hrs with Vehicle control (Veh) or 10µM of 2-ME, 17β-estradiol (E2), 4-hydroxyestradiol (4-OHE) and 16α-hydroxyestradiol (16-OHE) and analyzed by western blot hybridization.

**Fig.4. 2-ME regulates β-catenin levels.** Cytoplasmic (A) and nuclear extracts (B) prepared from MG63 cells following 24hrs of Veh and 2-ME (10µM) treatment were analyzed by western blot using anti-β-catenin, anti-actin and anti-lamin B (Santa Cruz Biotechnology) antibodies. Nuclear extracts from Veh and 2-ME-treated cells were precipitated with anti-TCF4 antibody (Cell
Signaling Technology) or non-immune IgG controls (Dako, Carpineria, CA), and analyzed by western blot hybridization using anti-β-catenin antibodies (C).

**Fig.5. 2-ME regulates β-catenin-dependent TCF signaling.** MG63 cells transfected with TOP flash and FOP flash luciferase reporter plasmids were treated with Veh, 5 μM 2-ME and 5 mM lithium chloride (LiCl) and analyzed for luciferase expression after 48 hrs. The cells were pre-treated for 24hrs with LiCl (2-ME+preLiCl) or co-treated with LiCl (2-ME+LiCl) for 48hrs. *p<0.01 vs Veh; # p<0.01 vs LiCl.

**Fig.6. siRNA-mediated inhibition of Frzb expression reverses 2-ME-mediated anti-tumor effects.** MG63 cells were transfected with siRNAs directed against Frzb and non-specific RNAs (NS) and treated with Veh and 2-ME (5 μM) for 48hrs. Protein levels were measured by western blot hybridization (A &D). Apoptosis was determined by Hoechst staining (C). Quantitation of β-catenin levels were carried out by densitometry (D). *p<0.01 vs Veh; #p<0.01 vs 2-ME in NS siRNA-transfected.

**Fig.7. Frzb down regulation blocks 2-ME-induced autophagosome formation.** MG63 cells were transfected with siRNAs directed against Frzb and non-specific RNAs (NS) and treated with Veh (a & b) and 2-ME (5 μM) (d & e) for 48hrs and analyzed using electron microscopy. TEM images from NS siRNA-transfected (a & c) and Frzb siRNA- transfected (b & e) cells. Arrows indicate autophagosomes.
Fig.8. Effect of Frzb downregulation on LC3 protein levels. MG63 cells were transfected with siRNAs directed against Frzb and control non-specific (NS) siRNAs and treated with Veh and 2-ME (5 μM) for 48hrs and analyzed by western blot. A) Western analysis; B) Quantitation of the blot.
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