Mol. Cells 2016; 39(5): 389-394
http://dx.doi.org/10.14348/molcells.2016.2300

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A Receptor Tyrosine Kinase Inhibitor, Dovitinib (TKI-258), Enhances BMP-2-Induced Osteoblast Differentiation *In Vitro*

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Dovitinib (TKI258) is a small molecule multi-kinase inhibitor currently in clinical phase I/II/III development for the treatment of various types of cancers. This drug has a safe and effective pharmacokinetic/pharmacodynamic profile. Although dovitinib can bind several kinases at nanomolar concentrations, there are no reports relating to osteoporosis or osteoblast differentiation. Herein, we investigated the effect of dovitinib on human recombinant bone morphogenetic protein (BMP)-2-induced osteoblast differentiation in a cell culture model. Dovitinib enhanced the BMP-2-induced alkaline phosphatase (ALP) induction, which is a representative marker of osteoblast differentiation. Dovitinib also stimulated the translocation of phosphorylated Smad1/5/8 into the nucleus and phosphorylation of mitogen-activated protein kinases, including ERK1/2 and p38. In addition, the mRNA expression of BMP-4, BMP-7, ALP, and OCN increased with dovitinib treatment. Our results suggest that dovitinib has a potent stimulating effect on BMP-2-induced osteoblast differentiation and this existing drug has potential for repositioning in the treatment of bone-related disorders.

INTRODUCTION

In vertebrates, the balance between osteoblastic bone formation and osteoclastic bone resorption controls bone homeostasis. However, decreased osteoblastic activity and increased osteoclastic activity lead to a reduction in bone mineral density and, consequently, increases in the risk of fractures and metabolic skeletal diseases, like osteoporosis (Boyle et al., 2003; Harada and Rodan, 2003). Osteoblasts are the main cells developed from mesenchymal stem cells that contribute to bone mineral density and produce markers of osteoblasts, such as alkaline phosphatase (ALP), and bone matrix proteins such as osteocalcin (OCN) and osteopontin (OPN) (Katagiri et al., 1994; Long, 2012). The induction of these markers has been regulated by the degree of cross-talk among mitogen-activated protein kinase (MAPK) pathways such as ERK, JNK, and p38 (Candeliere et al., 2001; Chae et al., 2002; Suzukim et al., 2002; Wu et al., 2006). Among the bone formation stimulating factors, bone morphogenetic proteins (BMPs), which are members of the transforming growth factor-β (TGF-β) superfamily, exert stimulating effects on osteoblast differentiation and bone mineralization (Phimphilai et al., 2006). In particular, BMP-2 is a main regulator of osteoblastogenesis and bone regeneration (Rosen, 2009; Wagner et al., 2010).

Many studies have investigated methods for balance recovery between bone formation and resorption. Thus, anabolic agents that stimulate osteoblast differentiation or antiresorptive activity have been developed to attempt to treat bone diseases (Garces and Garcia, 2006; Rosen and Bilezikian, 2001). However, since existing anabolic agents for bone diseases have some limitations in the administration methods and costs, new effective drugs are needed (Garrett, 2007). In recent years, many companies began searching existing drugs to reduce research and development (R&D) spending and adjust these drugs for other indications *via* the drug repositioning process (Longman, 2004; Son et al., 2013; Stuart, 2004). This process provides many pharmaceutical companies or investigators an effective way to reduce developmental costs and risks of failure in drug developmental processes such as pharmacokinetic/pharmacodynamic studies and clinical trials.

Dovitinib (TKI258) is a small-molecule multi-kinase inhibitor in phase I/II/III clinical trials for the treatment of gastric cancer, pancreatic cancer, advanced breast cancer, multiple myeloma, urothelial cancer, and renal cell carcinoma (ClinicalTrials.gov) (André et al., 2013; Hashinoff et al., 2012). Moreover, dovitinib has a safe and effective pharmacokinetic/pharmacodynamic profile (André et al., 2013; Sarker et al., 2008; Wang et al., 2013). Dovitinib was first designed and synthesized as a multi-targeted kinase inhibitor (Trudel et al., 2005). In many studies, dovitinib exerted anticancer activity and antiangiogenic activity through the inhibition of fibroblast growth factor receptor (FGFR) and platelet-derived growth factor receptor (PDGFR) (Lee et al., 2005). This inhibition effect led to an in-depth investigation of dovitinib as a new anticancer drug, but there currently are no reports evaluating bone homeostasis or skeletal diseases.

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Received 27 October, 2015; revised 16 February, 2016; accepted 25 February, 2016; published online 30 March, 2016

Keywords: ALP, BMP-2, Dovitinib, MAPK, osteoblast differentiation, Smad1/5/8

eISSN: 0219-1032

Table 1. Sequences of the primers used in this study

Target gene	Forward (5′-3′)	Reverse (5′-3′)
BMP-4	CCTGGTAACCGAATGCTGAT	AGCCGGTAAAGATCCCTCAT
BMP-7	CGATACCACCATCGGGAGTT	AAGGTCTCGTTGTCAAATCG
ALP	CACTCAAGGGAGAGGTCCAG	CTGCCCAAGAGAGAAACCTG
Osteocalcin	CTCTGTCTCTCTGACCTCAC	GTTTGGCTTTAGGGCAGCAC
GAPDH	GAGTCAACGGATTTGGTCGT	GATCTCGCTCCTGGAAGATG

In this study, we investigated whether dovitinib can regulate the BMP-2-mediated signaling pathway and exert anabolic effects in osteoblast differentiation. Our results indicated that dovitinib has potent stimulating effects in the induction of ALP and mRNA transcription of BMPs and osteogenic markers including ALP and osteocalcin (OCN) through activation of ERK1/2, p38 MAPKs, and phosphorylation of Smad1/5/8. In this study, we evaluated the effect of dovitinib as a potential anabolic agent in osteoblast differentiation of bi-potential mesenchymal precursor C2C12 cells.

MATERIALS AND METHODS

Materials

Dovitinib was purchased from Selleck Chemicals (USA) and recombinant human bone morphogenetic protein (BMP)-2 was purchased from R&D systems, Inc. (USA). All cell culture materials including fetal bovine serum (FBS), DMEM, and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) were purchased from HyClone (UK). Antibodies against p-ERK1/2, ERK1/2, p-p38, p38, and p-Smad1/5/8 were purchased from Cell Signaling Technology, Inc. (MA, USA). An antibody against actin-horse radish peroxidase (HRP) and Smad1/5/8 were purchased from Santa Cruz Biotechnology, Inc. (USA). The ALP staining kit, MEK inhibitor PD98059, and p38 inhibitor SB202190 were purchased from Sigma Aldrich (USA).

Cell culture and differentiation

Murine bi-potential mesenchymal precursor C2C12 (mouse myoblast cell line) cells were purchased from ATCC (USA) and maintained in DMEM containing 10% FBS and 1% antibiotics in a humidified atmosphere of 5% CO_2 at 37°C. For osteoblast differentiation, C2C12 cells were seeded in a 96-well plate for 72 h and then treated with BMP-2 (50 ng/ml) in culture media containing 5% FBS. The medium was changed every 3 days.

Cell viability assay

The C2C12 cells (4×10^3 cells/well) were seeded in a 96-well plate for 24 h. Cells were treated with BMP-2 (50 ng/ml) alone or combined with dovitinib in culture media containing 5% FBS for 72 h. Cell viability was assessed using the Cell Counting Kit (CCK)-8 assay kit (Dojindo Molecular Technologies, Inc., Japan) according to the manufacturer's instructions. The absorbance was measured using the Hidex sense beta plus microplate reader (HIDEX, Finland).

Alkaline phosphatase (ALP) activity assay

The C2C12 cells (4×10^3 cells/well) were seeded in a 96-well plate and incubated for 24 h. Cells were treated with BMP-2 (50 ng/ml) alone or combined with dovitinib in culture media containing 5% FBS for 72 h. After incubation, cells were fixed with 3.7% formaldehyde, rinsed with PBS, and stained with the ALP staining kit according to the manufacturer's instruction. The ALP-positive cells were visualized by phase-contrast light microscopy (Olympus Optical, Japan). The ALP activity was evaluated using the 1-Step™ PNPP Substrate solution (Thermo Fisher scientific, USA) and the absorbance was measured using the Hidex sense beta plus microplate reader (HIDEX, Finland) at 405 nm. Relative ALP activity was normalized by the absorbance of dovitinib only treated group.

Western blot analysis

After treatment with BMP-2 (50 ng/ml) alone or combined with dovitinib, cytoplasmic and nuclear fractions of lysates were prepared as described previously (Son et al., 2013). Lysates of cytoplasmic fractions and nuclear fractions were loaded on 8-15% polyacrylamide gels and transferred to nitrocellulose membranes. Specific primary antibodies were used to detect the expression of proteins. After incubation with HRP-conjugated secondary antibodies, membranes were developed using Signal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, US) with the LuminoGraph (Atto, USA) chemiluminescent imaging system.

Quantitative real-time PCR (qRT-PCR) analysis

The C2C12 cells were treated with BMP-2 (50 ng/ml) alone or combined with dovitinib in culture media containing 5% FBS for 72 h. Total RNA was isolated from C2C12 cells using TRIzol reagent (Life Technologies, USA), and cDNA was synthesized from 1 μg of total RNA using the *Enzynomics™* Reverse Transcriptase Kit (Enzynomics, Korea), according to the manufacturer's instructions. Quantitative real-time PCR was performed using the iQ™ SYBR® Green Supermix (Bio-Rad, USA) and the CFX96™ Real-Time System (Bio-Rad, USA). The primer sequences used in this study are shown in Table 1. All reactions were run in triplicate, and data were analyzed using the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001). The internal standard was GAPDH and statistical significance was determined with the Student's *t*-test using GAPDH-normalized $2^{-\Delta\Delta C}{}_T$ values (Livak and Schmittgen, 2001).

Statistical analysis

Data are represented as the mean ± SEM of at least three independent experiments. Significant differences were evaluated by the Student's *t*-test: $^{\#}P < 0.01$; $^{\#\#}P < 0.001$ (versus the control); $^{*}P < 0.05$; $^{**}P < 0.01$ (versus cells treated with BMP-2 alone); $^{\S}P < 0.05$; $^{\S\S}P < 0.01$ (versus cells treated with BMP-2 and dovitinib)

RESULTS

Dovitinib enhances BMP-2-induced osteoblast differentiation via the Smad1/5/8-mediated signaling pathway in C2C12 cells

First, to determine the optimal concentration of dovitinib (Fig. 1A)

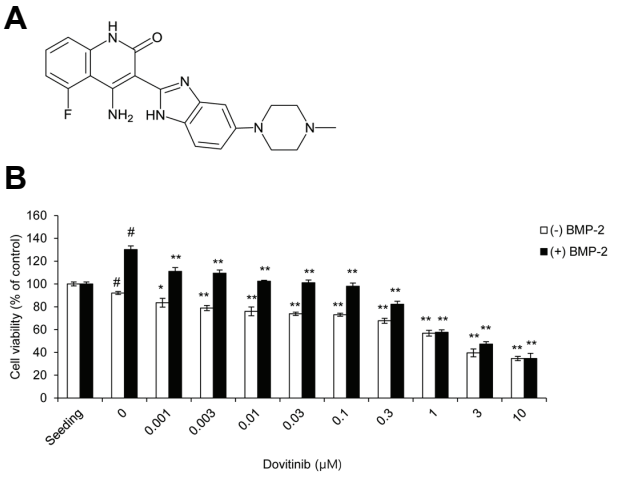

Fig. 1. The effect of dovitinib on the viability of BMP-2-stimultated C2C12 cells. (A) Chemical structure of dovitinib. (B) The C2C12 cells were treated with BMP-2 (50 ng/ml) alone or combined with dovitinib for 72 h. Cell viability was evaluated using the CCK-8 assay. Detailed experimental procedures are described in the Materials and Methods. All experiments were performed in triplicate.

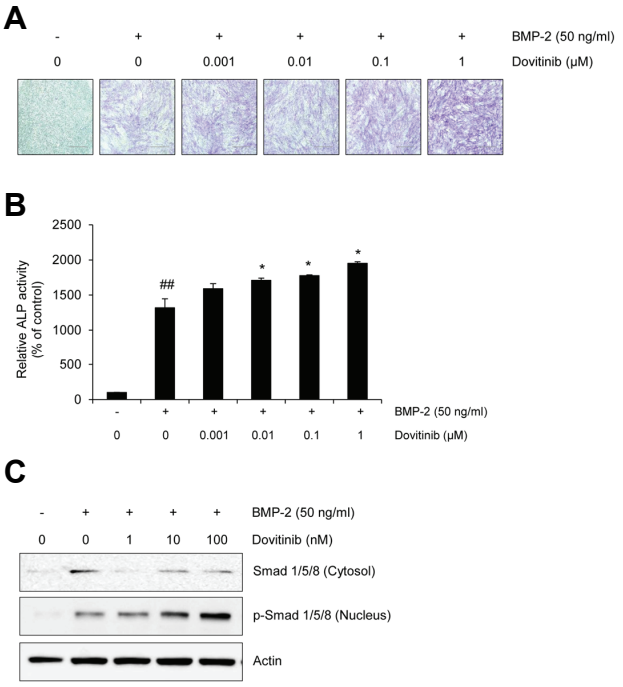

Fig. 2. Dovitinib stimulates the induction of ALP in BMP-2-induced osteoblast differentiation through activation of the Smad1/5/8-mediated signaling pathway. (A) The effect of dovitinib on BMP-2-induced osteoblast differentiation was detected by visualizing the induction of ALP in C2C12 cells. Scale bars represent 100 μm. (B) The activity of ALP, when cells were treated with BMP-2 alone or combined with dovitinib, was measured by a microplate reader. All experiments were performed in triplicate. (C) The C2C12 cells were treated with BMP-2 (50 ng/ml) alone or combined with dovitinib for 2 days. After 3 days, cells were lysed and fractionated to the cytosol and nuclear portions. The phosphorylation and translocation of Smad1/5/8 into the nucleus were measured by Western blot analysis. Actin was used as a loading control.

for investigating its effect on BMP-2-induced osteoblast differentiation, we assessed its cytotoxicity using a CCK-8 assay. As shown in Fig. 1B, dovitinib exhibited significant cytotoxicity at concentrations of more than 3 μM, but BMP-2 treated group did not show cytotoxicity at 0.1 μM. Therefore, non-cytotoxic dosages of dovitinib were used in the following experiments. Next, to evaluate the effect of dovitinib on BMP-2-induced osteoblast differentiation, we examined the expression of ALP, a representative marker for osteoblast differentiation, in C2C12 cells (Franceschi and Iyer, 1992) (Fig. 2A). Treatment with BMP-2 induced the expression of ALP and this was enhanced by dovitinib in a dose-dependent manner. This result was confirmed by an ALP activity assay. As shown in Fig. 2B, BMP-2 stimulated the activity of ALP and dovitinib enhanced BMP-2 induced ALP activity in a dose-dependent manner. Next, to investigate the mechanism of BMP-2-induced osteoblast differentiation, we examined the related signaling pathways. We found BMP-2 activates phosphorylation of Smad1/5/8, which then interacts with Smad4 and subsequently translocates into the nucleus (Cao and Chen, 2005). Dovitinib stimulated the BMP-2-mediated accumulation of phosphorylated Smad1/5/8 in a dose-dependent manner (Fig. 2C). However, dovitinib only did not show a stimulating effect on the ALP activity (Supplementary Fig. S1) and phosphorylation of Smad1/5/8 (Supplementary Fig. S2A). These results demonstrated that dovitinib enhances BMP-2-induced osteoblast differentiation through the activation of the Smad1/5/8-mediated signaling pathway.

Dovitinib stimulates the MAPK signaling pathway in BMP-2-induced osteoblast differentiation of C2C12 cells

Additionally, BMP-2 activates non-Smad signaling such as MAPKs, including ERK1/2, p38, and JNK, in the osteoblast differentiation process (Guicheux et al., 2003; Reilly et al., 2005). Dovitinib enhanced the phosphorylation of ERK1/2 and p38 (Fig. 3A), but JNK were not changed (data not shown). To confirm the relevance between BMP-2-induced osteoblast differentiation and MAPK activation, we detected the ALP activity in the presence of dovitinib and the MEK inhibitor PD98059 and p38 inhibitor SB202190. The number of ALP-positive cells was increased by dovitinib treatment, but PD98059 and SB202190 suppressed the ALP activity in a dose-dependent manner (Figs. 3B and 3C). However, dovitinib only did not show dose-dependent enhancing effect on the phosphorylation of ERK1/2 and p38 (Supplementary Fig. S2B). These results support that dovitinib exerts a stimulating effect on BMP-2-induced osteoblast differentiation of C2C12 cells through the activation of MAPK signaling pathways, as well as the Smad-mediated signaling pathway.

Dovitinib activates mRNA expression of osteoblast differentiation marker genes

During BMP-2-mediated osteoblast differentiation, BMP-2 induces transcription of endogenous BMPs in C2C12 cells. Approximately 20 BMPs of vertebrates originally have osteogenic activity, as well as various other physiological activities (De Biase and Capanna, 2005). Among different BMPs, BMP-2, BMP-4, BMP-5, BMP-6, and BMP-7 are most commonly discovered as osteogenic BMPs. We further investigated whether mRNA expression of these osteogenic BMPs and osteogenic

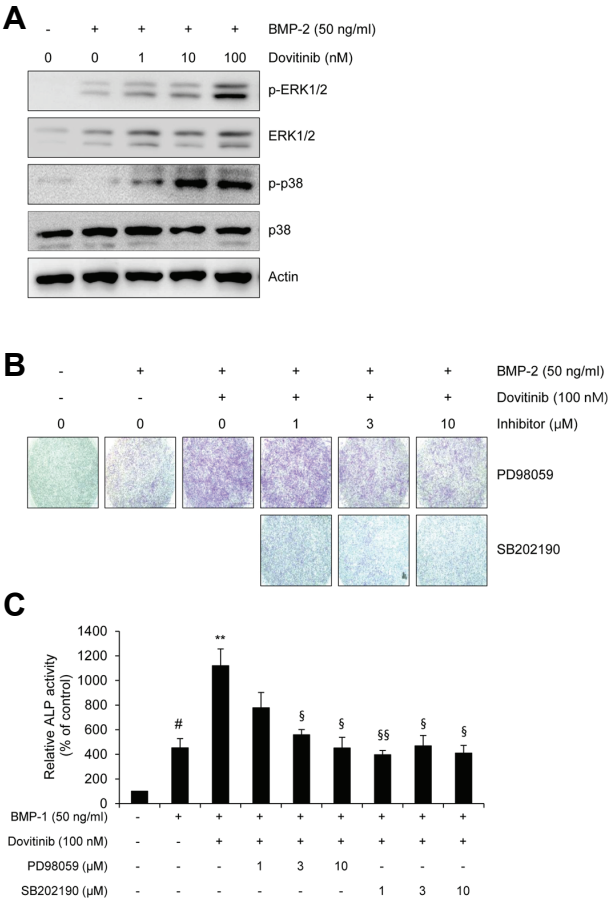

Fig. 3. Dovitinib activates MAPK signaling pathways in BMP-2-induced osteoblast differentiation. The C2C12 cells were treated with BMP-2 (50 ng/ml) ± dovitinib for 10 minutes. (A) The phosphorylation of ERK1/2 and p38 was measured by Western blot analysis. Actin was used as a loading control. (B, C) The C2C12 cells were treated with PD98059 or SB202190 in the presence of BMP-2 (50 ng/ml) and dovitinib (100 nM) for 3 days. The effect of inhibitors on the BMP-2-induced osteoblast differentiation was detected by the induction of ALP activity in C2C12 cells. Experiments were performed in triplicate. Scale bars represent 100 μm.

markers such as ALP, Osteocalcin (OCN), and Runx2 is involved in the osteogenic stimulating effect of dovitinib using real-time PCR analysis. Among osteogenic BMPs, the mRNA expression levels of BMP-4, BMP-7, ALP, and OCN were increased by the combination of BMP-2 and dovitinib in a dose-dependent manner (Fig. 4), but the expression levels of other BMPs and Runx2 were not changed (Supplementary Fig. S3). This result indicated that dovitinib enhances the mRNA transcriptional activity of osteogenic markers BMP-4, BMP-7, ALP, and OCN in the BMP-2-induced osteoblast differentiation process.

DISCUSSION

In this study, we investigated the osteogenic effect of dovitinib in the BMP-2-mediated osteoblast differentiation of C2C12 cells. Dovitinib (TKI-258) was originally developed as a multi-targeted receptor tyrosine kinase (RTK) inhibitor and has a potent

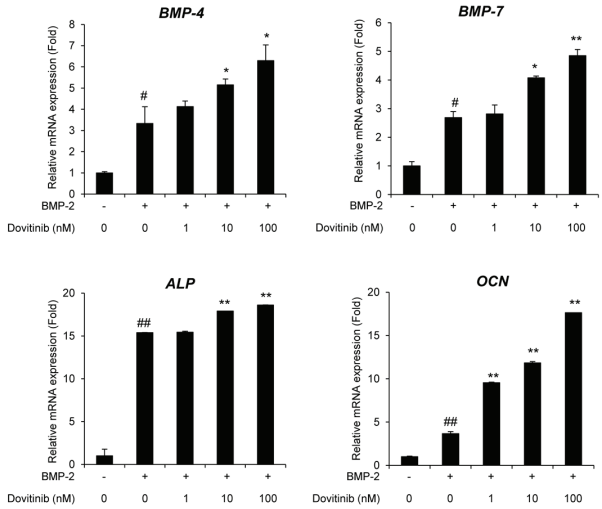

Fig. 4. Dovitinib enhances BMP-2-induced transcriptional activity of osteogenic BMPs. The C2C12 cells were treated with BMP-2 (50 ng/ml) alone or combined with dovitinib in culture media containing 5% FBS for 72 h. The mRNA expression levels of BMP-4, BMP-7, ALP, and osteocalcin were measured by qRT-PCR analysis, with GAPDH used as an internal control. Detailed experimental procedures are described in the "Materials and Methods".

inhibitory effect on the activities of the Class III (FLT3/c-Kit), Class IV (FGFR1/3), and Class V (VEGFR1-4) RTKs (Lopes de Menezes et al., 2005; Porta et al., 2015). In the previous reports, dovitinib has been used as an anti-cancer agent undergoing preclinical or clinical trials (Angevin et al., 2013; Eritja et al., 2014; Kim et al., 2011; Milowsky et al., 2014).

Until now, dovitinib showed anti-cancer activity in many types of human cancers, but there were no specific reports against bone disease. Although dovitinib shows wide ranges of IC50 in many types of cancer cells, this study demonstrated that relatively low concentrations of dovitinib could exert a stimulating effect on the BMP-2-induced osteoblast differentiation of C2C12 cells without significant cytotoxicity. As mentioned in previous reports, dovitinib showed anti-cancer activity through inhibition of FGFRs, PDGFRs, and VEGFRs (Angevin et al., 2013; Eritja et al., 2014; Kim et al., 2011; Milowsky et al., 2014). However, in C2C12 cells, BMP-2 increased the proliferation of C2C12 cells and dovitinib did not exert significant cytotoxicity. This result implies that relatively low concentrations of dovitinib could not inhibit the activity of FGFRs or PDGFRs to affect the cell viability of BMP-2-treated C2C12 cells. Although, osteoblast differentiation can be mediated by FGF/FGFR or PDGF/PDGFR signaling, multiple signaling networks including TGF-β/BMP, MAPK, Smad, Akt/mTOR, and Wnt signaling and transcription factors tightly regulate osteogenesis or bone formation (Caverzasio et al., 2013; Guicheux et al., 2003; Hipskind and Bilbe, 1998; Kobayashi et al., 2015; Marie et al., 2012; Rahman et al., 2015). Additionally, among the FGFs, FGF-8 suppresses BMP-2-induced osteoblast differentiation through the inhibition of ERK pathway (Katsuyama et al., 2015). This report also support our results that dovitinib might act as a FGF-8 inhibitor to activate BMP-2-induced osteoblast differentiation. In this regard, we suggest that dovitinib showed synergistic effect on osteoblast differentiation process of C2C12 cells via BMP-2-induced

MAPK and Smad signaling cascades.

The biopharmaceutical industry has rapidly grown and invested a lot of money for the R&D of new drugs; however, the number of approved new drugs has not kept pace with the increase in R&D spending. To reduce the R&D expenditures and increase the success rate, many companies apply various strategies in drug development. Of these cost-reducing approaches, drug repositioning is a new method to search existing developed drugs (Ashburn and Thor, 2004; Chong and Sullivan, 2007). Drug repositioning allows companies to develop new drugs through changing the scope of the original medical indication, leading to faster processing, while reducing the risks of failure in drug discovery and development. In a previous report, we investigated the potential of the anti-cancer drug CX-4945 as a regulator of osteoblast and osteoclast differentiation (Son et al., 2013). This report provided a possibility that anti-cancer drugs in clinical trials or FDA approved can be used for other indications.

Based on the concept of drug repositioning, we also evaluated the effect of dovitinib on BMP-2-induced osteoblast differentiation. Recent reports provide new information about therapeutic applications of anti-cancer agents for the treatment of another disease or synergistic effects with exiting drugs (Beeharry et al., 2014; Bharadwaj et al., 2015; Hanusova et al., 2015; Pemovska et al., 2015; Song et al., 2015). Published reports mainly represent that dovitinib exerts anti-cancer activity through inhibition of FGFRs, PDGFRs, and VEGFRs in various types of cancers (Angevin et al., 2013; Eritja et al., 2014; Kim et al., 2011; Milowsky et al., 2014). Targeted therapies, associated with specific types of diseases, have improved in drug discovery and development process. However, increasing resistance rates of tumor cells against targeted chemotherapies induce fail in the development of new drugs. Compared with the previous reports, our study did not show dovitinib induced cytotoxicity and inhibitory activity on the expression of FGFRs or PDGFRs in C2C12 cells. Although the biochemical activity of dovitinib was unpredictable and not consistent with published results, our results represent a novel effect of dovitinib in BMP-2-mediated signaling. Our key findings indicated that BMP-2 induces osteogenic metabolisms in C2C12 cells through activation of Smad1/5/8 and MAPK mediated signaling and the transcriptional activity of osteogenic BMPs, such as BMP-4 and BMP-7. This process of BMP-2-induced osteoblast differentiation was enhanced by combined treatment with dovitinib, without a significant cytotoxic effect.

Bone homeostasis is regulated by the ongoing balance between osteoclastic bone resorption and osteoblastic bone formation (Harada and Rodan, 2003). The physiological imbalance in bone remodeling between the differentiation of osteoblasts and osteoclasts results in the bone mass decrease and skeletal disorders such as osteoporosis (Goltzman, 2002). While most of approved drugs regulating bone metabolism mainly suppresses bone resorption through the inhibition of osteoclast differentiation, development of anabolic drugs that induces osteoblast differentiation is not sufficient yet (Goltzman, 2002). In this regard, our study provides a potent opportunity that tyrosine kinase inhibitors can be used for the treatment of osteoporosis. But in fact, we did not evaluate another effect of dovitinib yet whether it has enhancing activity on *in vivo* bone formation (or mineralization) and inhibitory activity on the osteoclast differentiation. Since bone homeostasis is regulated by osteoblast differentiation and osteoclast differentiation, we thought that further investigations for the identification of dovitinib activity for bone homeostasis will be needed in future.

Here, we showed the potential for an existing drug through repositioning. This could help reduce the time to discover a novel drug candidate and the optimization of pharmacological characteristics. This study utilized an efficient method for the development of new drugs that has a cost-saving effect on future R&D processes.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

ACKNOWLEDGMENTS

This research was supported by Eulji University in 2014 and the Basic Science Research Program through the National Research Foundation (NRF) of Korea, funded by the Ministry of Science, ICT & Future Planning (NRF-2014R1A1A1002349).

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