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Protein kinase inhibitor SU6668 attenuates positive regulation of Gli proteins in cancer and multipotent progenitor cells

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ABSTRACT

Observations that Glioma-associated transcription factors Gli1 and Gli2 (Gli1/2), executers of the Sonic Hedgehog (Shh) signaling pathway and targets of the Transforming Growth Factor β (TGF-β) signaling axis, are involved in numerous developmental and pathological processes unveil them as attractive pharmaceutical targets. Unc-51-like serine/threonine kinase Ulk3 has been suggested to play kinase activity dependent and independent roles in the control of Gli proteins in the context of the Shh signaling pathway. This study aimed at investigating whether the mechanism of generation of Gli1/2 transcriptional activators has similarities regardless of the signaling cascade evoking their activation. We also elucidate further the role of Ulk3 kinase in regulation of Gli1/2 proteins and examine SU6668 as an inhibitor of Ulk3 catalytic activity and a compound targeting Gli1/2 proteins in different cell-based experimental models. Here we demonstrate that Ulk3 is required not only for maintenance of basal levels of Gli1/2 proteins but also for TGF-β or Shh dependent activation of endogenous Gli1/2 proteins in human adipose tissue derived multipotent stromal cells (ASCs) and mouse immortalized progenitor cells, respectively. We show that cultured ASCs possess the functional Shh signaling axis and differentiate towards osteoblasts in response to Shh. Also, we demonstrate that similarly to *Ulk3* RNAi, SU6668 prevents *de novo* expression of Gli1/2 proteins and antagonizes the Gli-dependent activation of the gene expression programs induced by either Shh or TGF-β. Our data suggest SU6668 as an efficient inhibitor of Ulk3 kinase allowing manipulation of the Gli-dependent transcriptional outcome.

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1. Introduction

Misregulation of cellular signaling pathways, that are important in embryonic development and maintaining adult homeostasis, leads to inherited as well as sporadic diseases. One of such pathways, where a clear correlation between abnormal pathway activation and disease progression has been observed, is the Sonic Hedgehog (Shh) signaling pathway [1]. Disruption or misregulation of the Shh pathway results in various developmental abnormalities including holoprosencephaly, Pallister–Hall syndrome, Gorlin syndrome, Greig cephalopolysyndactyly, Rubinstein–Taybi syndrome and different types of cancer (basal cell carcinoma, medulloblastoma, glioma, breast, pancreatic, prostate cancers and more). Similarly important is the TGF-β signaling pathway, with its role in various types of cancer, vascular diseases and fibrosis [2,3].

The Shh pathway utilizes Gli proteins (Gli1, 2, 3) as transcriptional effectors. According to the widely accepted paradigm, differentiated regulation of Gli proteins occurs in an Hh signal dependent way. In the absence of the ligand, *Gli1* is transcriptionally repressed; full-length Gli2 and Gli3 (Gli2/3FL) proteins are bound by a putative cytoplasmic complex called Hedgehog signaling complex (HSC). HSC may consist of a number of proteins including Suppressor of Fused (Sufu), kinesin-like protein Kif7, unc-51-like kinase 3 (Ulk3), and Gli2/3FL transcription factors [4–8]. Gli2/3FL proteins bound by HSC are phosphorylated for degradation and processing into the transcriptional repressor forms (Gli2/3REP) [9–12]. Activation of Shh pathway leads to rapid stabilization and activation of Gli2/3FL probably through yet uncharacterized phosphorylation events, their relocation to the nucleus and up-regulation of their target genes, for instance *Ptch1* and self-amplifying *Gli1* [7,12]. *Gli2* has been also suggested as a transcriptional target of

Abbreviations: AP, alkaline phosphatase; ASCs, adipose tissue derived stromal cells; GliACT, transcriptional activator form of Gli proteins; GliFL, full-length Gli proteins; Ptch, patched; RNAi, RNA interference; Shh, Sonic Hedgehog; Smo, Smoothened; shRNA, short hairpin RNA; siRNA, small interfering RNA; Sufu, Suppressor of Fused; TGF-β, Transforming Growth Factor β; Ulk3, unc-51-like kinase 3; WB, Western blot; HSC, Hedgehog signaling complex; qRT-PCR, quantitative real-time PCR

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Shh signaling in mouse CNS during embryonic development [13]. Although both proteins, Gli2 and Gli3, may be involved in primary mediation of Shh activities, the role of Gli2 activator is more crucial, whereas Gli3 acts mainly as a transcriptional repressor [14–16].

Gli proteins are known to be regulated independently of Hh ligands on both transcriptional and post-translational levels. Mouse Gli1 protein can be activated *via* Erk1/2 kinases, and *Gli2* is shown to be up-regulated in the epidermis of mice over-expressing TGF-β1 [17,18]. Also, the TGF-β1/SMAD3/TCF4/β-catenin signaling axis controls human *GLI2*, and consequently *GLI1*, expression [18,19]. Regulation of Gli2 in bone metastases and tumor-induced osteolysis also occurs independently of the canonical Shh pathway [20].

Most of the small molecule inhibitors of the Shh pathway identified so far target trans-membrane SMO oncoprotein responsible for triggering the intracellular signaling cascade following the ligand binding to another trans-membrane protein PTCH1. In addition, several inhibitors of GLI proteins and Shh itself have been identified (reviewed in Ref. [21]). However, no inhibitors targeting the activity of either HSC complex or protein kinases required for activation of GLI proteins have been reported. The latter might be effective not only in Shh pathway inhibition, but also in alleviating TGF-β/GLI dependent signaling events.

SU6668 ((Z)-5-[(1,2-dihydro-2-oxo-3H-indol-3-ylidene)methyl]-2,4-dimethyl-1H-pyrrole-3-propanoic acid; TSU68) has been shown to inhibit several tyrosine and serine/threonine protein kinases in an ATP competitive manner [22,23]. The affinity chromatography experiment using a resin covalently bound with SU6668 has revealed that additionally to the previously known targets, SU6668 is capable to bind a number of other protein kinases including ULK3 [23]. We have recently identified Ulk3 as an important Gli regulator. However, a mechanism of regulation of the *Ulk3* gene and possible interrelations between endogenous Ulk3 and Gli proteins remains unclear.

Adipose tissue derived stromal cells (ASCs, also known as mesenchymal stem or progenitor cells) have been extensively investigated during the last decade. These heterogeneous cell populations have evoked a great interest for regenerative medicine due to their non-immunogenic phenotype and capacity to respond to appropriate inducers by increasing expression of markers specific for different mesodermal lineages, such as adipocytes, chondrocytes or osteoblasts [24–26]. The Shh signaling pathway has not been extensively characterized in human ASCs, although one research group has reported that activation of Shh signaling negatively regulates differentiation of ASCs towards osteoblasts triggered by osteogenic cocktail [27]. However, these studies were conducted using Shh-conditional media or SMO agonists added to ASCs in the presence of osteogenic inductors, whereas influence of Shh itself on native ASCs has not been analyzed. In contrast, the osteogenic capacity of Shh in mouse ASCs and C3H10T1/2 is well documented [28,29]. Differentiation of osteoprogenitors occurs under control of Runx2, a factor essential for bone formation and skeletal development [30,31]. *Runx2* is expressed from two alternative promoters at least in two isoforms. Both *Runx2* isoforms are expressed in osteoblasts and participate in differentiation [30,32]. Osteogenesis is characterized by expression of lineage-specific proteins, such as early markers Sp7 and alkaline phosphatase (AP) and late markers osteopontin (Opn) and osteocalcin (Bglap) [29,33,34]. Gli2/3 proteins as mediators of Hh activities participate not only in positive regulation of osteogenesis but also in early chondrogenesis in mice [35–37], whereas adipogenesis is inhibited by activation of the Shh signaling [28,38]. Expression and activities of GLI1/2 proteins in human ASC tri-lineage differentiation programs have not been described.

The current study aims to investigate whether the mechanism of activation of Gli1 and Gli2 (Gli1/2) proteins has similarities regardless of signaling pathway evoking that. In answering this question, we examine SU6668 as a small molecule inhibitor able to prevent activation of Gli1/2 proteins in both Shh and TGF-β signaling pathways in an Ulk3 dependent manner. Finally, we provide novel data in the field of stem cell biology relating to possible roles of Shh signaling and GLI1/2 proteins in ASC differentiation programs.

2. Materials and methods

2.1. Ethic statement

Donors of the primary cells provided written informed consent to participate in this study in accordance with the approval for research with human materials No 159 from 14th of February, 2013 by Ethics Committee of National Institute for Health Development, Tallinn, Estonia.

2.2. Proteins and chemicals

FLAG-tagged ULK3 and GLI2, Shh and His-tagged ULK3-Ubi proteins were purified as previously described [6,39,40]. SU6668 was dissolved in DMSO (both from Sigma-Aldrich, Steinheim, Germany) and stored at −70 °C prior use. Human recombinant TGF-β1 and TGF-β3 were purchased from PeproTech (Rock Hill, NJ, USA). Human insulin, dexamethasone (DEX), IBMX, indomethacin and ascorbate-2-phosphate were purchased from Sigma-Aldrich, while ITS supplement was purchased from Gibco, Invitrogen (Carlsbad, CA, USA).

2.3. Cell culture

Peripheral blood mononuclear cells (PBMCs) and human ASCs were isolated and characterized as previously described [41]. The donors of primary cells are described in Supplementary Table 1. Freshly isolated PBMCs were frozen in D-MEM containing 1 g/L glucose (Gibco), supplemented with 50% of Fetal Bovine Serum (FBS) (PAA, Pasching, Austria) inactivated at 56 °C for 30 min (HI-FBS) and 10% DMSO and stored in liquid nitrogen prior to use. The C3H10T1/2 cell line was a generous gift from Prof. Rune Toftgård's lab (Centre for Nutrition and Toxicology, Karolinska Institute, Sweden). The MDA-MB-231 and 3T3-L1 cell lines were purchased from ATCC. MDA-MB-231 and 3T3-L1 cells were propagated in D-MEM containing 4.5 g/L glucose (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin mix (PEST) (Gibco). C3H10T1/2 and ASCs were propagated in D-MEM containing 1 g/L glucose supplemented with 10% of HI-FBS and 1% PEST. The cells were grown at 37 °C and 5% CO_2. All treatments of the cells except inductions of ASCs towards chondrocytes were conducted in the respective base growth medium supplemented with 3% of FBS or HI-FBS, 1% PEST, 12 nM Shh or 10 ng/ml of TGF-β3 and different concentrations of SU6668, if indicated. Adipogenic differentiation was induced by 10 μg/ml of human insulin, 1 μM dexamethasone, 0.5 mM IBMX and 10 μM indomethacin. Chondrogenic differentiation was conducted in DMEM-high glucose containing 10 ng/ml of TGF-β1, 1× ITS supplement, 100 μM ascorbate-2-phosphate, 1 μM DEX and 1% PEST. Media were replenished every 2 d.

2.4. Alkaline phosphatase activity

C2H10T1/2 cells were washed with PBS and lysed in Lysis Solution (Tropix, Bedford MA, USA). Alkaline phosphatase (AP) activity was measured using CSPD substrate with Sapphire-II™ Enhancer (Invitrogen) and Genios Pro combined fluoro-and luminometer (Tecan Group Ltd., Männedorf, Switzerland). Total protein concentrations were measured using BCA Protein Assay kit (Pierce Biotechnology Inc., Rockford, IL, USA) and used for normalization of AP activity values.

2.5. Over-expression studies

Synthetic Negative siRNA (Silencer Negative Control 2, Neg. siRNA), Silencer® Select siRNAs S89965 and S89966 against mouse *Ulk3* and S24886 and S24887 against human *ULK3* were purchased from Ambion (Austin, TX, USA). Synthetic siRNAs were delivered to cells using

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