Role of Nodal in Prostate Cancer Progression

BaoHan T. Vo and Shafiq A. Khan
Center for Cancer Research and Therapeutic Development and Department of Biological Sciences
Clark Atlanta University, Atlanta, Georgia 30314

ABSTRACT

Nodal is an embryonic morphogen belonging to the TGF-β superfamily that has been demonstrated to inhibit differentiation and to maintain the pluripotency of human embryonic stem cells. Like other members of the TGFβ family, Nodal signals through type I and type II serine/threonine kinase receptor complexes and intracellular Smad proteins which translocate to the nucleus where they regulate gene expression. In addition, Cripto, a member of the epidermal growth factor-Cripto-FRL-Cryptic (EGF-CFC) protein family, has also been implicated in Nodal signaling. So far, Nodal has been studied in breast, ovarian, and melanoma cancer cell lines; however, very little is known about Nodal in prostate cancer progression. Therefore, the purpose of this study was to determine Nodal expression and its biological effects in human prostate cell lines. RT-PCR and Western Blot analysis were performed to analyze the expression of Nodal and Nodal receptors and its effect phosphorylation of Smad 2/3 in prostate cell lines. Effects of human recombinant Nodal (rhNodal) on proliferation of DU145, LNCaP, PC3, and WPE cells were determined by MTT assay and ³H-Thymidine incorporation assay. We observed that Nodal protein is highly expressed in DU145, LNCaP-C81, and WPE (stem) cells and weakly expressed in RWPE1 and RWPE2 cells; however, Nodal protein expression was undetectable in PC3 and PC3M cells. Nodal receptor mRNAs were expressed, at varying levels, in prostate cell lines. Treatment with exogenous, Nodal caused a dose-dependent inhibition of proliferation in DU145 and WPE cells, but Nodal had no effects on the proliferation of LNCaP and PC3 cells. These findings demonstrate that Nodal is expressed in some prostate cancer cell lines and prostate stem cells. Nodal induced a dose and time dependent phosphorylation of Smad 2/3 in DU145 cells. In conclusion, we demonstrated for the first time that Nodal is expressed in prostate cancer cell lines and it inhibits proliferation of DU145 cells and WPE (stem) cells. These effects may be mediated via a Smad 2/3 dependent mechanism.

INTRODUCTION

Nodal like transforming growth factor-β (TGFβ), activins, and inhibins exerts its biological effects by signaling through a cell surface serine/threonine kinase receptor complex of types II and types I receptors and intracellular Smad proteins [1, 2]. There are five members of type II (ActRII, ActRIIB, ActRII, ActRIIB) and seven members of type I receptors (Activin receptor like kinase (ALK) 1-7). Upon phosphorylation by the type II receptor, the type I receptor phosphorylates and activates Smads. Smad2 and Smad3 respond to Nodal, TGFβs, activin, and inhibins, whereas Smad1, Smad5, and Smad8 mediate BMP signaling [3, 4]. After receptor is activated, phosphorylated Smads are released from the receptors and form complexes with the co-Smad, Smad4 and translocate to the nucleus to regulate the transcription of target genes.

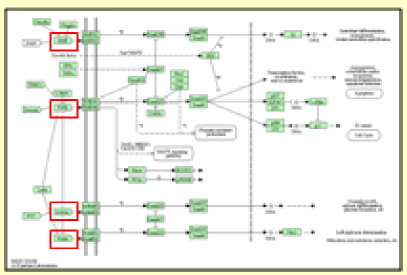

Figure 1. TGFβ signaling pathways.

Nodal was first cloned from a 7.5 day post-coitum mouse embryo cDNA library [5]. Based on studies in the mouse, zebrafish, and Xenopus, Nodal plays an important role in the induction of dorsal mesoderm, anterior patterning and formation of left-right asymmetry during early embryo development [6]. It has been suggested that Nodal regulates human placental development [6]. Basically, Nodal is an important regulator of embryonic development. Little is known about Nodal in prostate cancer progression. Therefore, the purpose of this study was to determine Nodal expression and its biological effects in human prostate cell lines.

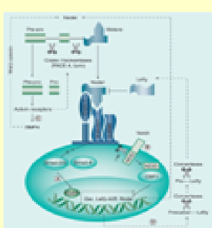

Figure 2. Nodal signaling pathways [6].

Table 1. Primers used for the detection of target mRNAs

Gene	Primer	Sequence (5'→3')	bp
Nodal	Forward Reverse	[illegible] [illegible]	[illegible]
ALK4	Forward Reverse	[illegible] [illegible]	[illegible]
ALK7	Forward Reverse	[illegible] [illegible]	[illegible]
ActRII	Forward Reverse	[illegible] [illegible]	[illegible]
ActRIIB	Forward Reverse	[illegible] [illegible]	[illegible]
Smad2	Forward Reverse	[illegible] [illegible]	[illegible]
Smad3	Forward Reverse	[illegible] [illegible]	[illegible]
Smad4	Forward Reverse	[illegible] [illegible]	[illegible]
Smad7	Forward Reverse	[illegible] [illegible]	[illegible]
L-19	Forward Reverse	[illegible] [illegible]	[illegible]

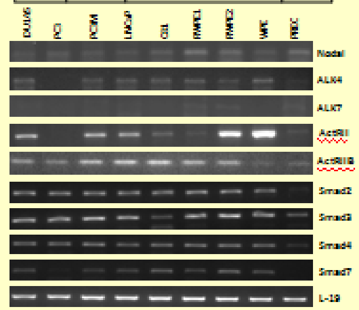

Figure 3. mRNA levels of Nodal, receptors, and Smads in nine prostate cell lines. Total RNA was isolated and semi-quantitative RT-PCR was performed. L-19 was used as an internal control.

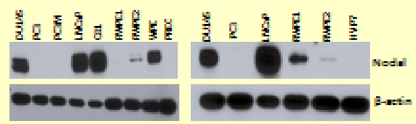

Figure 4. Western blot shows Nodal protein expression in prostate cell lines. Total proteins were prepared from different prostate cell lines. Cell lines were serum starved for 6 hours before collecting the total cellular proteins. Proteins were separated by SDS polyacrylamide gel electrophoresis and blotted using anti-Nodal, respectively.

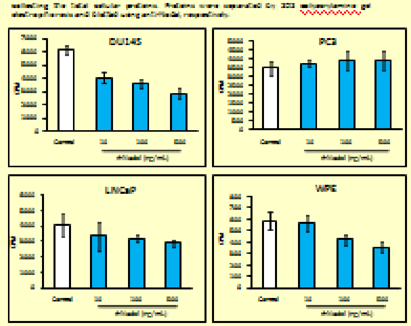

Figure 6. Effects of Nodal on proliferation of DU145, LNCaP, PC3, and WPE cells as determined by ³H-Thymidine incorporation assay. Cells were plated in 24 well tissue culture plates at a density of [illegible] cells/well. The cells were serum free media for 24 h and treated with rhNodal for 24 h in the presence of [illegible]. ³H-Thymidine was added with fresh media and incubated 4 hours. Following treatment, the cells were [illegible] and washed with distilled water. The number of cells were counted for 1 min using [illegible] program. Each bar represents the mean ±SD.

Table 2. Prostate cell lines used in this experiment

Cell line	Origin
DU145	Prostate carcinoma, derived from the brain of a 69-year old Caucasian male
PC3	Prostate adenocarcinoma, derived from the bone of a 62-year old Caucasian male
PC3M	Highly metastatic subline derived from a hepatic metastasis of PC3
LNCaP	Prostate carcinoma, derived from the left supraclavicular lymph node of a 50-year old Caucasian male
C81	LNCaP cells at passage 81
RWPE1	Epithelial cells from histologically normal adult human prostate
RWPE2	Epithelial cells were derived from RWPE-1 cells by transformation with Ki-ras using the Kirsten murine sarcoma virus (Ki-MuSV)
WPE	Stem cells were derived from the RWPE-1 cell line after two consecutive cycles of single cell cloning. The cells show high expression of cytokeratin 5 and 14 and MMP-2 but low expression of cytokeratin 18. They are androgen-independent for growth and survival.
PREC	Primary prostate epithelial cells

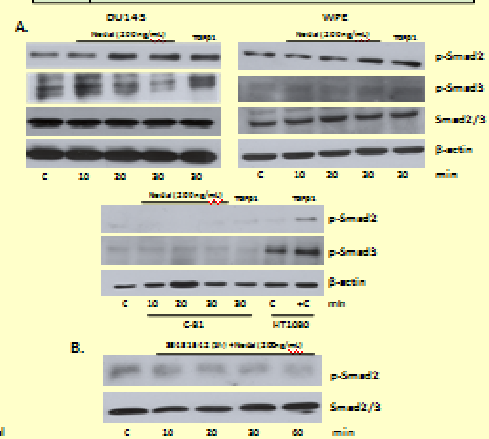

Figure 5. Nodal-mediated signaling in prostate cell lines. A. Western blot analyses of phosphorylated Smad2/3, total Smad2/3, and β-actin in DU145, WPE, and C81 at different time periods after treatment with rhNodal (100ng/mL). TGFβ (10ng/mL) was used as a positive control. B. Western blot analyses of phosphorylated Smad2 and total Smad2/3 in DU145 cells pretreated with ALK4/5/7 inhibitor (SB431542, 10μM) for 1 h and then treated with rh Nodal (100ng/mL) for 10, 20, 30 and 60 min.

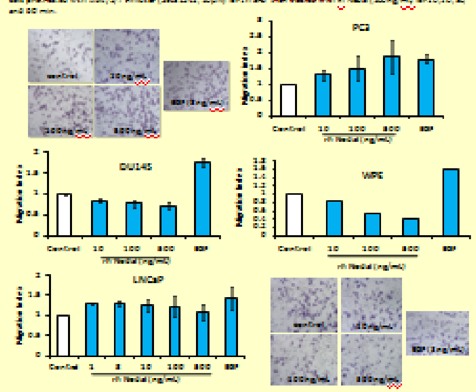

Figure 7. Effects of Nodal on cell migration in DU145, PC3, LNCaP, and WPE cells. Cell culture inserts were coated with rat tail collagen as described in material and methods. The cells were plated inside the cell culture insert and then loaded inserts into the 24-well plate containing rh Nodal (10, 100, 500ng/mL). EGF (5ng/mL) was used as a positive control. The transwell insert-loaded plate was incubated for 5 h. The inserts were then fixed, stained, and counted in five fields. Each treatment was performed in duplicate.

MATERIALS AND METHODS

Cell Culture: DU145, PC3, and PC3M were maintained in MEM supplemented with 5% FBS. LNCaP-C81 was maintained in RPMI 1640 medium supplement with 10% FBS. RWPE1, RWPE2, WPE, and PREC were maintained in keratinocyte serum free medium (KSFM). These medium were supplemented with antibiotics. The cells were maintained in a 37°C incubator with 5% CO_2 and 100% humidity.

RT-PCR: Total RNA was isolated from the all cell lines and analyzed using RT-PCR. Expression of L19, a ribosomal protein was used as a loading control.

Western Blot: The protein concentration in cell lysates were determined using the DC protein assay kit (Bio-Rad) and spectrophotometer. Equal amounts of proteins were separated by SDS polyacrylamide gel electrophoresis and blotted onto PVDF. Proteins were then probed using anti-Nodal, anti-pSmad2/3, anti-Smad2/3, and β-actin as an internal control.

Cell Proliferation: Cell proliferation assays were performed using two techniques: ³H-Thymidine incorporation/DNA synthesis assay and MTT assay. The cells were plated accordingly and then treated with three doses of rh Nodal (10, 100, and 500 ng/mL).

Cell Migration Assay: The outside and inside transwell insert membrane was coated with 50 μl of rat tail collagen (50 μg/ml). Chemoattractant solutions were made by diluting rh Nodal (10, 100, 500 ng/mL) or EGF (5 ng/ml) into MEM/RPMI/KSFM supplemented with 0.2% BSA. EGF was used as a positive control. Cell density was adjusted 5 × 10⁴/mL and loaded into transwell inserts. The transwell insert-loaded plate was incubated for 5 h. Cells inside transwell inserts were removed by cotton swabs and fixed with 4% paraformaldehyde (pH 7.5) for 20 minutes at room temperature. Cells on the outside of the transwell insert membrane were stained using HEMA 3 staining kit.

Statistical Analysis: The results are presented as means ± S.D. of three replicate cultures within each treatment group and are presented for a single representative experiment. Observations were confirmed in at least three independent experiments using different cell preparations.

SUMMARY

Nodal mRNA expression was detected in all nine prostate cell lines; however, Nodal mRNA was significantly elevated in normal and primary prostatic epithelial cells compare to the metastatic prostate cell lines. Western blot analyses showed that Nodal protein only expressed in DU145, LNCaP-C81, and WPE (stem) cells and weakly expressed in RWPE1 and RWPE2 cells.

Nodal receptor mRNAs were expressed, at varying levels, in prostate cell lines.

When DU145 and WPE cells were treated with human recombinant Nodal (rhNodal) at 100ng/mL, Smad2 was phosphorylated in a dose-dependent manner.

Treatment with exogenous, Nodal caused a dose-dependent inhibition of proliferation and migration in DU145 and WPE cells, but Nodal had little effects on the proliferation and migration of LNCaP cells while Nodal stimulated cellular proliferation and migration in PC3 cells.

Inhibition of ALK 4/5/7, in a dose-dependent manner, blocked Smad-2 phosphorylation in DU145 cells.

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