Mol. Cells *29*, 63-69, January 31, 2010
DOI/10.1007/s10059-010-0017-z

Molecules and Cells

Downregulation of Wnt-Mediated ROS Generation Is Causally Implicated in Leprechaunism

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Although mutations in the insulin receptor have been causally implicated with leprechaunsim, the full pathophysiology of the syndrome cannot be accounted for by malfunction of this gene alone. We sought to characterize a connection between Wnt-mediated cell signaling and the production of reactive oxygen species (ROS) which revealed a novel mechanistic basis for understanding the pathogenesis of leprechaunism. To identify candidate genes involved in this process, a PCR-based subtractive hybridization was performed. Candidate genes were examined for interaction with the Wnt signaling pathway and ROS generation. We found that Dickkopf 1 (Dkk1), a Wnt inhibitor, is overexpressed in skin fibroblast cells derived from three leprechaunism patients and that the cells showed an impaired response to Wnt2 in terms of β-catenin-Tcf activation. Knockdown of Dkk1 in the patient cell lines rescued Wnt2-mediated Tcf activation. Concerted action of Wnt2 and knockdown of Dkk1 resulted in enhanced Nox4 expression and PDGF-induced ROS generation compared to parental patient cells. Furthermore, we found that NFATc2 was activated in response to Wnt2 stimulation and directly activates Nox4 expression. These data show a crosstalk between Wnt and ROS pathways which in turn provides new mechanistic insights at the molecular level into the pathogenesis of leprechaunism.

INTRODUCTION

Leprechaunism is a rare congenital syndrome characterized by various dysmorphic features and neonatal growth retardation (Longo et al., 1994; 2002; Musso et al., 2004; Takahashi et al., 1997). Several clinical studies have indicated that mutations in the insulin receptor, which lead to metabolic abnormalities including severe insulin resistance, represent the major risk factor for developing this syndrome. However, such insulin receptor based model cannot fully explain the range of clinical manifestations. For example, it was shown that skin fibroblasts from patients with leprechaunism failed to respond to various growth factors in addition to insulin. We previously demonstrated that NADPH oxidase 4 (Nox4) downregulation in patient cells resulted in impairment in generating reactive oxygen species (ROS) which in turn led to aberrant response to growth factors (Park et al., 2005). Compared to normal cells, significantly higher phosphatase activity resulting from defect in ROS generation was apparent in patient cells. Consequently, growth factors induced lower level tyrosine phosphorylation of cytosolic proteins (Park et al., 2005; Rhee et al., 2000). Importantly, ectopic expression of Nox4 into patient fibroblasts restored PDGF-dependent ROS generation and tyrosine phosphorylation (Park et al., 2005). These data provided novel mechanistic insights into dissecting the unexplained aberrant growth profile in leprechaunism.

The role of phagocytic NADPH oxidase (gp91*phox*, Nox2) in host defense had been well characterized (Babior, 2004; Geiszt and Leto, 2004). It was after the discovery of NADPH oxidase 1 (Nox1) as a homologue of gp91phox from non-phagocytic colon cells, that the identification of additional oxidases and their functional characterization in various cell types followed in rapid succession (Lambeth et al., 2007; Suh et al., 1999). The NADPH oxidase family now consists of seven human homologues, Nox1, gp91*phox* (Nox2), Nox3, Nox4, Nox5, Duox1, and Duox2. Activation of Nox isozymes requires various cytsolic proteins and unique regulatory mechanisms. For Nox2, the activation is initiated with phosphorylation of p47phox which recruits p67phox and Rac protein to form a multiprotein complex. Subsequent translocation of the complex to membrane-bound gp91phox (Nox2) initiates catalytic transfer of an electron to oxygen to generate superoxide anion (Babior, 2004; Geiszt and Leto, 2004; Lambeth et al., 2007). Regulation of non-phagocytic Nox1 is similar to that of Nox2 (Lambeth et al., 2007; Sumimoto, 2008). NoxO1, a homolog of p47phox, interacts with p22phox through SH3 domain and with NoxA1, a homolog of p67phox, via its proline-rich carboxy-terminal region, and NoxA1 interacts with GTP-Rac1. The complex of Nox1-NoxO1-NoxA1-Rac1 catalyzes the reaction of superoxide anion generation.

In contrast to Nox1 and Nox2, Nox4 isozyme does not require cytosolic accessory proteins and appears to be constitutively active (Geiszt et al., 2000; Lambeth et al., 2007). Although a recent report has suggested that membrane protein p22phox is required for the Nox4 activity (Martyn et al., 2006), multiple lines of evidence indicate that the level of ROS genera-

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Received July 16, 2009; revised October 8, 2009; accepted October 14, 2009; published online December 18, 2009

Keywords: dickkopf, leprechaunism, NADPH oxidase, NF-AT, Wnt

tion by Nox4 is likely determined by the expression level of Nox4 itself (Geiszt et al., 2000; Lambeth et al., 2007; Mahadev et al., 2004; Park et al., 2004). Nox4 was initially identified from kidney but it was subsequently shown to be widely expressed in various cell types, including neuronal cells, smooth muscle cells, and adipocytes. Numerous stimuli including growth factors induce Nox4 expression in diverse cell types as a part of signaling process (Higashi et al., 2003; Hwang et al., 2003; Moe et al., 2006; Pedruzzi et al., 2004; Sturrock et al., 2006). Of note, Nox4 plays an important role in the insulin-mediated signaling cascade resulting in increased IRS-1 tyrosine phosphorylation and glucose uptake (Mahadev et al., 2004).

Wnt proteins are a group of secreted molecules implicated in multiple aspects of vertebrate development and homeostasis (Moon, 2005; Niehrs, 2006). Wnt proteins bind to the family of receptor proteins, Frizzled (Fz) which leads to stabilization of a downstream protein β-catennin through inactivation of glycogen synthase kinase 3 (GSK3). β-catennin protein subsequently accumulates in the nucleus and interacts with LEF/TCF transcriptional factors culminating in activation of target genes. Other proteins participate in this signaling pathway. For example, lipoprotein receptor-related protein 5/6 (LRP5/6) serves as a co-receptor for Wnt by forming a ternary complex of Wnt and Fz proteins to regulate their signaling. Dickkopf-1 (Dkk1) belongs to a family of secreted proteins consisting of 4 members (Dkk1, 2, 3, 4) in vertebrates (Bafico et al., 2001; Glinka et al., 1998; Guder et al., 2006; Jin, 2008; Kawano and Kypta, 2003). Dkk1 modulates Wnt signaling by competing with Wnt for LRP5/6 binding and thereby blocking Wnt signaling (Bafico et al., 2001; Glinka et al., 1998; Guder et al., 2006; Jin, 2008; Kawano and Kypta, 2003).

A previous study reported identification of genes responsible for growth retardation in leprechaunism patients using microarray analysis (Melis et al., 2003). The result showed that approximately 20% of genes involved in cell growth and differentiation were downregulated in the leprechaunism cells. However, functional significance of up- or down-regulation of various genes in leprechaunism cells has yet to be determined in detail. We carried out a subtractive hybridization screen to identify candidate genes responsible for growth retardation in leprechaunism cells and report a novel connection between ROS generation and Wnt-mediated cell signaling that has implications in the pathogenesis of leprechaunism.

MATERIALS AND METHODS

Cell culture

Primary skin fibroblasts obtained from skin biopsies of normal individuals and patients were maintained in high glucose DMEM supplemented with 20% fetal bovine serum and 1% antibiotics at 37°C in a 5% CO_2 and air atmosphere. IRB approved number (file number: 2009-08-082) was obtained from IRB of Samsung Hospital.

Preparation of conditioned medium

To prepare conditioned medium (CM) containing Wnt2, NIH3T3 cells were seeded in 6-well plates at 2×10^5 cells/well and transfected with 2 μg of Wnt2 cDNA/well using Superfect reagents (Qiagen, USA). CM was collected 24 h after transfection, centrifuged and filtered prior to application.

PCR-based cDNA subtraction and southern blot analysis

PCR-based cDNA subtraction was performed as described in the PCR-select cDNA subtraction kit (Clontech, USA). Leprechaunism patient skin fibroblasts were used as the tester, while normal skin fibroblasts were used as the driver. The subtracted cDNAs were inserted into a pGEM-T Easy Vector (Promega, USA) and transformed into *E. coli* DH5α to generate the patient cell-specific cDNA library. Candidate gene fragments were resolved on a 1.2% agarose gel, transferred simultaneously to two nylon membranes and subjected to a Southern blot analysis with ^{32}P-labeled total cDNA preparations from normal and leprechaunism patient cells as probes.

Assay for the intracellular H_2O_2 production

Intracellular production of H_2O_2 was detected after stimulation of cells with PDGF (100 ng/ml) in serum-free Dulbeccos's modified Eagle's medium as previously described (Park et al., 2004). Dishes of confluent cells were washed with Hanks' balanced salt solution and incubated for 5 min in the dark at 37°C with the same solution containing 5 μM 2′,7′-dichlorofluorescin diacetate (DCF-DA; Molecular Probes, USA). DCF-DA is oxidized by H_2O_2 to the highly fluorescent 2′,7′-dichlorofluorescein (DCF). The cells were examined with a laser-scanning confocal microscope (model LSM 510, Carl Zeiss, Germany) equipped with an argon laser tuned to the excitation wavelength of 488 nm and with an LP505 emission filter (515-540 nm). Images were digitized and stored at a resolution of 512 by 512 pixels. Five groups of cells were randomly selected from each sample, and the mean relative fluorescence intensity for each group of cells was measured with a Zeiss vision system (LSM510, version 2.3) and then average value of all groups was determined. All experiments were repeated at least five times.

Real time PCR based analysis

Total RNA was isolated from the indicated cultured cells, reverse-transcribed, and quantified by real-time PCR. We purchased Dkk1, Nox4, and GAPDH primers from Applied biosystems (USA). TaqMan analysis was performed using an ABI prism 7700 according to manufacturer's instruction. Dkk1 and Nox4 values are expressed as copy numbers per 10^{11} copies of GAPDH measured in the same sample. ($\times 10^7/10^{11}$ copies GAPDH). Data are means ± SE of values from 5 independent experiments.

Small interfering RNA of *Dkk1*

A sequence of 19 nucleotide residues in length (GATGAGTACTGCGCTAGTC) specific to the human Dkk1 cDNA (nucleotide residues, 419-437) and another as the control scrambled sequence (CTAAGGGAGTCGTCATGCT) were selected for synthesis of siRNA (Brummelkamp et al., 2002). pSUPER vector for siRNA was purchased from Oligoengine (USA). The depletion of endogenous human *Dkk1* gene by siRNA was confirmed by RT-PCR.

Reporter assay

To determine the transcriptional activity of β-catenin, transient transfections were performed with Fugene transfection reagent for the TOP-FLASH plasmid containing three copies of the β-catenin/T-cell factor (TCF)-binding sites upstream of a minimal herpesvirus thymidine kinase promoter driving the firefly luciferase expression (Roche Diagnostics, USA). Thirty-thousand cells were plated in 24-well plates 30 minutes before the addition of a mixture containing 20 μl of serum-free DMEM, 0.6 μl of Fugene, 0.4 μg of the TOP-FLASH reporter construct, and 0.8 μg of the Renilla luciferase vector phRG-TK (Promega). Cells were subjected to stimulation with Wnt2 for 24 h and lysed in 50 μl of lysis buffer, and the luciferase activity was determined with a luminometer using the Dual Luciferase Assay system (Promega) using 20 μl of lysates. The firefly luciferase activity

was normalized to the activity of the renilla luciferase. The activity of the TOP-FLASH reporter construct was expressed as normalized relative luminoscence units (RLUs).

Nuclear protein extraction and electrophoretic mobility shift assay

To prepare nuclear extracts, cells were rinsed with and scraped into phosphate-buffered saline and then isolated by centrifugation at 1500 × *g* for 5 min. The resulting pellet was resuspended in a solution containing 10 mM HEPES-KOH (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT) and 0.5 mM PMSF (phenylmethylsulfonyl fluoride). Nonidet P-40 was then added to the cells on ice to a final concentration of 0.5%, and the tube was mixed vigorously for 30 s. The nuclei were separated by centrifugation and extracted with a solution containing 50 mM HEPES-KOH (pH 7.9), 420 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 20% glycerol and 0.5 mM PMSF. Electrophoretic mobility shift assays for NFATc was performed using 10 μg nuclear extract. Double stranded oligonucleotide probes were labeled by using [α-^{32}P] dATP (> 6,000 Ci/mmol; PerkinElmer Life And Analytical Sciences, Inc) and DNA polymerase. Each radioactive probe was incubated with 10 μg of nuclear proteins in 20 μl of 20 mM Tris-HCl (pH 7.5) containing 1 μg of poly(dI-dC), 50 mM NaCl, 0.1 mM DTT, and 10% glycerol at room temperature. The following oligonucleotides were used as probes: NFATc 5′-CGCCCAAAGAGGAAAATTTGTTTCATA-3′, NFATc mutant 5′-CGCCCAAAGCTTAAAATTTGTTTCATA-3′ (mutation site underlined) (Liu et al. (2004)). For the supershift assays, 2 μg of the appropriate Abs (Santa Cruz Biotechnology, USA) were added to the reaction mixtures (30 min at room temperature) before addition of the labeled probe.

Northern blot analysis

Aliquots of mRNA (1 μg) from normal or patient cells were used for northern blot analysis as described previously (Sambrook and Russell, 2001). Isolated mRNA was separated on a formaldehyde-agarose gel and transferred to a nylon membrane (Hybond; Amersham Biosciences, USA). Hybridization was performed with Dkk1 or LRP6 or GAPDH cDNA probes amplified by PCR and labeled by [^{32}P] dCTP (PerkinElmer Life And Analytical Sciences, Inc., USA). The histogram represents means ± SE (n = 3) of the relative intensity of mRNA isolated from patient cells relative to that of mRNA isolated from normal cells. The symbol, *, denotes that two groups are significantly different from each other at $p < 0.005$.

Statistical analysis

Data are represented as the mean ± SE. The statistical analyses were performed by unpaired Student's *t*-test. Differences were considered significant when $p < 0.05$.

RESULTS

Overexpression of Dickkopf 1 in leprechaunism

Although mutations in insulin receptor gene have been found in most leprechaunism patients, the complex phenotypes featuring developmental defects and growth retardation cannot be fully explained by such genetic alterations only (Longo et al., 1994; 2002; Musso et al., 2004; Takahashi et al., 1997). In order to identify additional candidate genes involved in molecular pathogenesis of leprechaunism, we carried out a PCR-based subtractive hybridization using fibroblast cells from leprechaunism patients and normal individuals (Supplementary Tables 1 and 2). *Dickkopf1* (*Dkk1*) gene was found to be upregulated in patient fibroblasts (Supplementary Table 1 and Fig. 1). Quantitative real-time RT-PCR assays confirmed that the expression level of Dkk1 in fibroblasts from three patients was higher than that of normal cells (Table 1). The expression of LRP6, a Wnt co-receptor (Fig. 1) and Wif1 and SFRP1, two secreted Wnt inhibitors did not show any difference between leprechaunism patient cells and normal cells (data not shown).

Table 1. The expression of Dkk1 in Leprechaunism

Normal	RMS	NZ	Mt.Sinai
271 ± 6.8	1644.2 ± 113.8	675.9 ± 52.2	728.5 ± 54.1

Total RNA was isolated from the indicated cultured cells, reverse-transcribed, and quantified by real-time PCR. DKK1 values are expressed as copy numbers per 10^{11} copies of GAPDH measured in the same sample. ($\times 10^7/10^{11}$ copies GAPDH). Data are means SE of values from independent experiment (n = 5).

It has been reported that Dkk1 is expressed in a spatiotemporally specific manner in various neural and mesenchymal tissues during vertebrate embryogenesis and acts as a secreted Wnt inhibitor by interfering with Wnt-LRP5/6 interaction (Bafico et al., 2001; Guder et al., 2006; Jin, 2008; Kawano and Kypta, 2003). Thus, in order to determine the significance of elevated expression of Dkk1, we examined Wnt-dependent β-catenin activation in patient cells. The cells were transfected with a TOP-FLASH reporter designed to measure β-catenin/TCF activity. Incubation of cells (RMS, Mt.Sinai, and NZ-1) from three patients with tissue culture medium containing Wnt2 resulted in a minimal β-catenin/TCF activation while cells from normal individuals showed a robust response indicating that patient cells expressing the elevated level of Dkk1 could not respond to Wnt signaling (Fig. 2A). To verify that this was due to Dkk1 overexpression in patient, we subjected RMS fibroblast cells to a transient transfection with pSuper-Dkk1 encoding a small interfering RNA (siRNA) specifically targeting *Dkk1* mRNA. The cells transfected with the pSuper-Dkk1 exhibited a marked reduction in the endogenous Dkk1 transcript level compared with cells transfected with the scramble pSuper vector (Fig. 2C). Skin fibroblast cells from RMS patient transfected with the pSuper-Dkk1 vector showed β-catenin activation in response to Wnt2 stimulation, whereas cells transfected with scramble pSuper exhibited an impaired Wnt-dependent β-catenin/TCF activation. These results demonstrate that overexpression of Dkk1 in patient fibroblasts is the main reason for the poor response to Wnt signaling (Fig. 2B).

Wnt-dependent Nox4 expression through NF-AT

We have previously shown an impairment of reactive oxygen species (ROS) generation in cells from leprechaunism patients. We also showed that this was due to downregulation of Nox4 in these cells (Park et al., 2005). We asked if Wnt signaling is coupled to ROS signaling via regulation of Nox4 expression. In the promoter region 5′ to Nox4 gene, two AP-1 and two NF-AT binding sites but no TCF binding sites were discovered, (Fig. 3A) suggesting that expression of Nox4 isozyme is potentially regulated by the non-canonical Wnt pathway. It has been established that AP1 and NFAT activities are regulated by non-canonical Wnt pathway in several cellular and developmental contexts (Pandur et al., 2002). We found that Wnt stimulation led to significant activation of NF-AT but not AP-1 in normal skin fibroblast cells (Fig. 3B). Importantly, we observed that the extent of NFAT activation by Wnt stimulation was significantly lower in all three patient fibroblast cell lines compared to normal

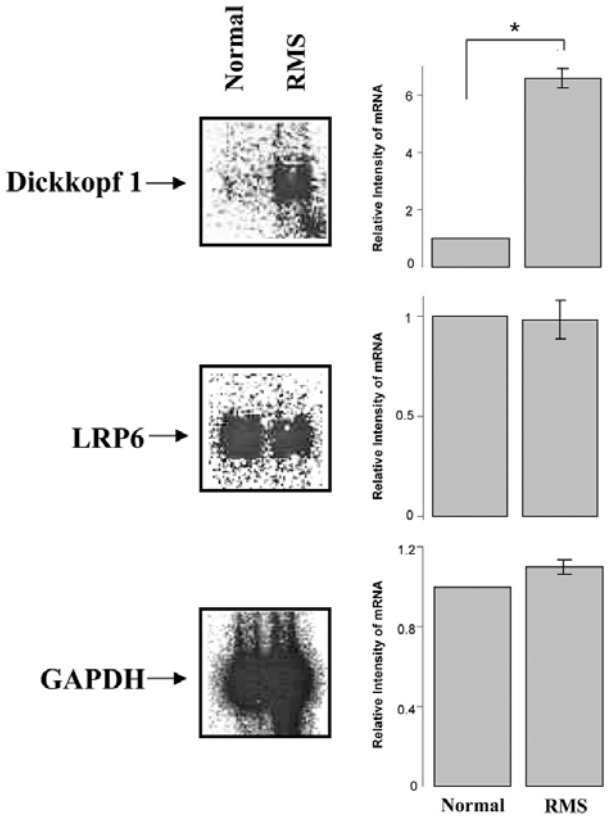

Fig. 1. Overexpression of Dickkopf 1 in leprechaunism. Isolated mRNA from normal or RMS cells were used for northern blot analysis as described in the "Materials and Methods". Membrane was hybridized with Dkk1, LRP6 or GAPDH cDNA probes amplified by PCR and labeled by [^{32}P] dCTP.

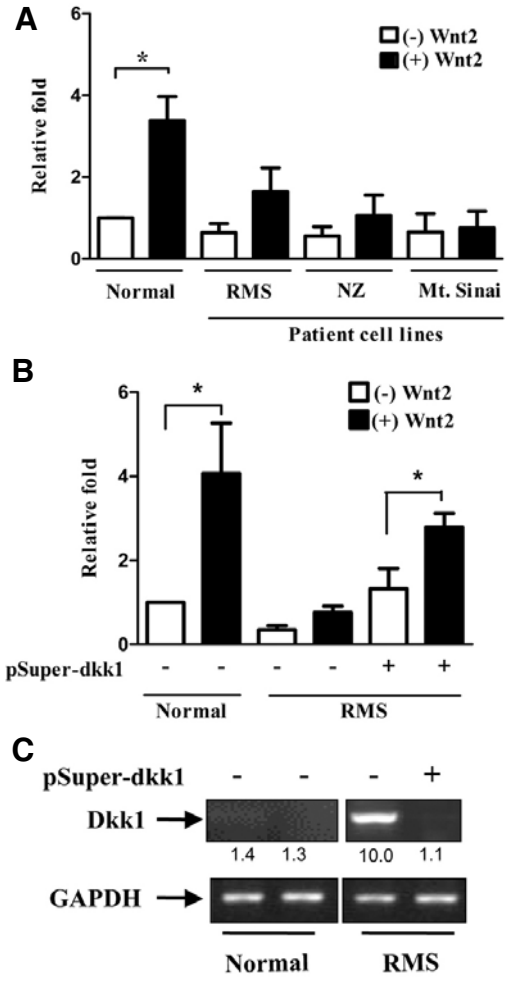

Fig. 2. Downregualtion of Wnt signaling in leprechaunism. (A) To determine the transcriptional activity of β-catenin, transient transfections were performed for the TOP-FLASH plasmid containing three copies of the β-catenin/T-cell factor (TCF)-binding sites upstream of a minimal herpesvirus thymidine kinase promoter driving the firefly luciferase expression. Cells were subjected to stimulation with Wnt2 for 24 h, the cells were lysed and used for the luciferase activity. (B) Cells were trasnfected with pSuper-Dkk1 siRNA or pSuper-scrambled siRNA (-) and TOP-FLASH plasmids. Luciferase activity was measured as in (A). The firefly luciferase activity was normalized to the activity of the renilla luciferase. The activity of the TOP-FLASH reporter construct was expressed as normalized relative light units (RLUs). The data in A (n = 5) and B (n = 4) are presented as means + S.E. of separate experiments. *, $p < 0.05$ by Student's *t*-test. (C) Total RNA was prepared from each sample and used for RT-PCR. Dkk1 expression was demonstrated by RT-PCR. GAPDH served as internal control for normalization purposes. Dkk1 mRNA levels were normalized to GAPDH then to RMS transfected with control vector to determine the relative intensities.

cells (Fig. 3C). We performed electorphoretic mobility shift assay (EMSA) to determine whether NF-AT interacts with promoter region of Nox4 and if so which of the NF-AT isozymes is involved in this process. Nuclear extracts from Wnt stimulated normal cells readily showed NF-AT specifically interacting with the promoter region of Nox4 (Figs. 3D and 3E). Binding to consensus NFAT oligonucleotides could be inhibited by the addition of a 100-fold excess of unlabeled NFAT probe (Fig. 3D), and mutated versions of the NFAT consensus failed to generate DNA-protein complex (Fig. 3E). Moreover, incubation with antibodies against NFATc2 but not against other NF-AT isozymes resulted in a supershift of the protein-DNA complex (Figs. 3D and 3E). These results together suggest a molecular connection between Nox4 expression and Wnt signaling through the transcriptional factor NFATc2.

Effect of Dickkopf 1 on ROS generation

We next examined whether overexpression of Dkk1 in patient cells is responsible for downregulation of Nox4. The patient cells were transfected with pSuper-Dkk1or with pSuper vector alone. A substantial increase in Nox4 expression was seen specifically with pSuper-Dkk1. In fact, the level of Nox4 expression was similar to that of normal cells (Table 2). Stimulation of patient cells transfected with pSuper-Dkk1 with Wnt2 resulted in a marked increase in Nox4 transcript compared to cells transfected with the scramble pSuper vector (Table 2).

Next we explored whether the enhanced Nox4 expression resulting from knockdown of Dkk1 contributes to PDGF-mediated ROS generation. The generation of ROS by patient fibroblast cells was measured using the oxidation of 2′,7′-dichlorofluorescin diacetate (DCF-DA) to 2′,7′-dichlorofluorecein (DCF) with laser-based confocal microscope. Transfection of patient cells with pSuper-Dkk1 vector resulted in increased generation of ROS upon PDGF treatment as revealed by an

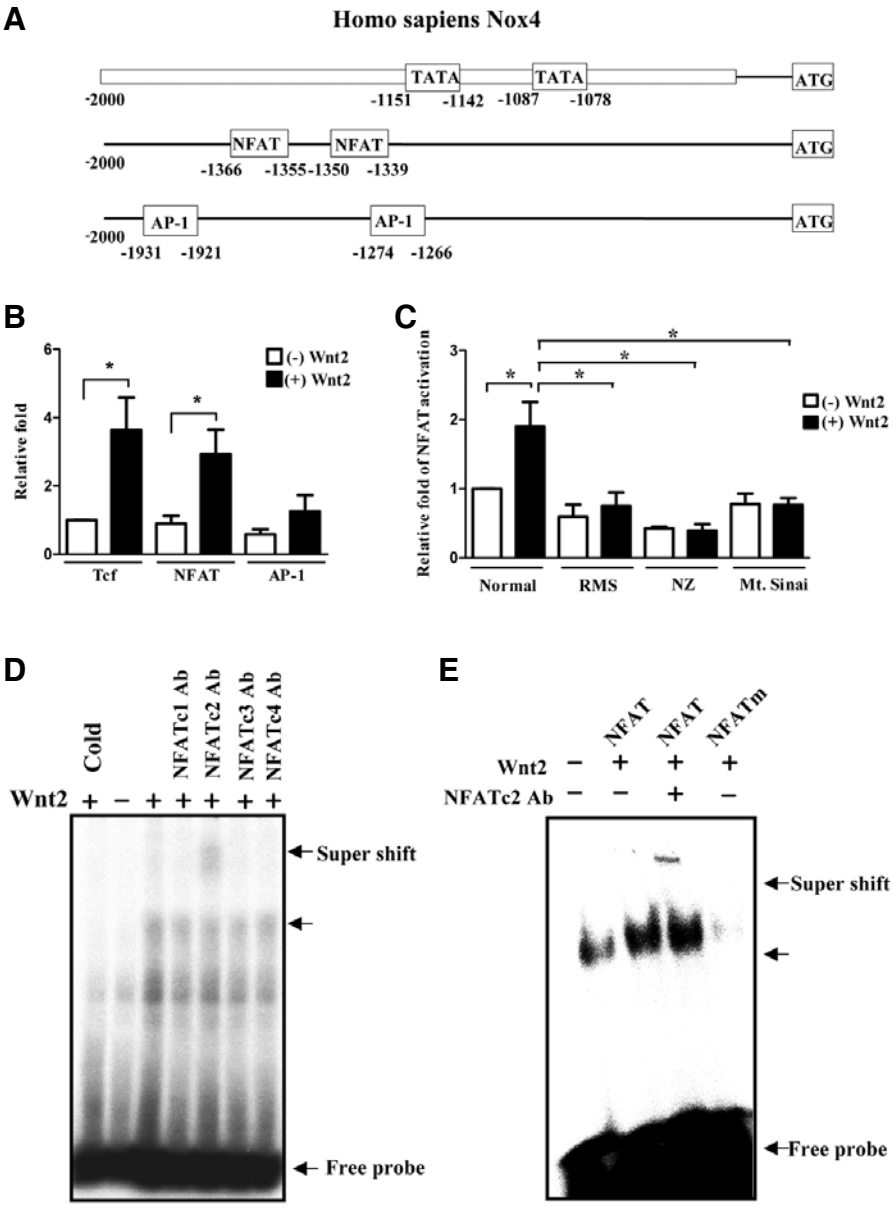

Fig. 3. NFAT activation was abolished in leprechaunism in response to Wnt stimulation. (A) Schematic representation of 5′-promoter region of Nox4 gene and putative binding sites of transcription factors. (B, C) Cells were transfected with plasmids for the indicated reporter assays and then incubated with Wnt2 for 24 h. After incubation, cell lysates were used for the Luciferase activity assay. The data in B (n = 5) and C (n = 4) are presented as means + S.E. of separate experiments. *, $p < 0.05$ by Student's *t*-test. (D, E) Electrophoretic mobility shift assays were performed using oligonucleotides with specific NFAT binding sequence or with mutant sequence (NFATm) and nuclear extracts (10 μg) of patient cells with or without Wnt2 treatment (6 h). Competition assays were performed using a 100-fold excess of unlabeled NFAT oligonucleotides. DNA-protein binding reaction was carried out in the absence or presence of specific antibodies to NFATc1-4 (D) or NFATc2 (E). For supershift analysis, 2 μg of the indicated antibodies were preincubated with the nuclear extracts for 30 min prior to the addition of labeled oligonucleotide. Data shown in the figure are representative of three independent experiments with similar results.

Table 2. The effect of Dkk1 on Nox4 expression in response to Wnt2a

	Normal	RMS	RMS + pSUPER-Dkk1
Wnt2a (-)	345 ± 20.2	168.8 ± 31.4	379.3 ± 27.1
Wnt2a (+)	512 ± 30.3	238 ± 43.8	1183 ± 219.4

Total RNA was isolated from the indicated cultured cells, reverse-transcribed, and quantified by real-time PCR. Nox4 values are expressed as copy numbers per 10^{11} copies of GAPDH measured in the same sample. ($\times 10^6/10^{11}$ copies GAPDH). Data are means ± SE of values from independent experiment (n = 5)

increase in DCF fluorescence whereas the cells transfected with scramble pSuper showed lower amount of ROS generation than normal cells (Fig. 4A). The result indicates that down-regulation of Nox4 by Dkk1 overexpression contributes the aberrant responses of cultured cells to various growth factors.

DISCUSSION

Wnt proteins play important roles in various mammalian developmental processes including cell polarity determination, lineage commitment, differentiation, and proliferation (Moon, 2005; Niehrs, 2006). Through binding to the members of the seven transmembrane Frizzled receptor family and coreceptor LRP5/ 6, Wnt proteins can stimulate the canonical β-catenin-Tcf pathway and/or the non-canonical PLCβ-calcium-calcineurin-NFAT pathway (Ma and Wang, 2007; Pandur et al., 2002). We have found that Dickkopf1 (Dkk1), a secreted Wnt inhibitor is overexpressed in fibroblast cell lines from leperchaunism patients (Fig. 1). Such overexpression of Dkk1 apparently results in reduction of Wnt-mediated cell signaling which in turn leads to inhibition of both the canonical and noncanonical pathways (Ma and Wang, 2007). Developmental defects resulting from

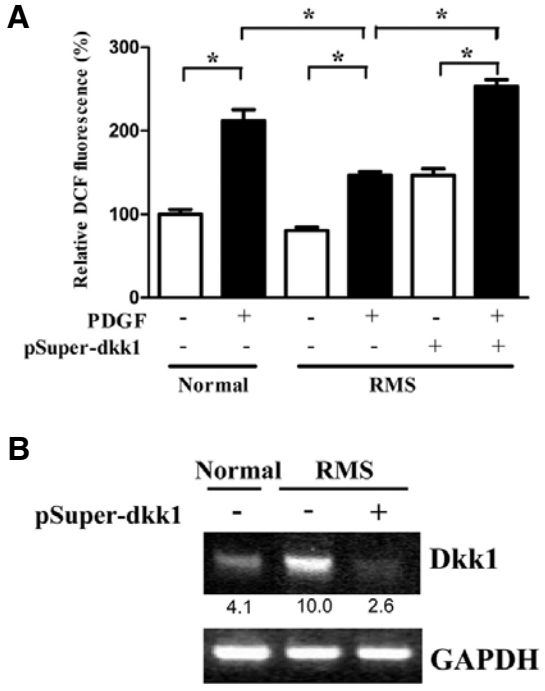

Fig. 4. Silencing of Dkk resulted in recovery of ROS generation in response to PDGF. Normal and RMS fibroblast cells were transfected with either pSuper-scrambled siRNA (-) or pSuper-Dkk1 siRNA. After 48 h, the cells were deprived of serum for 12 h. The generation of H_2O_2 was measured on the basis of DCF fluorescence after 10 min PDGF (100 ng/ml) treatment. Data are means ± SE of values from three independent experiments. *, $p < 0.05$ by Student's *t*-test. (B) Total RNA was prepared from each sample and processed for RT-PCR. Dkk1 expression was demonstrated by RT-PCR. GAPDH served as a loading control. Dkk1 mRNA levels were normalized to GAPDH then to RMS transfected with control vector to determine the relative intensities.

the loss of Dkk1 gene have been reported in Drosophila and Xenopus as well as in human holoprosencephaly patients all of whose phenotypes are consistent excessive Wnt signaling (Mukhopadhyay et al., 2001; Piccolo et al., 1999; Roessler et al., 2000). The developmental defects in leprechaunism are well characterized and include sparse subcutaneous fat, coarse face, dentitional abnormalities, and rectal prolapse. Given that Wnt signaling is key controlling component in developments, the failure of Wnt signaling by overexpression of Dkk1 may be causally related to the developmental defects of leprechaunism. Several lines of evidence indicate that Dkk1 blocks LRP6-mediated Wnt signaling by interacting with C-terminal domain of LRP6 (Bafico et al., 2001). The stimulation of Wnt signaling by LRP6 has been shown to be mediated through both the canonical and noncanonical pathways (Pandur et al., 2002). The critical element of the latter pathway is the NFAT transcription factor complex which plays an important role in T-cell activation and neuronal morphogenesis (Graef et al., 2001a; 2001b). Members of NFATc display diverse expression patterns and partially degenerate DNA binding specificities and are thus ascribed with distinct functions. While normal fibroblast cells showed NFATc2 activation in response to Wnt stimulation, those from patients failed to do so (Fig. 3C). These data suggest that Wnt-NFATc2-dependent gene expression may be involved in pathophysiology of leprechaunism.

We have previously found that low level tyrosine phosphorylation by growth factors in the leprechaunism patient cells results from impaired ROS generation (Park et al., 2005). We also showed that it was Nox4 downregulation in patient cells that was responsible for deficient ROS generation. Consistently, the cells with defects in ROS generation and phosphorylation were rescued by the ectopic expression of Nox4. Up- or down-regulation of Nox4 expression was shown to be controlled by various agonists (Higashi et al., 2003; Hwang et al., 2003; Moe et al., 2006; Pedruzzi et al., 2004; Sturrock et al., 2006). However, responsible transcriptional factors for Nox4 expression were not determined. In this report, we showed for the first time that NFATc2 directly stimulates the expression of Nox4 in response to Wnt2 (Figs. 3 and 4; Table 2). Specifically, the promoter region of Nox4 contains two NFAT binding sites, and Nox4 expression and ROS generation in response to PDGF are increased by knockdown of Dkk1 (Fig. 4; Table 2). Based on these results, a novel regulatory network connecting Wnt signaling pathway and ROS signaling pathway can be drawn. More importantly, our study provides new insights into the underlying causes of the many developmental defects manifested in leprechaunism.

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

ACKNOWLEDGMENTS

This work was supported by the National Core Research Center (grant R15-2006-020-00000-0) and World Class University program (R31-2008-000-10010-0) of the Ministry of Education, Science and Technology/Korea Science and Engineering Foundation through Ewha Womans University and by National Research Laboratory program (grant number ROA-2007-000-20004-00). J.W.P, H.S.K., and H.C. are a recipient of Brain Korea 21 sc-holarship.

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