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Defining the Effects of GDF-2 on TGF_B Signaling in Tumorigenic Epithelial Cells

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DEFINING THE EFFECTS OF GDF-2 ON TGFB SIGNALING IN TUMORIGENIC EPITHELIAL CELLS

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ABSTRACT

Cancer cells can be viewed as such cells, which have disrupted/aberrant signaling pathways for maintaining cellular homoeostasis. Identifying such altered signaling mechanisms can help us target these pathways in a better way. GDF-2 (Growth and differentiation factor – 2) or BMP9 (Bone morphogenetic protein – 9), a multifunctional cytokine, is a member of the Bone Morphogenetic Protein subfamily, under TGFβ superfamily, with roles distinct from BMP2/4/7. While GDF-2 is known to be one of the most potent ectopic inducers of bone and cartilage formation, it rose to significance with the identification of its receptor ALK1 in endothelial cells. TGFβ's role in cancer progression could be described as both agonistic and antagonistic. It maintains tissue homeostasis and prevents incipient tumors from progressing down the path to malignancy. But cancer cells have the capacity to avoid the suppressive influence of TGFβ pathway. Pathological forms of TGFβ signaling promote tumor growth and invasion, evasion of immune surveillance and cancer cell metastasis. Apart from the C-terminal phosphorylation of smad2/3 by TGFβ, it can also phosphorylate the linker region. We find that GDF-2 can regulate smad phosphorylation by phosphorylating smad1/5 at the C-terminus to regulate cell survival [1-5]. Interestingly, GDF2 can also mediate linker phosphorylation of smad2 and not smad1 in a subset of epithelial cancer and non-cancerous cells. My Studies

indicate that GDF-2 mediated linker smad2 phosphorylation can antagonize TGFβ signaling implicating that the balance in the level of growth factors is an important factor in mediating downstream signaling pathways.

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LIST OF ABBREVIATIONS

ActR II ..... Activin Receptor type II

ALK ..... Activin Receptor like Kinase

BMP .....Bone Morphogenetic Protein

BMPR II ..... Bone Morphogenetic Protein Receptor II

CDK2 ..... Cyclin Dependent Kinase 2

CDK4 ..... Cyclin Dependent Kinase 4

ECM ..... Extra Cellular Matrix

ERK ..... Extracellular Signal Regulated Kinase

FBS ..... Fetal Bovine Serum

FGF..... Fibroblast Growth Factor

GDF..... Growth and Differentiation Factor

HHT2 ..... Hereditary Hemorrhagic Telangiectasia

JNK ..... Jun Amino terminal Kinase

MAPK ..... Mitogen Activated Protein Kinase

PAI-1 ..... Plasminogen Activator Inhibitor 1

PI3K ..... Phosphoinositide 3-Kinase

ROCK ..... Rho Associated Protein Kinase

R-SMAD ..... Receptor Regulated SMAD

SARA ..... Smad Anchor for Receptor Activation

TGF ..... Transforming Growth Factor

VEGF .....Vascular Endothelial Growth Factor

TAK1 ..... TGFβ Activated Kinase 1

CHAPTER 1

INTRODUCTION

1.1 IMPORTANCE OF GDF-2

TGFβ superfamily members have various roles in tumorigenesis. They promote cancer in its advanced stage as well as inhibit it in its early stages [6]. BMPs comprise an extensive group of conserved and related growth factors, of which approximately 20 have been identified so far. BMPs were first identified in the extract of bones and were known for their ability to be involved in direct ectopic bone formation [7]. Subsequently, apart from being involved in bone formation, BMPs were found to be involved in many developmental processes like embryonic patterning and early skeletal formation. BMPs also regulate tooth, hair, kidney, muscle, skin, and hematopoietic and neuronal development and also maintain the iron metabolism and vascular homeostasis *in vivo* [7]. BMPs are divided into 4 subgroups according to the similarity of their amino acid sequences and functions. The BMP-2/4 subgroup includes BMP-2, BMP-4 and the Drosophila dpp, functioning in the embryonic development. The BMP-7 subgroup is composed of BMP-5, 6, 7, 8 and the Drosophil gbb. The third is GDF-5 subgroup, including GDF-5, 6 and 7. These three subgroups are

important for keeping up normal tissue functions. BMP 9 and BMP 10 are the member of a fourth subgroup whose functions are now being discovered [8]. BMPs act through two different type II and type I serine/threonine kinase receptors, which are required for downstream signal transduction. The serine/threonine kinase domains of type II receptors are constitutively active, and phosphorylate Gly-Ser (GS) domains in the type I receptors upon ligand binding, leading to the activation of type I receptor kinases. BMPs bind to three different type II receptors, i.e. BMP type II receptor (BMPR II), activin type II receptor (ActR II) and activin type IIB receptors (ActR IIB). Regarding type I receptors, BMPs bind to three different type I receptors, called activin receptor like kinase (ALK) 2, ALK3 and ALK6 [9].

1.2 OVERVIEW OF THE SIGNALING PATHWAY

The *gdf-2* gene encodes BMP-9, also known as GDF-2. The role of GDF-2 in vascularization and angiogenesis has revealed roles in suppressing angiogenesis and promoting proliferation in endothelial cells [1, 2, 10, 11]. There is little information on the effects of GDF-2 in epithelial cells. The significance of GDF-2's role in epithelial cells comes from the facts that it is predominantly expressed in liver, although it's expression has also been detected in skin and heart as well. It also acts as a differentiation factor in the central nervous system [12] and it promotes proliferation of hepatocytes [3, 4, 13]. GDF-2 is also shown to be a suppressor of breast cancer *in vivo [5].* GDF-2 signaling in endothelial cells is initiated when it binds to the heteromeric type I/type II receptors. Two distinct type I receptor serine threonine kinases have been shown to bind GDF-2:

ALK1 in endothelial cells [1, 14] and ALK2 in other cell types [1, 4, 15] and also ALK3 and ALK6, in the absence of ALK1 and low ALK2. There are three distinct type II receptors, which bind to GDF-2: BMPR II, ActRII and ActRII B [1, 15]. But the binding of GDF-2 to type I and type II receptors varies in different cell types. Once the ligand binds the receptors, they are activated which in turn activates the receptor regulated smads (R-SMADs) smad1, smad5 and smad8, which in turn forms a complex with the co-smad smad4. This whole complex gets trans located into the nucleus and along with other transcription factors, promotes target gene expression and regulation [16].

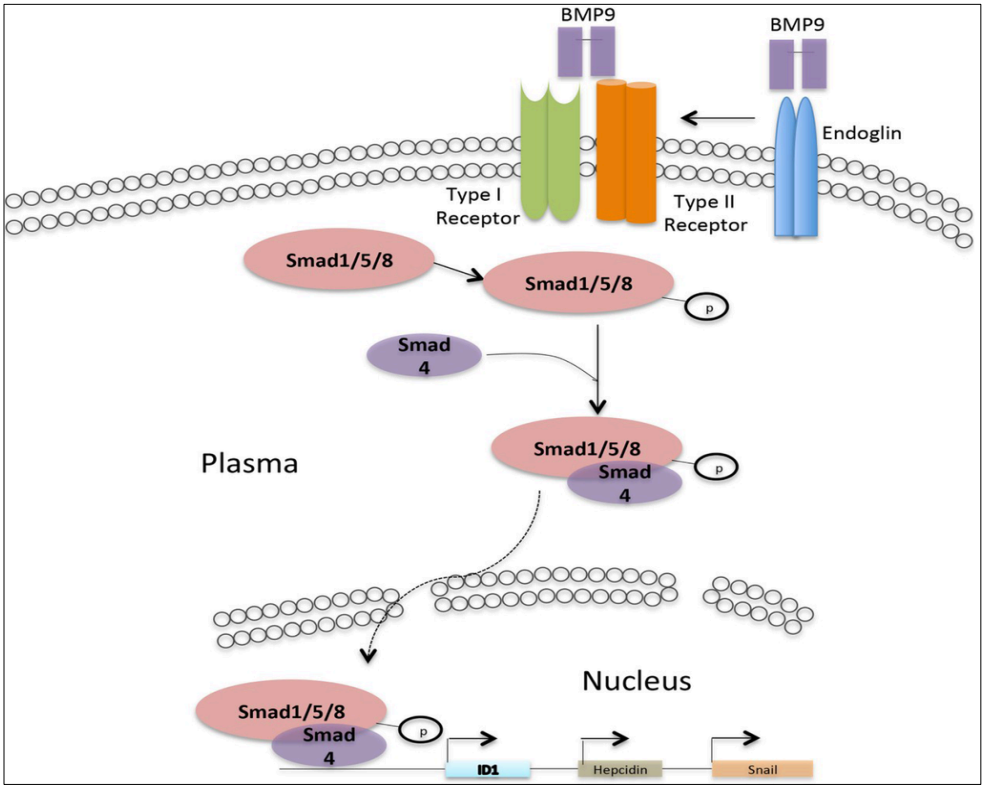

Figure 1.1 Overview of the BMP signaling pathway; *Int. J. Mol. Sci.* 2014, *15*(11), 20656-20667

1.3 STRUCTURAL AND FUNCTIONAL PROPERTIES OF GDF-2

Like all other TGFβ ligands, GDF-2 is synthesized as a large pre-pro-protein. Once dimerization occurs, the pro domain is cleaved from the active GDF-2; however, it still remains attached to GDF-2 through non-covalent interactions [14]. Expression of GDF-2 in different organs has been studied and it has been found that hepatocytes are by far the best producers of GDF-2 and moreover, it has been observed that similarly to TGFβ, GDF-2 circulates in active and inactive forms [17]. However, unlike TGFβ, the pro-domain of GDF-2 does not bind to ECM, meaning that GDF-2 is not inhibited to enter the circulation, resulting in a much higher concentration of GDF-2 in the serum [17]. The estimated level of GDF-2 in the blood is around 2-20 ng/ml, which is much higher than the EC_{50} for ALK1 activation. Indeed, aortic endothelial cells showed endogenously phosphorylated smad1/5/8 proteins, presumably due to sustained activation by circulating GDF-2 molecules [17].

The functional properties of GDF-2 are not very well understood. In fact, GDF-2 knockout mice did not show defects in angiogenesis [18], even though multiple studies have identified GDF-2 as an important factor for vasculature maintenance [1]. Knockout of the GDF-2 receptor, ALK1, is embryonically lethal, while haploinsufficiency in ALK1 leads to a genetic disorder called hereditary hemorrhagic telangiectasia type 2 (HHT2), which is characterized by abnormal vessel formation [19].

Early studies have described GDF-2 as a vascular quiescence factor [20]. Subsequent studies have shown that GDF-2 inhibits VEGF-induced angiogenesis and endothelial cell proliferation [1]. Recent studies, however, have reported the ability of GDF-2 to mediate proliferation of multiple types of endothelial cells both *in vitro* and *in vivo*, most likely by up regulating VEGF receptors and Angiopoetin-1/Tie2 expression [2]. These findings are not surprising, as ALK1 signaling up regulates ID1 and ID3 protein expression [21], which are strong promoters of angiogenesis [22]. Interestingly, the soluble form of ALK1 was recently reported to inhibit tumor growth in mouse models [23]. As with other TGFβ superfamily ligands, the functional role of GDF-2 remains controversial and is likely extremely cell and context dependent.

Apart from angiogenesis, GDF-2 is also known to have many other effects *in vitro* and *in vivo*. As most other BMPs, GDF-2 can function as an osteogenic and chondrogenic factor [24]. Additionally, GDF-2 can also regulate metabolism, by inhibiting glucose production and up regulating important enzymes of lipid homeostasis [25].

The most intriguing part of GDF-2 signaling is the contradiction of outcomes in its studies. In breast cancer cells, administering GDF-2 has shown to decrease HER-2 protein and transcript levels and reduce the tumor volume in nude mice [26]. On the other hand, GDF-2 acts as a pro-proliferating factor through the ALK2/SMAD1/SMAD4 pathway in epithelial ovarian cancer cells [15]. This pro-tumorigenic or anti-tumorigenic role of GDF-2 is still a subject of

investigation and the gap, whether GDF-2 is a critical factor in cancer progression remains unexplored

CHAPTER 2

COMPONENTS OF THE SIGNALING PATHWAY

2.1 SMAD FAMILY

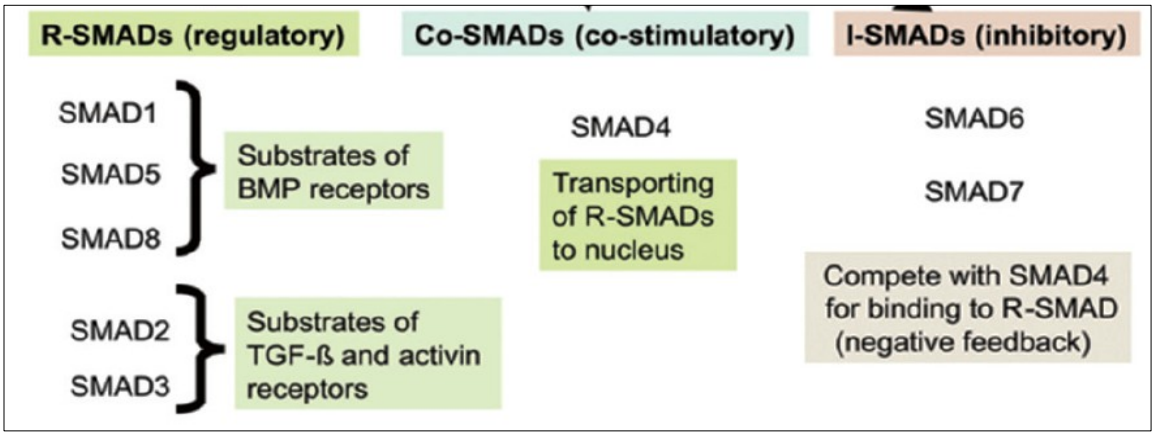

Figure 2.1 The smad family of proteins; *Indian Journal of Cancer,* Vol. 48, No. 3, July-September, 2011, pp. 351-360

Smads are intracellular proteins, which relay signaling from TGFβ ligands to the nucleus. There are three types of smads: R-smad (receptor regulated), Co-smad (common mediator) and I-smad (inhibitory). Smads 1, 2, 3, 5, 8 are R-smads and can be phosphorylated by type I receptors in the C- terminus. Binding of smad proteins to the type I receptors is aided by the presence of smad anchor for receptor activation (SARA) protein. Once phosphorylated, smads dissociate

from the receptors and form heteromeric complexes with smad4 [27]. Upon phosphorylation, smad complexes translocate to the nucleus, where they are able to interact with transcription factors and be recruited to specific promoter elements. Usually, each type I receptor is associated with only a subset of smads. Out of ALK 4, 5, 7, all phosphorylate smad2 and smad3, while ALK 1, 2, 3 and 6 induce smad 1, 5, 8 phosphorylation [28].

2.2 NON-SMAD PATHWAYS

2.2.1 P38 PATHWAY

The TGFβ and BMP receptor family not only induces activation of smad proteins, but also are also capable of activating other signaling molecules such as MAPKs, ERK, p38 and others. Perhaps the most recognized non-SMAD pathway initiated by TGFβ superfamily is p38 MAPK pathway. p38 exists at the third level of MEK phosphorylation, meaning that there are at least two sequential phosphorylation events before p38 can become phosphorylated. It is thought that p38 MAPK is activated through MKK3/6 [29]. Further upstream, MKK3/6 is activated through a TGFβ kinase (TAK1). Multiple studies have shown that TAK1 deficient cells are unable to signal through p38 pathway [30, 31]. Interestingly, p38 pathway is completely independent from smad signaling, as smad2/3 or smad4 deficient cells are prefectly capable of activating these pathways [32]. Recent studies have revealed that TGFβ receptors cannot only be phosphorylated on their serine/threonine residues, but can also be activated through phosphorylation of tyrosine residues [33]. TβRII cytoplasmic domain contains three tyrosine residues, which upon phosphorylation can recruit scaffold

proteins, which in turn initiate non-SMAD signaling pathways [34].

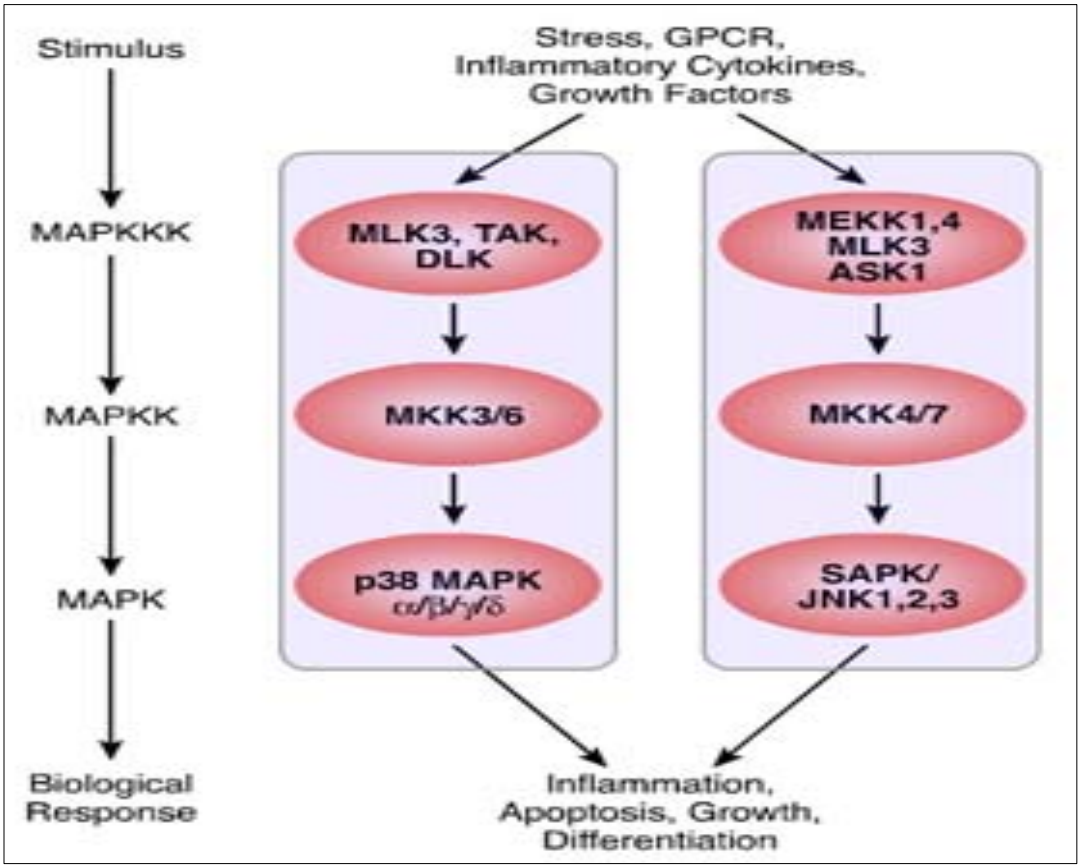

Figure 2.2 Overview of the MAPK pathway; *Can J Ophthalmol*. 2009 Aug; 44(4): 431-6

2.2.2 ERK PATHWAY

Several studies have shown that TGFβ can induce ERK activation. Interestingly, in some cells ERK phosphorylation occurs rapidly within minutes of TGFβ binding, suggesting a direct method of recruitment [35]. In contrast, other cells show a delayed response, happening hours after stimulation, implying that protein synthesis is required for signaling to occur [36]. As in the case of p38 signaling, phosphorylation of tyrosine residues on TβRII plays a crucial role in

ERK signaling. Moreover, like p38, smad involvement is not needed to induce ERK phosphorylation [37].

2.2.3 GDF-2 SIGNALING

GDF-2 can also activate non-smad pathways [38]; however, no extensive research has been done to elucidate the exact mechanisms of smad-independent pathways. It is likely that GDF-2 initiates non-smad signaling similarly to TGFβ, by phosphorylating tyrosine residues allowing for protein docking [38]. Several studies have shown that BMPs, including GDF-2 can induce MAPK pathways, through TAK1, leading to p38 MAPK or JNK activation [39]. Additionally, BMPs are also shown to induce ERK phosphorylation [39]. Interestingly, GDF-2 has also been found to have an inhibiting effect on PI3K/Akt pathway, which is activated by TGFβ signaling, implying that ALK1 and ALK5 pathways could function in opposition to each other [40].

CHAPTER 3

EXPLORING THE LINKER REGION OF SMADS

The R-smads consist of two conserved globular domains, namely the MH1 and MH2 domains connected by a more divergent linker region [41].

The MH1 domain binds DNA, whereas the MH2 domain binds the membrane receptors for activation, nucleoporins for nuclear translocation, and other smads and nuclear factors to form transcriptional complexes [42]. The diversity of the linker region, which consists of several serine and threonine residues, allows for regulation of R-smads by multiple signaling inputs. The linker region of smad1 consists of four MAPK phosphorylation sites (Ser-187, 195, 206 and 214), whereas smad2/3 consists of four SP/TP sites for proline-directed kinases. In response to mitogens, Erk MAPK mediates the phosphorylation of these sites *in vivo* [43, 44]. CDK2 and CDK4 have also been known to mediate the phosphorylation of some of the linker residues in smad2/3 in addition to residues at the N-terminus of smad2/3 [45]. p38 MAPK and JNK also phosphorylate the linker region of smad2/3 and regulate their transcriptional activity [46, 47]. The MAPK mediated phosphorylation of the linker region

generally results in the inhibition of smad1 activity [43, 48] and attenuation of nuclear accumulation of smad1 [43]. Similarly, MAPK mediated attenuation of smad2 activity has been attributed to smad2 linker phosphorylation [44, 49]. In *Xenopus* embryogenesis, linker phosphorylation of smad1 through MAPK plays an important role in inhibiting BMP signaling, which results in neural induction [48]. Linker phosphorylation of smad2/3 during *Xenopus* embryogenesis results in cytosolic retention of smad2/3 and inhibition of TGFβ signaling [49].

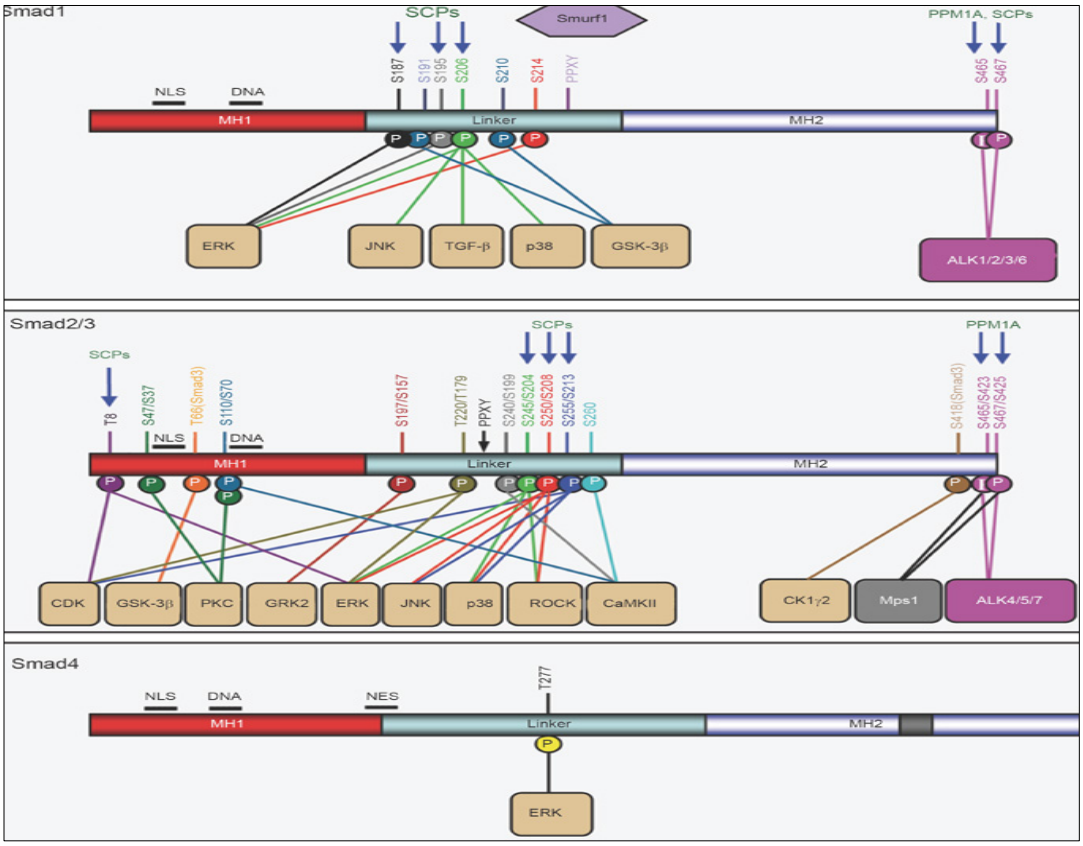

Figure 3.1 Different phosphorylation sites of the linker region of smad proteins; *Cell Research* (2009) 19:8–20

The TGFβ and BMP pathways are intensely regulated by inputs that adjust pathway activity according to contextual status. Antagonists such as

fibroblast growth factor (FGF) and epidermal growth factor (EGF) and cell stress signals act through MAPKs to cause phosphorylation of a region that links the DNA binding and transcriptional domains of the smads [43, 48-50]. Linker phosphorylation of smads in the basal state leads to their cytoplasmic retention and ubiquitin ligase-driven; proteasomal degradation [51, 52], with an attendant decrease in the responsiveness of cells to BMP and TGFβ signals [43, 44, 48, 49]. Smad linker phosphorylation by antagonists provides a critical counterbalance to TGFβ and BMP signaling. This has led to postulates that in the canonical pathways C-tail phosphorylation activates smad signaling and linker phosphorylation inhibits it [52, 53]. However, this dichotomy is not so tidy. BMP induced smad1 linker phosphorylation that has been reported previously [52], has revealed unexpected facets of the canonical TGFβ and BMP pathways. Unlike linker phosphorylation by antagonistic signals, which is cytoplasmic and MAPK mediated, agonist induced linker phosphorylation occurs during or directly prior to the assembly of smad proteins into transcriptional complexes and is mediated by CDK8 and CDK9 [54].

MAPK mediated linker phosphorylation appears to have a dual role in smad2/3 regulation. Mitogens and hyperactive Ras result in extracellular signal regulated kinase (ERK)-mediated phosphorylation of smad3 at Ser 204, 208 and Thr 179 and of smad2 at Ser 245/250/255 and Thr 220. Mutation of these sites increases the ability of smad3 to activate target genes, suggesting that MAPK phosphorylation of smad3 is inhibitory [44, 55]. However, in contrast, ERK dependent phosphorylation of smad2 at Thr 8 enhances its transcriptional activity

[56]. Phosphorylation of smad3 by p38 MAPK and ROCK (Ser 204, Ser 208 and Ser 213) and c-Jun N-terminal kinase (JNK) (Ser 208 and Ser 213; analogous to Ser 250 and Ser 255 in smad2) may enhance smad2/3 transcriptional activity, suggesting that smads and the p38/ROCK/JNK signaling pathways might cooperate in generating a more robust TGFβ response [46, 47, 57]. A significant increase in Ser 208/Ser 213 phosphorylation of smad3 is associated with late stage colorectal tumors, suggesting that the linker-phosphorylated smad3 may mediate the tumor-promoting role of TGFβ in late tumorigenesis.

CHAPTER 4

THESIS OBJECTIVES

4.1 RATIONALE

There are many studies where it has been shown that GDF-2 acts through ALK1 in endothelial cells [1, 10, 58]. GDF-2 can also act via ALK2 [15] in other cell lines, where ALK1 is absent or present in very low levels. A recent study from our lab has shown that GDF-2 can also activate smad1/5 signaling by increasing a complex formation between ALK3 and 6 and type II receptor BMPRII (unpublished data). As these receptors all induce the smad1/5/8 pathway, it would suggest that smad independent signaling pathways are also involved. BMPs are also known to regulate a variety of smad independent pathways including the p38 and ERK MAPK signaling pathways [9]. Our data suggests that GDF-2 is also able to induce smad2 but not smad1 linker phosphorylation, which in turn retain smad2 in the cytoplasm and not allow it to go into the nucleus, which would further suggest that non smad pathway(s) (p38, ERK) might be involved upstream of smad2, since canonical smad pathways usually denote C-

terminal phosphorylation of smads and that leads to nuclear translocation of smads.

4.2 HYPOTHESIS

GDF-2 phosphorylates the linker region of smad2 and blocks its nuclear translocation and it also suppresses TGFβ mediated smad2 signaling.

4.3 SPECIFIC AIMS

- To test whether GDF-2 mediates canonical smad1/5/8 signaling in tumorigenic ovarian epithelial cells
- To test whether GDF-2 is able to phosphorylate the linker region of smad2 and prevent its nuclear translocation and whether GDF-2 is able to suppress TGFβ mediated smad2 signaling.

CHAPTER 5

MATERIALS AND METHODS

5.1 CELL LINES AND THEIR CULTURE

Ovarian tumorigenic cell lines HEY, 4T1, BT 474, SKOV3 and OvCa 429 were cultured in RPMI supplemented with 10% FBS, L-glutamine and 100U penicillin/streptomycin. HMvEC cells were maintained in endothelial cell growth medium containing 10% FBS and MCF10A cells were maintained in DMEM/F12 medium supplemented with 5% horse serum, 20ng/ml EGF, 0.5mg/ml hydrocortisone, 100ng/ml cholera toxin, 10µg/ml insulin and 100U Pen/Strep. All cells lines were maintained at 37°C in a humidified incubator at 5% CO_2.

5.2 ANTIBODIES, REAGENTS AND PLASMIDS

Antibodies phosphosmad1/5 (#9516S), phosphosmad2/3 (#8828S), phosphosmad2 (#3104S), smad1 (#6944S), smad2 (#3103S) and p38 MAPK (#8690S), GAPDH (#2118S) and fibrillarin (#2639S) were from Cell Signaling Technology. Inhibitors SB203580 (#PHZ1253) and U0126 (#PHZ1283) were

from Invitrogen. GDF-2 and TGFβ were from R&D systems. Constructs expressing pE2.1 was a kind gift from Miyazono, K [59].

5.3 TRANSCRIPTION REPORTER LUCIFERASE ASSAY

Cells were grown in a 24 well plate and transfected with pE 2.1 vector containing the luciferase gene under 36 bp-pE2.1 element of PAI-1 gene [60-62] and the pRL-SV40 vector expressing Renilla luciferase under the control of SV40 promoter to control for transfection efficiency using Lipofectamine 2000. The cell were incubated with GDF-2 (10 ng/ml) and TGFβ (100 pM) for 24 hours, after 6 hours of serum starvation in SKOV3 and 24 hours of serum starvation in HEY. Cells were collected and lysed in 1x passive lysis buffer (Promega) according to manufacturer's instructions. To measure luciferase activity, 20µl of lysate was added to 25µl of Luciferase Reporter Assay Reagent (Promega) and luminescence was quantitated using a luminometer (Biotek).

5.4 SUB-CELLULAR FRACTIONATION

HEY cells were serum starved (overnight) and treated with the ligands for 30 minutes when they were 90-100% confluent. Then they were harvested from 90-100% confluent P10 dish and subjected to sub-cellular fractionation, according to the manufacturer's protocol from the Cell Signaling (#9038S).

5.5 WESTERN BLOTTING

Protein samples were heated to 90°C for 5 minutes and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10 or 12.5%

acrylamide gels, followed by transfer to nitrocellulose membranes for 1 hour at 10 volts. Membranes were blocked for 30 minutes in 5% non-fat dried milk in Tris-buffered saline, after which they were incubated overnight with primary antibody in 5% bovine serum albumin in Tris-buffered saline with 0.1% Tween 20, followed by 1 hour with fluorescent secondary antibody.

5.6 IMMUNOFLUORESCENCE AND MICROSCOPY

Cells were fixed in 4% paraformaldehyde, permeabilized in 0.3% TX-100 and blocked with 1% BSA in PBS. Primary antibody (1:200) incubation for an hour was followed by 30 minutes incubation with Alexa Fluor® 488 (H+L) (#A11008 Life Technologies). After washing, cells were stained with 4, 6-diamidino-2-phenylindole (DAPI; Roche). Imaging was carried out using a Zeiss LSM700 confocal microscope.

5.7 STATISTICAL ANALYSIS

Statistical analysis was performed by Student's *t*-test analysis.

CHAPTER 6

RESULTS

6.1 GDF-2 activates smad1/5 signaling in both normal and tumorigenic epithelial cells

It has been known that GDF-2 is a ligand for ALK1 in endothelial cells [10], but its role in normal and oncogenic epithelial cells that don't have ALK1 receptor is still not clear. To find out whether GDF-2 would also mediate downstream signaling pathways in non-tumorigenic and tumorigenic epithelial cell lines, a time dependent analysis of smad1/5 phosphorylation was carried out in both tumorigenic (BT 474, OvCa 429, HEY and SKOV3) and non-tumorigenic (HMvEC and MCF10A) cell lines. Robust smad1/5 signaling was seen as early as 5 minutes or 15 minutes after GDF-2 treatment **[Figure 6.1]**

Since previous studies have indicated that BMP superfamily members (BMP 2/4) can activate the smad2/3 pathways as well [63], I examined smad2/3 signaling in two of these cell lines. I found out that GDF-2 did not phosphorylate smad2/3 and subsequently did not mediate smad2/3 signaling **[Figure 6.2]**.

Consistent with smad1/5 activation in response to GDF-2 treatment, I observed clear nuclear translocation of smad1/5 **[Figure 6.3]** in 4T1 cells, a mouse mammary epithelial model**.** Therefore, I concluded that GDF-2 exclusively mediates smad1/5 phosphorylation and signaling.

6.2 GDF-2 induces phosphorylation of linker region of smad2 but not smad1 and this might be via the MAPK pathway

It has been previously observed that BMPs induce smad1 linker phosphorylation at four different MAPK phosphorylation sites (Ser-187, 195, 206 and 214) [52]. The MAPK mediated phosphorylation of the linker region generally results in the inhibition of smad1 activity [43, 48] and attenuation of the nuclear accumulation of smad1 [43]. Similarly, TGFβ induced MAPK mediated attenuation of smad2 activity has been attributed to smad2 linker phosphorylation [44, 49]. Therefore, to find out whether GDF-2 is also inducing smad1 linker phosphorylation, I examined smad1 linker phosphorylation in different tumorigenic cell lines **[Figure 6.4]**.

Surprisingly, I observed that GDF-2 did not induce smad1 linker phosphorylation, although it very clearly induces smad1/5 phosphorylation in both tumorigenic and non-tumorigenic cell lines. Historically, it has been known that GDF-2 is not able to induce smad2/3 phosphorylation and subsequently, it's signaling [10] and it has always been associated with smad1/5 signaling. But since, I did not observe any smad1 linker phosphorylation in any of the cell lines, I opted to see if GDF-2 could induce smad2 linker phosphorylation and

surprisingly, I observed smad2 linker getting phosphorylated by GDF-2 in a time dependent manner **[Figure 6.5]**. This observation is novel and there are no previous reports of smad2 linker region getting phosphorylated by GDF-2. Smad1 linker region gets phosphorylated by MAPKs [43, 48, 52] and although I could not observe any smad1 linker phosphorylation by GDF-2, I could clearly conclude that GDF-2 is phosphorylating the smad2 linker. .

To find out whether MAPK pathway is involved in this phenomenon, I took the help of two inhibitors: U0126 (MEK inhibitor) and SB203580 (p38 MAPK inhibitor). It was again surprising to observe that the two inhibitors, individually, and in a combinatorial way, were able to suppress smad2 linker phosphorylation mediated by GDF-2 **[Figure 6.6]**.

Thus, I was able to make the conclusion that GDF-2 induces smad2 but not smad1 linker phosphorylation in a subset of tumorigenic epithelial cells and it might be mediating that via MAPK pathway.

6.3 GDF-2 mediates the retention of linker-phosphorylated smad2 in the cytoplasm and it does not allow the nuclear translocation of C-terminal phosphorylated smad2

Linker phosphorylation of smads in the basal state leads to their cytoplasmic retention and ubiquitin ligase-driven; proteasomal degradation [51, 52], with a concomitant decrease in the responsiveness of cells to BMP and TGFβ signals [43, 44, 48, 49]. Smad linker phosphorylation by antagonists provides a critical counterbalance to TGFβ and BMP signaling. This has led to postulates that in the canonical pathways, C-tail phosphorylation activates smad

signaling and linker phosphorylation inhibits it [52, 53]. Also, TGFβ is historically known to phosphorylate smad2/3 and mediate it's signaling [64] and it also promotes the nuclear translocation of smad2/3 for target gene regulation. To find out whether GDF-2 exhibits the same effects as TGFβ in retaining smad2 in the cytoplasm after it's linker region gets phosphorylated, I did subcellular fractionation in HEY cells and separated the cytosolic and nuclear fraction. Then the different fractions were blotted for phosphorylated smad2 linker and phosphorylated C-terminal of smad2 **[Figure 6.7]**. I observed that when the linker region of smad2 gets phosphorylated, surprisingly almost all of smad2 gets retained in the cytoplasm. Thus, it was clearly evident that GDF-2 acts in the same manner as TGFβ while phosphorylating the linker region of smad2 and then the retention of it in the cytoplasm. The interesting part of this observation is that although GDF-2 falls under the TGFβ superfamily, it is not at all similar to TGFβ and is expected to act in a manner similar to the other BMPs, since GDF-2 also induces smad1/5 phosphorylation.

Therefore, the reason why GDF-2 cannot induce smad2/3 C-terminal phosphorylation and mediates it's nuclear translocation might be due to the fact that GDF-2 induces smad2 linker phosphorylation and hence, inhibits it's downstream canonical signaling.

6.4 GDF-2 might be inhibiting the TGFβ mediated smad2/3 signaling pathway and it may be doing it via smad2 linker region phosphorylation.

The next step of my research was to find out the consequences of the smad2 linker phosphorylation by GDF-2. There have been many previous reports

of smad linker phosphorylation by TGFβ and BMPs, and all are related to the fact that the linker phosphorylation of the smads helps them to get retained in the cytoplasm and subsequently attenuates their nuclear accumulation, which in turn inhibits BMP or TGFβ signaling [43, 44, 48, 49]. I assumed that there might be some biological consequences of smad2 linker phosphorylation by GDF-2 due to the known fact that linker phosphorylation disrupts nuclear translocation of smad proteins. Since it was a novel observation that GDF-2 is able to phosphorylate smad2 and not smad1 linker, I proceeded to study the effects of GDF-2 mediated smad2 linker phosphorylation on TGFβ mediated signaling with the luciferase reporter assay, as TGFβ is known to be the ligand which phosphorylates smad2/3. HEY and SKOV3 cells were transfected with pE2.1-luciferase, a luciferase reporter gene under the control of TGFβ responsive PAI-1 based promoter and the GDF-2 mediated gene induction with and without TGFβ was assayed by measuring luciferase activity **[Figure 6.8].**

As evident from the figure above that gene induction by TGFβ is approximately 3- fold down in the presence of GDF-2 in HEY, and almost 1.5-fold down in SKOV3. This might be due to the fact that GDF-2 is suppressing TGFβ mediated signaling via smad2, by phosphorylating the linker region of smad2, which subsequently are not being able to translocate into the nucleus, thus lowering the target gene expression.

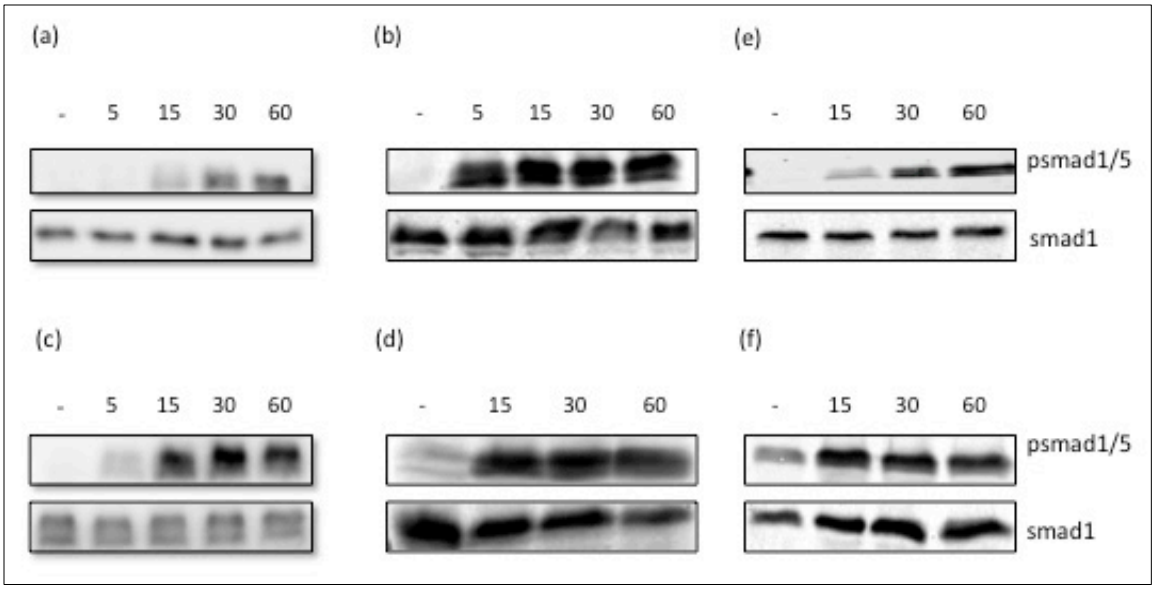

Figure 6.1 GDF-2 activates smad1/5 in tumorigenic and non-tumorigenic epithelial cells. *Western blotting of lysates from (a) BT 474, (b) SKOV3, (c) HEY, (d) OvCa 429, (e) MCF10A, (f) HMvEC, treated with GDF-2 (10ng/ml) for the indicated times (minutes) and immunoblotted for psmad1/5 and smad1.*

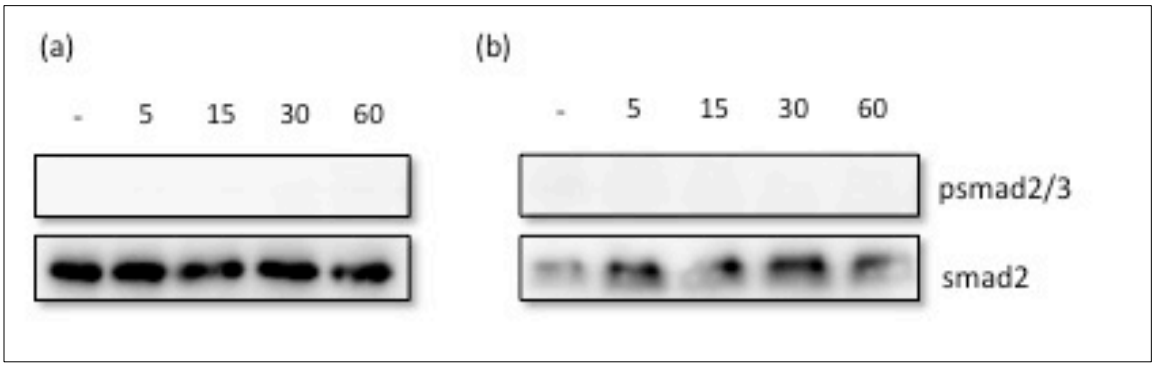

Figure 6.2 GDF-2 does not activate smad2/3 in epithelial cells. *Western blotting of lysates from (a) OvCa 429, (b) HEY, treated with GDF-2 (10ng/ml) for the indicated times (minutes) and immunoblotted for psmad2/3 and smad2.*

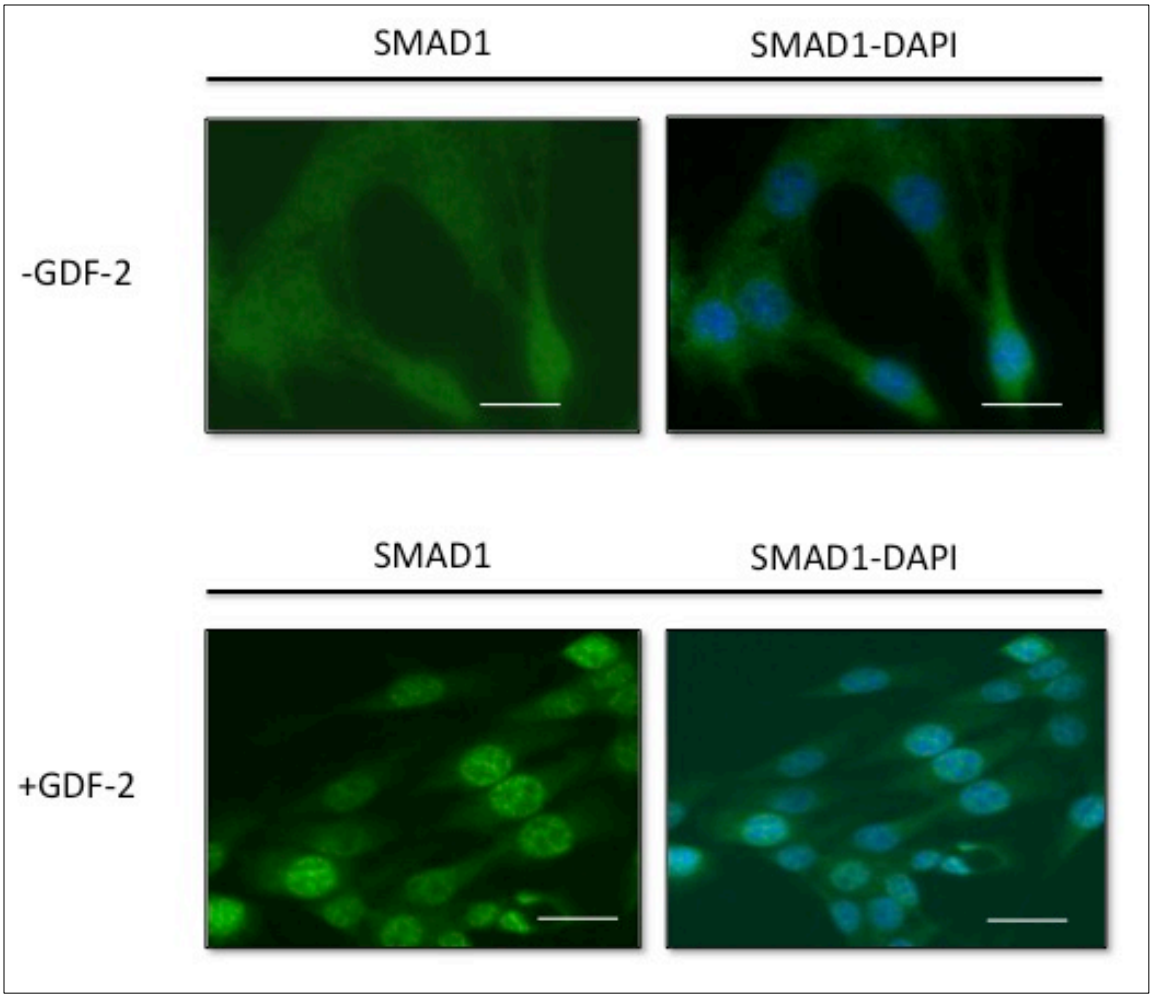

Figure 6.3 SMAD1 translocates into the nucleus. *Immunofluorescence images of 4T1 cells treated with GDF-2 for 30 minutes, followed by immunostaining for smad1*

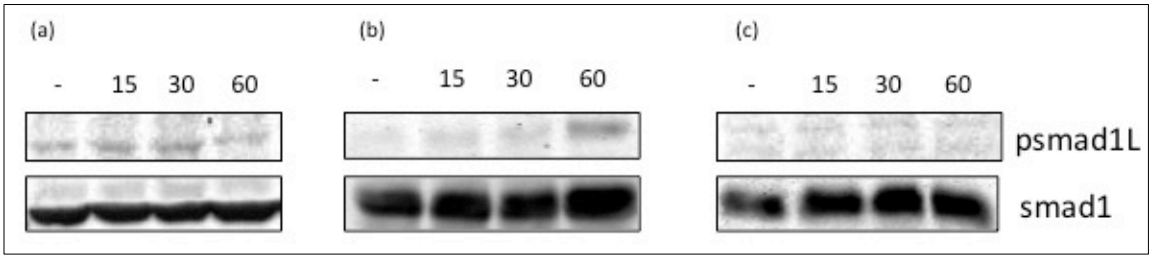

Figure 6.4 GDF-2 does not induce smad1 linker phosphorylation in tumorigenic epithelial cells. *Western blotting of lysates from (a) SKOV3, (b) HEY, (c) BT 474, treated with GDF-2 (10ng/ml) for the indicated times (minutes) and immunoblotted for psmad1 linker and smad1.*

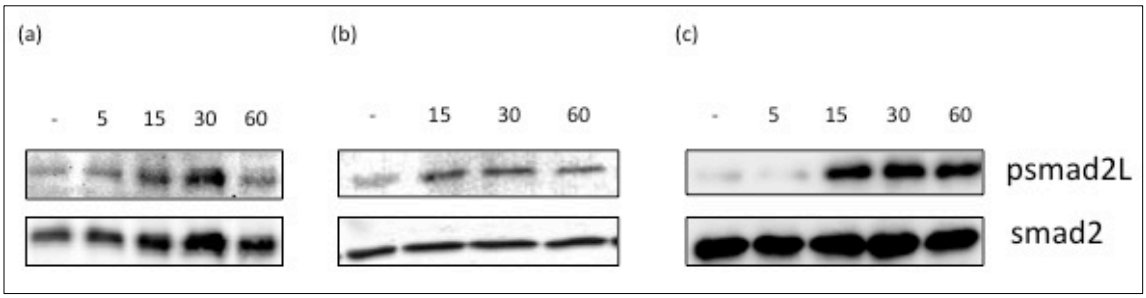

Figure 6.5 GDF-2 induces smad2 linker phosphorylation in tumorigenic epithelial cells. *Western blotting of lysates from (a) HEY, (b) SKOV3, (c) BT 474, treated with GDF-2 (10ng/ml) for the indicated times (minutes) and immunoblotted for psmad2 linker and smad2.*

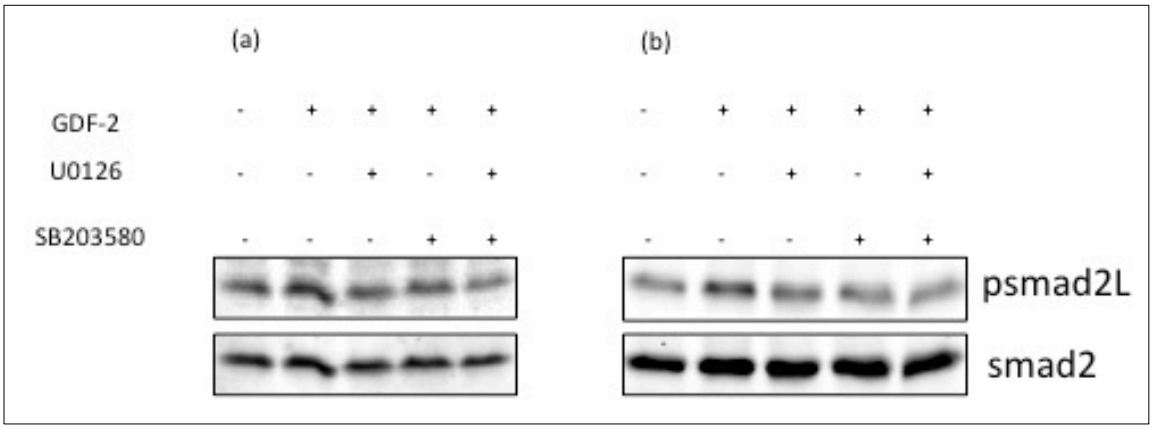

Figure 6.6 GDF-2 might be inducing smad2 linker phosphorylation via MAPK pathway. *Western blotting for psmad2L activation in (a) HEY and (b) SKOV3 in the presence and absence U0126 (10μM) and SB203580 (10μM) with and without GDF-2 (10ng/ml) as indicated.*

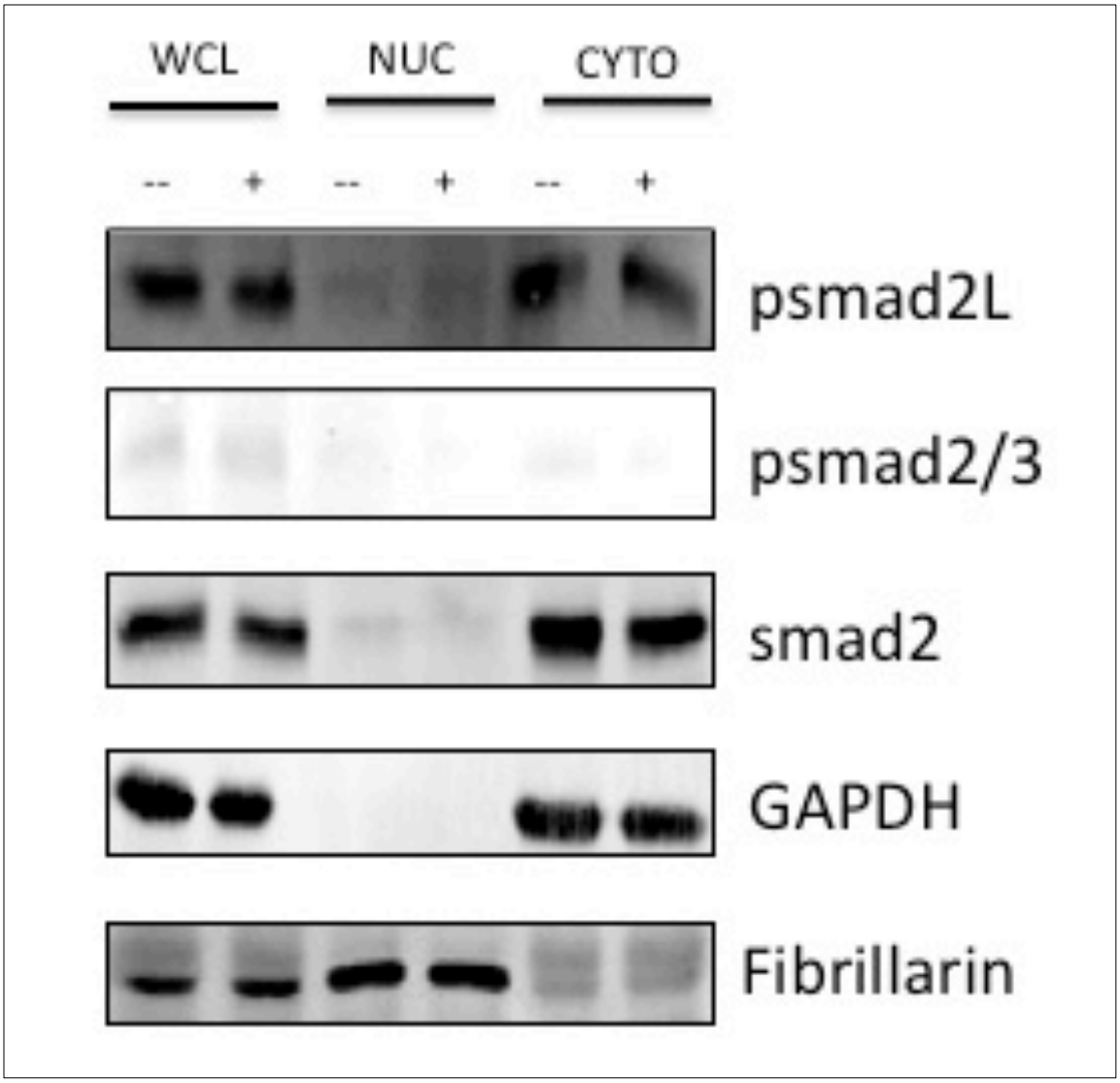

Figure 6.7 GDF-2 mediated smad2 linker phosphorylation inhibits smad2 to translocate into the nucleus. *HEY cells were treated with GDF-2 (10ng/ml) and fractionated into nuclear and cytoplasmic fractions. Twenty micrograms of protein were loaded from each fraction for western blotting and immunoblotted for psmad2 linker, psmad2/3 (C-terminal), smad2, GAPDH and fibrillarin. GAPDH and fibrillarin is cytoplasmic and nuclear marker, respectively.*

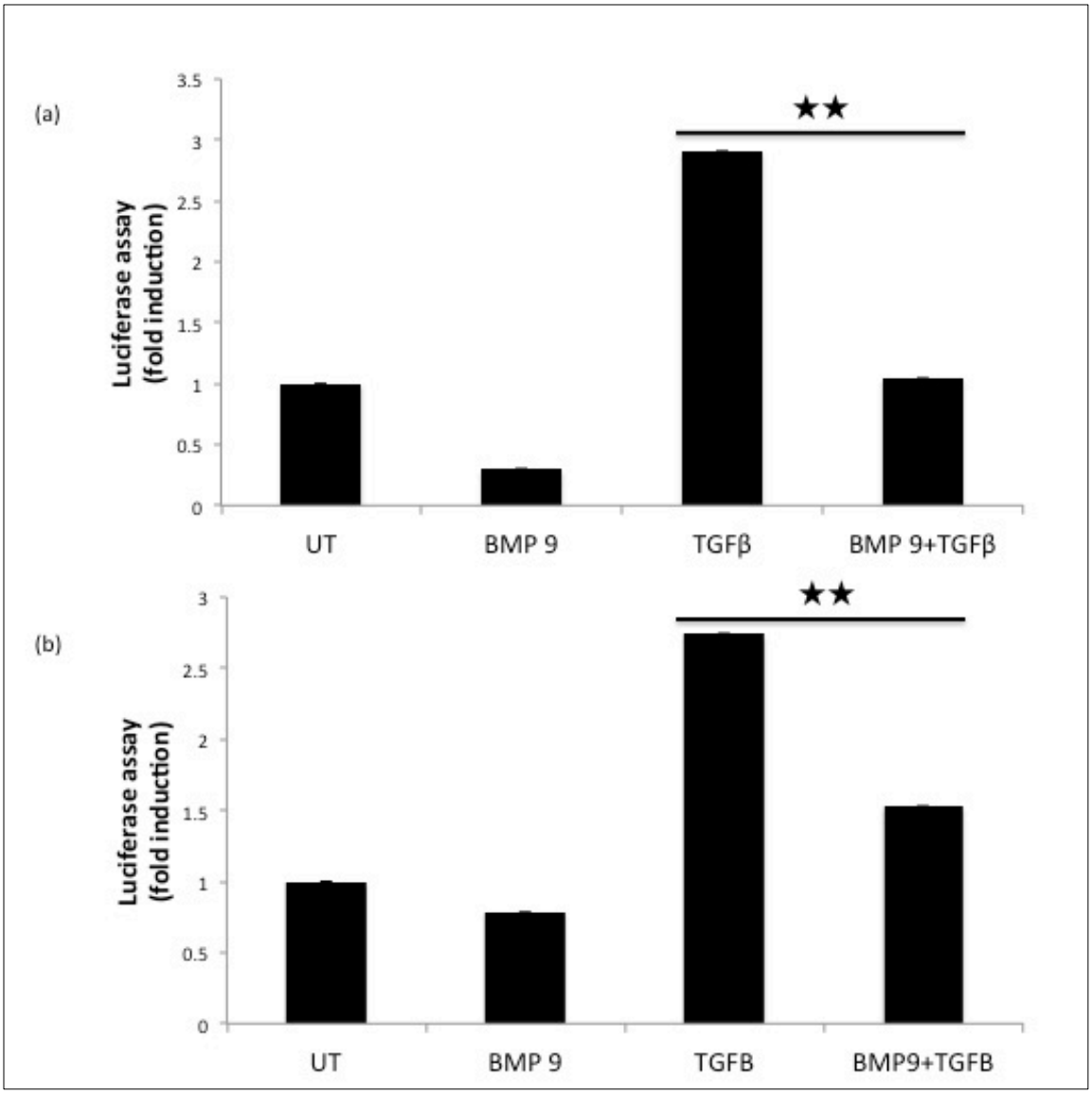

Figure 6.8 GDF-2 is able to suppress TGFβ mediated signaling. *(a) HEY and (b) SKOV3 cells were transfected with pE2.1-luciferase and treated with GDF-2 (10ng/ml) and TGFβ (100pM). The cells were then assayed by measuring luciferase activity. Data are shown as fold induction (relative to untreated cells). Statistical analysis was carried out using the paired t-test, ★★ = P<0.0001.*

CHAPTER 7

DISCUSSION

My study shows for the first time a role of GDF-2 in inducing smad2 linker phosphorylation in epithelial cells **[Figure 6.5].** It has been historically observed that BMPs and GDF-2 are able to induce smad signaling but it is somewhat restricted to smad1/5 **[Figure 6.1]**, and BMPs are also shown to induce smad1 linker phosphorylation but never smad2 or smad3 [1-5]. Therefore, my assumption was that GDF-2 would also induce smad1 linker phosphorylation and not smad2 linker. But I observed that GDF-2 is inducing smad2 linker phosphorylation instead of smad1 linker, which has not been reported before. I also noticed that GDF-2 is promoting the retention of smad2 in the cytoplasm via the phosphorylation of smad2 linker not allowing for it's nuclear translocation **[Figure 6.7].** The novelty in these observations is that GDF-2 is somehow mimicing TGFβ and showing the same effects on smad linker phosphorylation. I observed that similar to TGFβ's induction of linker phosphorylation via MAPK pathway [43, 44, 48, 49], GDF-2 is also able to activate the MAPK proteins and in

turn induce the linker phosphorylation of smad2 **[Figure 6.6]**. Another aspect of my study was to look for GDF-2's ability to suppress TGFβ mediated signaling. With the help of luciferase gene reporter assay, I was able to conclude that GDF-2 is indeed suppressing TGFβ mediated signaling. There are a lot of areas where this study can further go. The first of them would be to look for the different receptors (type I, II or III), which are responsible for bringing about the phosphorylation of linker region of smad2. One of the issues is that epithelial cells have very low levels of ALK1, which is known to be the specific receptor for GDF-2 in endothelial cells. Although previous studies have shown that GDF-2 can act via ALK2 [4, 15] or ALK3 (unpublished data from our group), and it's capacity to induce smad signaling is enhanced in the presence of Endoglin (a type III receptor) [65, 66], the question remains as to whether these receptors are also able to induce the linker phosphorylation in smads. There are different sites of phosphorylation in smads where MAPK proteins can phosphorylate. It is not known whether the same residues, which are getting phosphorylated by GDF-2, are the ones, which are responsible for TGFβ's suppression, by GDF-2. Site directed mutagenesis might be one of the ways to study this particular effect of GDF-2 on TGFβ. Linker phosohorylation of smads might be one of the key aspects of smad dynamics between the cytoplasm and nucleus. It's downstream biological consequences and relevance like cell proliferation, migration, invasion, might be used for therapeutical approaches.

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