Modulation of TGF-β Signaling by Hypoxia

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Abstract

A tumor is a fast-growing malignant tissue. This creates areas inside the tumor that are distant from local blood vessels to be able to get enough oxygen. This hypoxic condition activates a transcription factor called hypoxia inducible factor (HIF). HIF responses help a cell to adapt to decreased oxygen by activating glycolytic and angiogenesis pathways and by regulating apoptotic responses. Hypoxia drives the upregulation of a growth factor called transforming growth factor β (TGF-β). Similar to a hypoxia response, TGF is an important regulator of cell fate. TGF-β and HIF pathways regulate partially overlapping target genes. This regulation can also be cooperative. The TGF-β signal is initiated by activation of plasma membrane receptors that then activate effector proteins called small mothers against decapentaplegic (Smad) homologs. In healthy tissue, TGF-β keeps cell proliferation and growth under control. During cancer progression, TGF-β has shown a dual role, whereby it inhibits initial tumor formation but, conversely, in an existent tumor, TGF-β drives malignant progression. Along with HIF and TGF-β also protein dephosphorylation is an important regulatory mechanism of cell fate. Protein dephosphorylation is catalyzed by protein phosphatases such as Protein phosphatase 2A (PP2A). PP2A is a ubiquitous phosphatase that can exist in various active forms. PP2A can specifically regulate TGF-β signaling either by enhancing or inhibiting the receptor activity. This work demonstrates that during hypoxia, PP2A is able to fine-tune TGF-β signal by specifically targeting Smad3 effector in a Smad7-dependent manner. Inactivation of Smad3 in hypoxia leads to malignant conversion of TGF-β signaling.

KEY WORDS: Hypoxia, HIF, TGF-β, Protein phosphatase 2A, Smad3, Smad7, cancer

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Tiivistelmä

Syöpäkasvain on nopeasti kasvavaa kudosta, johon muodostuu hapenpuutteesta kärsiviä eli hypoksisia alueita, jotka ovat liian kaukana verisuonista, jotta happi pääsisi kulkeutumaan soluihin. Hypoksia johtaa HIF-transkriptiotekijän aktivoitumiseen. HIF aktivoi glykolyyttisten ja angiogeneettisten proteiinien tuottoa ja säätelee apoptoosia. Nämä mekanismit pyrkivät pitämään solun elossa hapen puutteesta huolimatta. Hypoksia lisää myös TGF-β –kasvutekijän tuottoa. HIF:n tavoin TGF-β:kin on oleellinen solun toimintojen säätelyssä. Molemmat edellä mainitut säätelevät osittain samoja geenejä, joko erikseen tai toistensa toimintaa tehostaen. TGF-β signalointi alkaa solukalvon reseptorin aktivoinnilla. Reseptori puolestaan aktivoi Smad:ksi kutsuttuja välittäjäproteiineja, jotka aktivoivat kohde geenien transkription. TGF-β signalointi estää terveen kudoksen liiallisen kasvun ja kudoksen solujen hallitsemattoman jakaantumisen. Syövässä TGF-β:lla on kuitenkin kahtalainen rooli. TGF-β estää aluksi kasvaimen syntymistä, mutta jo muodostuneessa kasvaimessa TGF-β tehostaa solujen kasvua ja muuntumista pahanlaatuisiksi. HIF:n ja TGF-β:n lisäksi proteiinien defosforylaatio on tärkeä säätelymekanismi soluissa. Defosforylaatiota katalysoivat fosfataasientsyymit, kuten proteiinifosfataasi 2A (PP2A). PP2A on yleisesti soluissa toimiva entsyymi, jolla on useita erilaisia aktiivisia muotoja. PP2A pystyy joko aktivoimaan tai estämään TGF-β -reseptorin toimintaa. Tässä väitöskirjassa näytetään, että PP2A defosforyloi hypoksiassa Smad3-proteiinia ja että tähän reaktioon vaaditaan Smad7-proteiini. Smad3-proteiinin inaktivaatio aiheuttaa TGF-β -signaalin muuntumisen pahanlaatuiseksi hypoksiassa.

AVAINSANAT: Hypoksia, HIF, TGF-β, Proteiinifosfataasi 2A, Smad3, Smad7, syöpä.

Table of contents

Abbreviations

ALK	Activin receptor-like kinase
ATP	Adenosine triphosphate
BMP	Bone morphogenetic protein
BrdU	Bromodeoxyuridine
CAMK II	Calcium/calmodulin-dependent protein kinase II
CDK	Cyclin-dependent kinase
co-Smad	Common mediator Smad
EGLN	Drosophila egg laying gene/protein
ERK	Extracellular signal-regulated kinase
GADD34	Growth-arrest and DNA-damage inducible protein 34
GDF	Growth and differentiation factor
Glut1	Glucose transporter 1
GSK3	Glycogen synthase kinase-3
HaCaT	Human epithelial cell line derived from *in vitro* spontaneously transformed keratinocytes from histologically normal skin
HeLa	Human malignant epithelial cell strain derived from a carcinoma of the cervix
HIF	Hypoxia-inducible factor
HPH	Hypoxia-inducible factor prolyl hydroxylase
HRE	Hypoxia response element
HUVEC	Human vein endothelial cells
i-Smad	Inhibitory Smad
ID1	Inhibitor of DNA binding 1
JNK	Stress-activated protein kinase JNK

LacZ	The *E. coli* lacZ gene encoding β-galactosidase and is often used as a reporter gene
MAPK	Mitogen activated protein kinase
MH	Mad-Homology domain
NMuMG	Normal mouse mammary gland cell line
P15	Cyclin-dependent kinase inhibitor 2B (CDKN2B)
P300	Histone acetyl transferase P300
PHD	Proline hydroxylase domain
PP1C	Protein phosphatase 1C
PP2A	Protein phosphatase 2A
PPM1A	Protein phosphatase M1A
PPP2###	Phospho protein phosphatase 2 (subunit ### e.g., PPPR5E)
PR65	Protein phosphatase 2 (formerly 2A), regulatory subunit A
pVHL	von Hippel-Lindau protein
ROCK	Rho-associated protein kinase 1
r-Smad	Receptor-regulated Smad
SBE	Smad binding element
SCC	Squamous cell carcinoma
SCP	Small C-terminal domain phosphatase
siRNA	Small interfering RNA
Smad	Small mothers against decapentaplegic homolog
TGF-β	Transforming growth factor *beta*
TGF-β[SF]	Transforming growth factor *beta* superfamily
UTSCC	University of Turku squamous cell carcinoma
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications (referred to with Roman numerals in the text):

I Heikkinen PT, Nummela M, Leivonen SK, Westermarck J, Hill CS, Kähäri VM, Jaakkola PM. Hypoxia-activated Smad3-specific dephosphorylation by PP2A. **Journal of Biological Chemistry**. 2010 Feb 5;285(6):3740-9.

II Heikkinen PT, Nummela M, Jokilehto T, Grenman R, Kähäri VM, Jaakkola PM. Hypoxic conversion of SMAD7 function from an inhibitor into a promoter of cell invasion. **Cancer Research**. 2010 Jul 15;70(14):5984-93.

1 Introduction

Multicellular life has evolved over time into highly complex systems. Sustaining multicellular life requires a finely-tuned regulation and surveillance of the behavior of each cell. Complex vertebrates need many highly specialized cell types with each having its own field of expertise, each serving its own purpose.

Around 200 different cell types constitute a human being. In theory, any type of cell can give rise to cancer if the surveillance mechanism for one reason or another fails. In practice, however, the sophisticated surveillance mechanism prevents these potentially malignant changes and many errors need to accumulate in one cell before the cell turns cancerous.

Hypoxia (*i.e.*, reduced tissue oxygen supply), transforming growth factor β (TGF-β), and protein kinases have all been areas of intensive investigation in the cancer research field for the past decades. Virtually all tumors are at least partially (spatially and temporarily) hypoxic because the rapidly growing cell mass quickly outruns the sufficient vasculature that provides the cells with oxygen (Gatenby et al. 2004). Hypoxia is known to strongly affect cellular gene expression, metabolism, angiogenesis, and cell survival. Hypoxic effects are thought to ultimately create a selection pressure that drives the malignant cancer's evolution. One of the most important factors of this development is TGF-β, the expression of which is also activated by hypoxia. Nevertheless, currently, there is only a limited amount of information about the hypoxic regulation of TGF-β signaling.

Normal cells produce TGF-β, which regulates cell proliferation and growth to maintain tissue homeostasis. Tumor cells also produce TGF-β but unlike normal cells, malignant cells do not respond to TGF-β growth limiting signals (Massague et al. 2000)**.** In fact, the normal growth limiting effect of TGF-β can be totally turned around in tumors. Tumor cells use TGF-β to drive their proliferation and growth while the surrounding normal tissue is inhibited and this gives a greater advantage to cancer cells (Massague et al. 2000).

Little by little also a role of protein phosphatases in regulating TGF-β signaling has emerged. Like many other pathways linked to cancer also TGF-β signaling needs kinase activity to function. Phosphatases are capable of turning off the kinase activated signals and therefore also phosphatases are proven to be essential in regulating cell fate.

TGF-β and hypoxia have long been known to be essential in carcinogenesis. Recent discoveries have demonstrated also the importance of phosphatases in tumorigenesis. So far, reports indicate phosphatases to be anti-tumorigenic. However, the phosphatases and their targets in TGF-β signaling under hypoxia are not yet fully understood. In this thesis, hypoxia and phosphatases in malignant conversion of TGF-β pathway are studied.

2 Review of the literature

2.1 Hypoxia in cancer

In year 2012 cancer was responsible of 8,2 million deaths worldwide according to the World Health Organization (WHO) (WHO 2015). The WHO estimation is that cancer will increse its portion of all deaths while the life expectancy increases. The current estimate is that in the year 2030 over 13.1 million individuals will die of cancer (Malvezzi et al. 2012) but to date, the most recent confirmed figures available are those from 2012 and are found at the WHO website (http://www.who.int/cancer/en/). This development can already be seen in developed countries where cancer incidence is significantly more frequent than in less developed areas (WHO 2015). The WHO also estimates that 40 percent of fatal cancer cases could be prevented by healthier lifestyles and patient screening.

Even though cancer therapies have been developing over the years, early diagnosis is the primary and most efficient treatment. The reason for the non-existence of a generic cancer drug is the fact that each cancer can be regarded as an individual disease. There are at least 100 different types of cancers. However, all cancers share common properties which make them superior to healthy tissue (Hanahan et al. 2011). These properties, however, are not easy for a cell to obtain.

To become cancerous, a cell has to gain self-sufficiency on growth signals, be able to escape from growth limiting factors, become resistant to apoptosis, harbor unlimited replication potential, activate constant angiogenesis and be able to form metastasis through invasion (Hanahan et al. 2011). A single cell that is able to avoid apoptosis and to proliferate limitlessly may serve as the origin of a tumor.

Uncontrolled cell proliferation in a tumor leads to a situation where oxygen diffusion from blood is no longer adequate and thus, leads to hypoxia within the tissue. Hypoxia is therefore an inevitable part of the tumor microenvironment. There is an evidence-driven hypothesis that hypoxia creates a selection pressure where only the strongest cells survive. This

selection favors only the most malignant cells that can cope with a hostile microenvironment (Semenza 2000; Vaupel 2004).

For cancer progression, the selection of the most malignant cell is of great importance. In order to metastasize, the cells need to gain several aggressive features. A metastasis forming cell has to be able to leave the primary tumor and to stay alive on the journey through blood circulation or the lymphatic system. Moreover, to form a metastasis, a cell needs to have means to leave the blood flow and to invade into tissue. Key events in metastasizing are breaking free from cellular tight junctions and acquiring the ability to move. Normal epithelial tissue is highly organized and cells are bound together by adhesion proteins, such as Cadherins (Yilmaz et al. 2010). To be able to migrate, a cell needs to modify its way of interacting with the extracellular matrix. Because all normal cells are highly specified for one purpose and designed to survive in a specific microenvironment, a metastatic cell has to be able to grow in a foreign and hostile environment. Primary tumors, if diagnosed early enough, can often be removed surgically and are unlike metastatic tumors that can reside at several places. That is why primary tumors rarely are lethal but metastases are (Leong 2013).

Hypoxia is a physiological condition where oxygen concentration is too low for energy production through oxidative phosphorylation and hence survival. On the other hand, hypoxia is an important factor during embryonic development where it regulates for example apoptosis for the fetus to form correctly (Chen et al. 1999). A fast-growing tumor reaches quickly the limit of oxygen diffusion from blood vessels. This in turn creates a hypoxic core where cells are under selection pressure potentiating the formation of a malignant tumor. (Graeber et al. 1996; Ryan et al. 1998). Without some means to sustain oxygen delivery, the tumor core will go into necrosis due to complete lack of energy (Gatenby et al. 2004). The lack of oxygen is overcome in the tumor microenvironment by hypoxia activated response at transcriptional level driven by the hypoxia inducible factors (HIFs) (Semenza 2000).

2.2 Mechanisms of hypoxic signaling

HIF is a dimer of α and β subunits (Jiang et al. 1996). HIF-α protein is mainly regulated posttranslationally while the mRNA levels of both α and β subunits are relatively stable (Wang et al. 1995; Hirose et al. 1996; Huang et al. 1996). The regulation of the α-subunit is based on steady state protein production and simultaneous degradation through proteasomes under normoxia, while the β subunit is stable (Figure 1). The constant production is costly energywise but it enables an immediate response for decreased oxygen and quick response to decreased energy production (Huang et al. 1998).

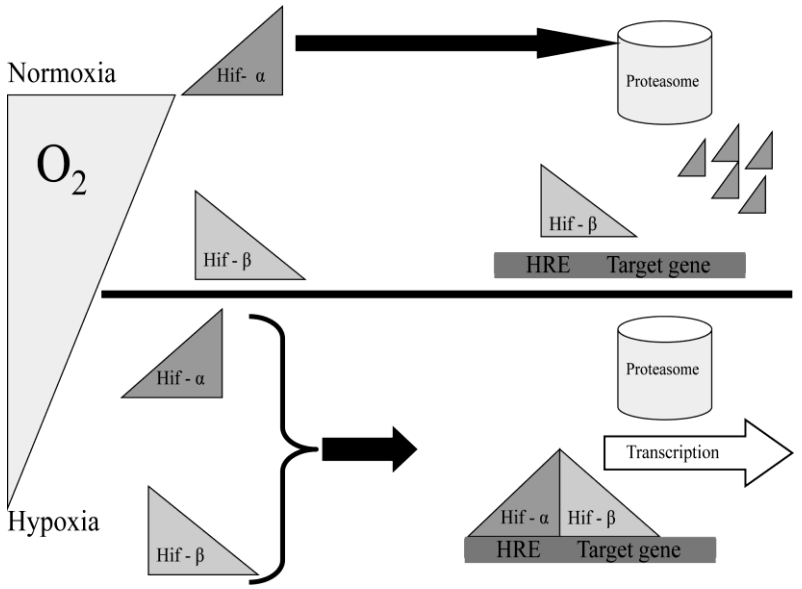

Figure 1 Hypoxic gene response is activated by low oxygen concentration. HIF transcription factor is regulated by proteosomal degradation of the α subunit under normoxia. Functional HIF can only be formed when both α and β subunits are present under hypoxia. When oxygen level decreases, the α subunit is stabilized and HIF can activate target gene transcription.

Thus far, three different HIFs, *i.e.* HIF1, HIF2 and HIF3, have been identified. The ubiquitous HIF1 is regarded as the main regulator of hypoxic genes. HIF2 is expressed in a limited set of tissues and it also has different although overlapping sets of target genes shared with HIF1. The function of HIF3 was

thought to be inhibitory for the other two HIFs but recent data indicates that it has transcriptional activity. (Loboda et al. 2010; Zhang et al. 2014)

The α subunit of HIF is targeted to proteasomes by hydroxylation and ubiquitination of specific proline residues. The hydroxylation is carried out by enzymes called PHDs (prolyl hydroxylase domain containing protein), HPHs (HIF prolyl hydroxylase) or EGLNs (homolog of *Drosophila egln-9*). Three different PHD isoforms are known (Bruick et al. 2001; Epstein et al. 2001). Out of these, PHD2 is regarded as the main regulator of HIF-1α stability (Jaakkola et al. 2001; Berra et al. 2003).

HIF regulation by the PHD hydroxylases is dependent on the availability of oxygen, 2-oxoglutarate, and ferrous iron (Fe^{2+}). The PHD-enzymes catalyze a reaction that transfers the oxygen atom from O_2 to two specific proline residues within HIF (prolines 402 and 564 in human HIF-1α protein) (Ivan et al. 2001; Jaakkola et al. 2001; Masson et al. 2001). The remaining oxygen is used to convert 2-oxoglutarate into CO_2 and succinate (Figure 2). This catalytic reaction is similar to collagen hydroxylases (Kivirikko et al. 1998). The hydroxylation reaction mechanism is therefore dependent on Krebs cycle functionality and can be inhibited by Krebs cycle intermediates (Koivunen et al. 2007). Also removing iron from the reaction results in stabilization of HIF and therefore mimics hypoxic response. This can be achieved by divalent ions such as cobalt or by iron chelators (Hirsila et al. 2005; Serra-Perez et al. 2010).

The hydroxylated proline residues in HIF-α amino acid sequences serve as binding sites for the von Hippel-Lindau protein (pVHL). VHL binding to hydroxylated proline residues initiates the formation of the E3 ubiquitin ligase complex (Maxwell et al. 1999). Ubiquitin ligase adds a chain of ubiquitins to HIF which targets HIF to proteasomes to be degraded (Figure 2) (Pause et al. 1997; Stebbins et al. 1999; Cockman et al. 2000; Kamura et al. 2000; Ohh et al. 2000; Tanimoto et al. 2000; Ivan et al. 2001; Jaakkola et al. 2001). Inactivation of pVHL function by deletion or mutation switches the HIF-pathway to a constant “on” mode. This is the case in the majority of renal clear cell carcinomas, which form typically highly vascularized tumors highlighting the importance of HIF signaling in angiogenesis (Kim et al. 2004).

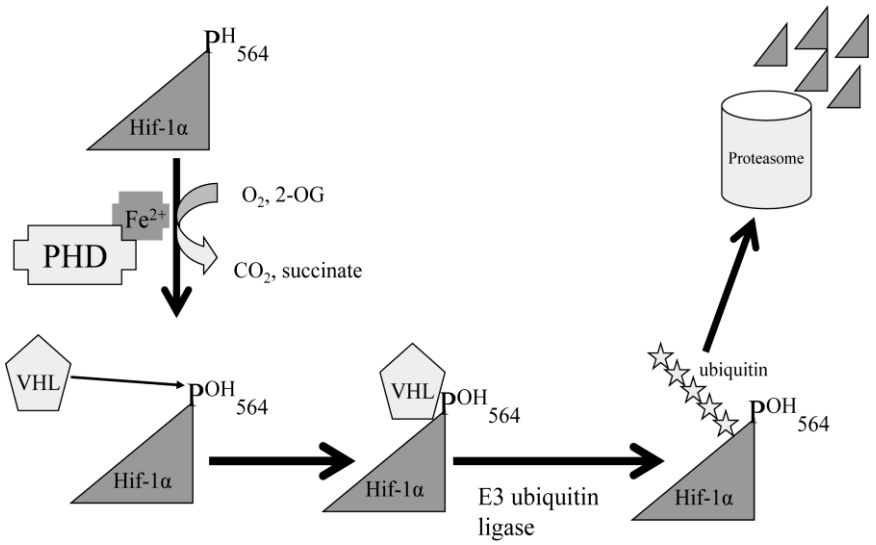

Figure 2 HIF is regulated by PHD enzymes, which require oxygen, 2-oxoglutarate, and iron as cofactors. HIF-1α is hydroxylated at proline 564 (or 402) by PHD-enzymes. The hydroxylated proline residue binds to pVHL and activates E3-ubiquitin ligase. This ubiquitination targets HIF-1α to proteasomes for degradation.

HIF regulates the expression of a large set of genes, approximately 200 that enable cells and tissues to respond to hypoxia. These genes are linked for example to survival, metabolism, and angiogenesis but also to apoptosis (Carmeliet et al. 1998; Chen et al. 1999; Sugishita et al. 2004). In fact, fully functional HIF signaling is an absolute requirement for embryonic development. Mouse studies have shown that HIF1 deficient mice are not viable (Wang et al. 1995; Ryan et al. 1998; Kotch et al. 1999). Unfortunately, such an important regulator of development and cellular survival is also important for developing cancer and advancing tumor (Ryan et al. 1998).

HIF-regulated genes include a majority of glycolytic enzymes coding genes such as *Glucose transporter 1 (Glut1)* and the activation of which can be used in cancer profiling (Semenza et al. 1994). Tumors often have a high glycolytic rate, which can be monitored by the expression of *Glut1* (Busk et al. 2008; Iida et al. 2008). The necessity of fast energy production for cancer is highlighted by the ability of cancer cells to facilitate aerobic glycolysis and excess lactate production known as the Warburg effect and to gain advantage over other healthy cells (Warburg et al. 1927; Warburg 1956; Vander Heiden et al. 2009).

Several of the HIF target genes such as vascular endothelial factor (*VEGF)* participate in angiogenesis (Flamme et al. 1997). Angiogenic signaling activates production of new blood vessels and ensures the energy transportation for the tumor (Carmeliet et al. 1998). Angiogenesis could also be regarded as part of survival response: anoxia or severe hypoxia causes necrosis, meanwhile a moderate hypoxia can drive tumor growth. Angiogenesis is, however, only one of the many responses to hypoxia. Before a tumor can make the most out of angiogenesis, it needs to escape apoptosis by adjusting the gene expression of anti- and pro-apoptotic genes and activating autophagy (El-Khattouti et al. 2013).

Finally, hypoxia also activates production of several growth factors, including transforming growth factor β (TGF-β). While TGF-β is regarded as a growth limiting growth factor in normal tissue. In cancer TGF-β can function as a growth promoter. The mechanism behind this dual role is not fully understood (Akhurst et al. 2001).

2.3 Transforming growth factor-β superfamily

The TGF-β superfamily (TGF-β[SF]) of multipotent growth factors regulates cell differentiation, proliferation, migration, and growth. Depending on the TGF-β[SF] growth factor, the signal can be read either by the secreting cell in autocrine signaling or by adjacent cells in paracrine signaling (Wrighton et al. 2009) (Figure 3). The TGF-β[SF] growth factors have been documented to participate in the regulation of several different pathways. For example, during inflammation reactions, some family members act as chemotactic agents for cells of the immune system (Poniatowski et al. 2015). In the wound healing process, the growth factor forms a gradient that guides macrophages to the wounded area and at the same time it activates keratinocyte migration, fibroblast contraction and production of extra cellular matrix proteins to effectively close the wound (Pakyari et al. 2013).

TGF-β[SF] growth factors form a large family of secreted regulatory proteins. The family is sectioned into different subfamilies: Transforming growth factor-βs (TGF-βs), bone morphogenetic proteins (BMPs), activins, and

inhibins. More distantly related family members are nodal, lefty as well as growth and differentiation factors (GDF). (Wrana 2013)

TGF-βs regulate cell growth, proliferation, migration, and survival. TGF-βs have also regulatory functions in angiogenesis. There are three isoforms of TGF-β, namely TGF-β1, 2, and 3. Unlike the rest of the growth factor superfamily members, the TGF-βs are secreted as latent forms that needs to be activated before TGF-βs can bind to its receptor (Figure 3) (Roberts 1998).

The BMPs are big group of related family members inside the TGF-β[SF]. The BMPs were first found to regulate bone development and were named after their function. Later the BMPs have been shown to regulate embryonic development and also cell fate in various cell types (Balemans et al. 2002).

The activins and inhibins form a group of growth factors that are regarded as close relatives with TGF-βs because they transduce their signals through the same downstream effectors as TGF-βs. Activins regulate reproduction and extracellular matrix production as well as cell fate. Inhibins are true to their name and inhibit activin signaling. (Loomans et al. 2014)

GDF, nodal, and lefty form the last group that is most distant from other members of the TGF-β superfamily. These growth factors regulate several processes such as: reproductive organ development, neuronal development, cell survival, spermatogenesis, embryogenesis and kidney development. (Knight et al. 2006; Robertson 2014; Shiratori et al. 2014)

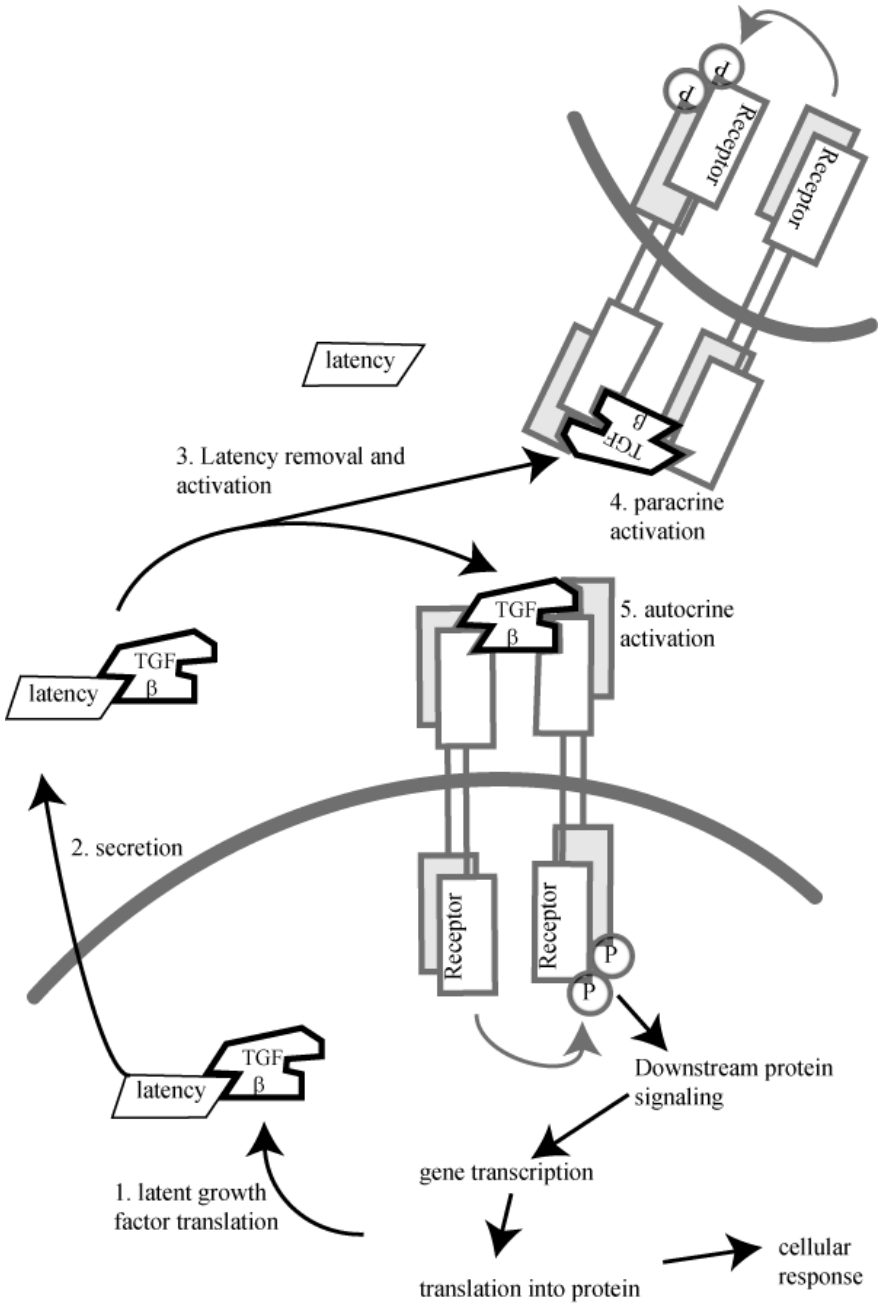

Figure 3 TGF-β -superfamily signaling principle. Translation of the growth factor protein **(**1). TGF-β1-3 are the only members of the growth factor superfamily that are secreted as latent and need to be activated. Growth factor is secreted into extracellular space (2). Removing latency activates TGF-β (3). After activation the signal can be used by neighboring cells in a paracrine manner (4) or locally in an autocrine manner (5).

2.4 TGF-β signaling

2.4.1 TGF-β Receptors

TGF-β[SF] signaling operates via membrane bound receptors that recognize the growth factors secreted by the cells. Each of the growth factor family member has a special set of receptors and different receptor combinations dictate which downstream proteins are utilized. These signal transducers are called receptor-activated Smads (r-Smad) (See section 2.4.2: Receptor-regulated Smads) (Wrana 2013).

There are three domains in the TGF-β receptor structure: extracellular, transmembrane, and cytoplasmic domains (Derynck 1994) (Figure 4). The extracellular domain serves as a docking site for activated growth factors. The transmembrane domain attaches the receptor to the cell membrane. The cytoplasmic tails are serine/threonine kinases that activate signal transducing r-Smads (Wrana 2013).

The receptors are divided into types 1-3. The type 1 receptors are also called Activin receptor kinase like (ALK) receptors (Kingsley 1994; Wakefield et al. 2013). There are seven type 1 receptors ALK1-7. Two of the type 1 receptors, ALK1, and ALK5, bind and transduce TGF-β ligand signals (de Caestecker 2004; Wakefield et al. 2013). BMP signals are transduced by ALK1, 2, 3, 4 and 6. Activins bind to ALK4, and ALK7 (Derynck et al. 1997; Wakefield et al. 2013). ALK7 and also ALK4 receptors serve as a docking site for nodal (Reissmann et al. 2001; Wakefield et al. 2013). Each cell type expresses specific set of receptors and the receptors present on cell surface dictate the cellular response to TGF-β[SF] growth factors (Wakefield et al. 2013).

The type 2 receptor has similar domains than type 1 receptors and it can also bind ligands. Even though both type 1 and type 2 receptors can bind to ligands, they need to form a complex to transduce the signal in the TGF-β pathway (Kingsley 1994; Wakefield et al. 2013).

The type 3 receptor, also termed Betaglycan, forms a complex with type 1 and 2 receptors and enhances ligand binding but it lacks enzymatic activity that is

needed for signal transduction (Gatza et al. 2010). The type 3 receptor is important during development as the knockout leads to embryonic lethality. In cancers the loss of TGF-β receptor type 3 is linked to malignancy. Interestingly, type 3 receptor expression is downregulated by TGF-β1 (Gatza et al. 2010).

As noted earlier, TGF-β is a special case inside the superfamily as the TGF-β is secreted as a latent form that needs to be activated prior to signal transduction (Figure 3) (Roberts 1998). Activated TGF-β binds to the extracellular domain of TGF-β-receptor type 2 which followed by complex formation with receptor type 1. The type 2 receptor then phosphorylates the type 1 receptor. This results in a fully activated receptor complex capable of phosphorylating and activating r-Smads (Huse et al. 2001; Wakefield et al. 2013) (Figure 4).

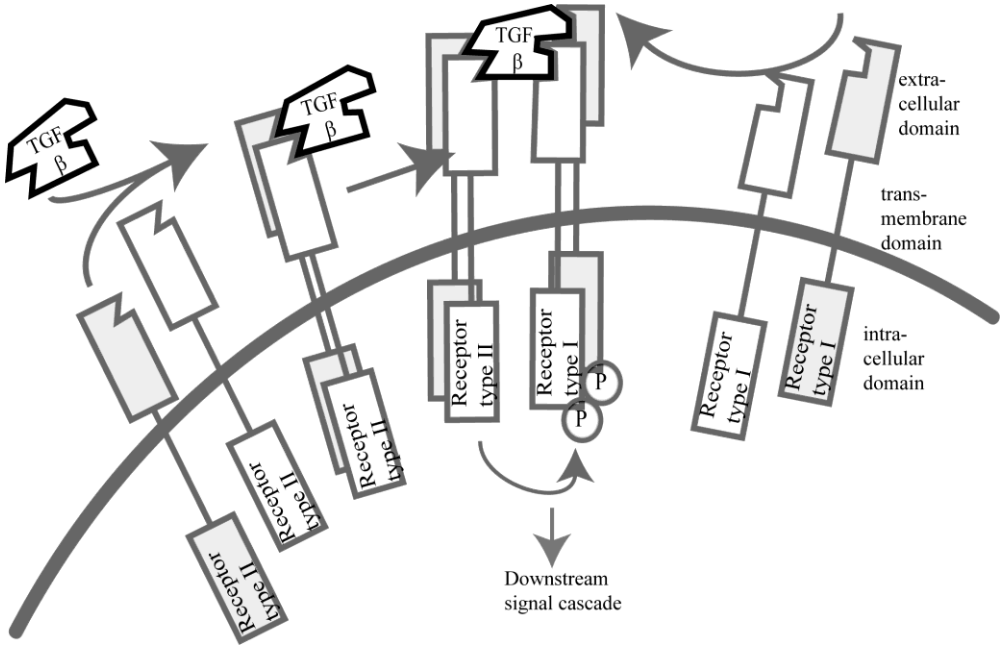

Figure 4 TGF-β receptor activation. Proteolytically activated TGF-β binds to the extracellular domain of the receptor type 1 and 2. The binding brings the intracellular domains of the two receptor types into close proximity with each other and enables the type 2 receptor to activate the type 1 receptor by phosphorylation. This receptor complex then initiates the intracellular signal cascade in response to TGF-β.

2.4.2 Receptor-regulated Smads

From the receptors, the signal is carried on by special proteins called Smads. The family of Smad proteins consists of eight closely related proteins (Smad1-8). Five out of the eight Smads are called receptor-regulated Smads (r-Smads), Smad1, 2, 3, 5, and 8. The r-Smads transduce the growth factor signal to the nucleus together with a co-Smad (Smad4), which is needed for nuclear localization. The remaining two are inhibitory Smads (i-Smads) (Smad6 and 7) that regulate the growth factor signal duration inside the cell. (Moustakas et al. 2001; Itoh et al. 2007; Macias et al. 2015). The cellular response to TGF-β-family growth factors depends on the activated r-Smads and their posttranslational modifications (Xu 2006). Furthermore the signal is affected by the availability of i-Smad and co-Smad (Itoh et al. 2007; Yan et al. 2009).

The r-Smads are conserved posttranslationally regulated transcription factor proteins and the main signal transducers in TGF-β family signaling. The r-Smads have three domains: a DNA binding MH1-domain, a linker-domain, and a receptor binding MH2-domain (Figure 6) (Wu et al. 2000; Macias et al. 2015). The TGF-β signal is transduced by receptor-mediated phosphorylation of specific C-terminal serine residues in the r-Smad MH2-domain. These receptor phosphorylated residues are: S422, S423 and S425 in Smad3 and S464, S465 and 467 in Smad2 (Abdollah et al. 1997; Chacko et al. 2001). Serine phosphorylation activates r-Smads and activated Smad2 or Smad3 can be mimicked by mutating the above mentioned C-terminal serines to glutamates (Funaba et al. 2000). C-terminally phosphorylated r-Smads carry the TGF-β signal from the plasma membrane to the nucleus, but cannot function alone. Instead, they depend on binding to a co-Smad to form an active transcription factor (Funaba et al. 2000). An active r-Smad-co-Smad-complex translocates to the nucleus and binds to a target sequence in the DNA to activate transcription. Which r-Smad is activated depends on the receptor combination and the growth factor in question. BMP, Activin and TGF-β activate different r-Smads in different cellular context (Shi et al. 2003). In epithelial cells, TGF-βs recruit mainly Smad2 and Smad3 to transduce the signal. Although in endothelial cells TGF-β can also activate Smad1 depending on cell surface receptor combinations Smad1, Smad5 and Smad8 are mainly the messengers for BMP growth factors (Miyazawa et al. 2002).

Despite of the high sequence homology, about 84% in total, and common regulatory biochemistry, Smad2 and Smad3 seem to have nonredundant although partially overlapping functions. (Alingments: http:// www. uniprot. org/ align/ with Smad3: http:// www. uniprot. org/ uniprot/P84022, Smad2: http:// www. uniprot. org/ uniprot/ Q15796) Evidence for this rises from Smad2 and Smad3 knockout and knockdown studies. In early embryo development, Smad2 knockout is lethal. Smad3 knockout is not lethal but knockout mice are smaller than wild type mice and develop metastatic tumors and have impaired immune response. These anomalies ultimately lead to death (Ashcroft et al. 1999; Chang et al. 2002; Roberts et al. 2003). However, Smad3 deletion results in an acceleration of wound healing and reduced inflammation in the wound, due to the activation of keratinocyte migration and inactivation of macrophage chemotaxis by TGF-β (Ashcroft et al. 1999; Ashcroft et al. 2000).

2.4.3 Regulatory Smads

Receptor-mediated activation of r-Smad is not enough for the signal to reach the nucleus. The nuclear transport of activated r-Smad is mediated by a special co-Smad (Smad4) protein. After receptor mediated phosphorylation, r-Smads form a complex with the co-Smad and this active complex is then translocates into the nucleus where it binds to a Smad binding element (SBE) in DNA to activate its target genes (Moustakas et al. 2001). The availability of co-Smad for complex formation is regulated posttranslationally by ubiquitination, sumoylation and phosphorylation (Roelen et al. 2003; Dupont et al. 2012; Heldin et al. 2012).

Because constant TGF-β signaling could be harmful, a cell needs to limit the duration of signal activation. Out of the eight Smads two, *i.e.* Smad6 and Smad7, are inhibitory Smads (i-Smads) (Bai et al. 2000; Yan et al. 2009). The best characterized function of i-Smads is to attenuate TGF-β signaling. Smad7 can inhibit all of the r-Smads from activating transcription and Smad6 is able to inhibit BMP signaling (Bai et al. 2000; Yan et al. 2009). Structurally, human i-Smads share homology in MH2 domain but in total only about 20% homology with human Smad2 and Smad3. The i-Smads lack the homology in MH1 domain and they lack the C-terminal phosphorylation sites. (Alingments: http:// www. uniprot. org/ align/ with Smad3: http:// www.

uniprot. org/ uniprot/ P84022, Smad2: http:// www. uniprot. org/ uniprot/ Q15796 and Smad7: http:// www. uniprot. org / uniprot/ O15105). (Hanyu et al. 2001). In principle this means that an i-Smad can bind to receptors but cannot be phosphorylated to carry on the growth factor signal. They compete with the r-Smads in receptor or co-Smad binding and therefore inhibit r-Smad transcriptional activity (Figure 5) (Yan et al. 2009). This competitive inhibition is one of the most fundamental mechanisms involved in the regulation of Smad signaling. Interestingly, i-Smads are targets for r-Smad activated transcription. In fact, the *Smad7* gene is among the very first genes to respond to a TGF-β stimulus creating an autoinhibitory effect of TGF-β signaling (Nakao et al. 1997).

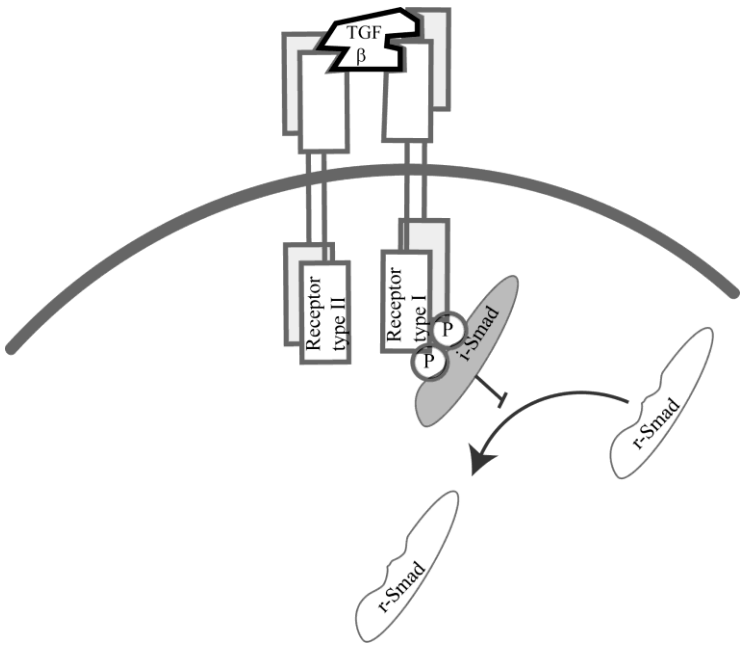

Figure 5 Receptor inhibition by i-Smad. Membrane bound receptor complex is activated upon TGF-β binding. Phosphorylated type 1 receptor serves as a docking site for the r-Smads. The i-Smad competes for binding to the activated receptor with r-Smads. The i-Smad cannot be activated and therefore it blocks r-Smad access to the receptor. The net result is inhibited TGF-β signaling.

Intense research in the TGF-β field has revealed several other functions for i-Smads. In addition to competitively substituting r-Smad from activated receptors the i-Smads target activated r-Smads or receptors for ubiquitination and proteasomal degradation (Yan et al. 2009). Smad7 can also inactivate

receptors by recruiting the protein phosphatase PP1C to dephosphorylate the activated receptor (Figure 5 and Figure 8) (Ebisawa et al. 2001; Shi et al. 2004).

2.5 Other regulators of TGF-β signaling

In addition to the above mentioned regulation of r-Smads they are posttranslationally regulated. Kinases such as mitogen activated kinases (MAPK), rho associated protein kinase (ROCK) and extra cellular regulated kinases (ERK) at the linker region, phosphateses such as small C-terminal domain phosphatases (SCPs) and protein phosphatase M1A (PPM1A) at linker and MH2 regions and acetylases such as histone acetyl transferace P300 (P300) at the MH1 and MH2 regions. (Figure 6) (Liu et al. 2010; Kamato et al. 2013). The i-Smads and co-Smad are regulated by ubiquitination, acetylation, phosphorylation and receptor binding enhancer proteins (Moustakas et al. 2001; Gronroos et al. 2002; Wrighton et al. 2008). The precise function of all the known posttranslational modifications are not fully understood. Dephosphorylation of r-Smad C-terminus has been identified to be an important modifier of Smad signaling (Duan et al. 2006; Lin et al. 2006; Wrighton et al. 2006). Moreover, linker region dephosphorylation by SCPs is known to regulate r-Smad functions. In the case of Smad4, the linker region phosphorylation is a part of the nuclear localization regulation (Roelen et al. 2003). The linker region phosphorylation of r-Smads seems to have rather versatile outcome. R-Smad linker modifications have been linked to Smad protein stability, interactions, and even transcriptional activity. Acetylation of Smad2 and Smad3 might enhance their signal transduction function (Wrighton et al. 2008).

Regardless of the existence of several r-Smads and layers of regulation, all the r-Smads share three common mechanisms inside the pathway: all r-Smads are activated by receptors, they all require a co-Smad to be able to activate target gene transcription, and they all can be inhibited by inhibitory Smads.

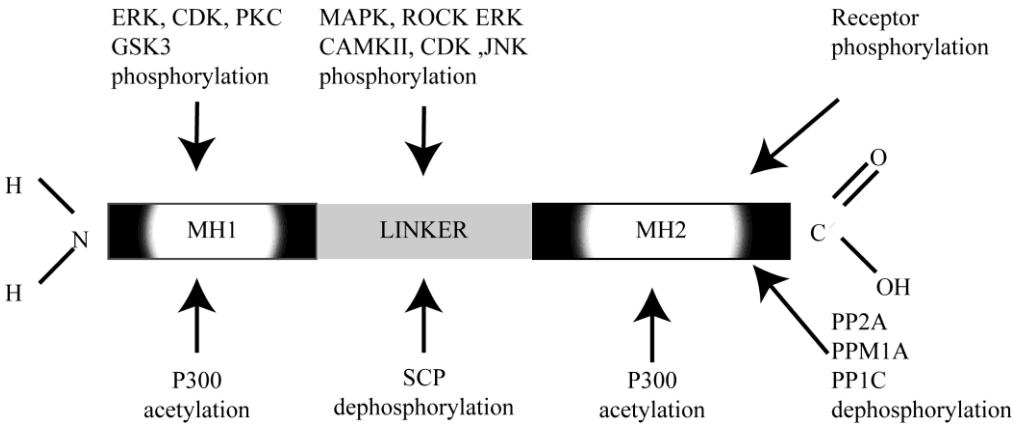

Figure 6 R-Smad structure and regulation. The r-Smads are subjects to phosphorylation, dephosphorylation and acetylation. The linker region phosphorylation is performed by several kinases and dephosphorylated by SCP's. C-terminus is dephosphorylated by PP2A, PPM1A and PP1C. The C-terminal phosphorylation in MH2 domain is regulated by receptor kinases. MH1 domain binds Smad binding element (SBE) in DNA for transcription initiation..

2.6 TGF-β signaling in cancer

TGF-β/Smad signaling has a versatile and controversial role in cancer. The dual role of TGF-β was demonstrated in a study by Cui *et al.* using a mouse model (Cui et al. 1996). They showed that TGF-β inhibits the outbreak of cancer by suppressing the formation of benign tumors. The function of TGF-β in normal tissue hinders the growth of epithelial cells and therefore TGF-β is considered to be a tumor suppressor. However, if a malignant tumor forms, then TGF-β function changes starting to enhance the aggressive metastasis formation and potentiate cancer growth (Cui et al. 1996). In line, secreted TGF-β creates a chemotactic signal for monocytes and other inflammation and immune response related cells in tumors. This has been shown to be Smad3 dependent in knockout mice (Ashcroft et al. 1999; Yang et al. 1999; Ashcroft et al. 2000; Arany et al. 2006). Interestingly, in tumor microenvironments, macrophages can enhance TGF-β production and activation of latent TGF-β (Wynn et al. 2010). Smad3 deficiency has also been linked to increased migration as shown by the detection of accelerated wound closure in Smad3 deficient mice (Ashcroft et al. 1999).

TGF-β signaling has several interacting pathways such as MAPK and ROCK1 that phosphorylate r-Smads at the linker region. There is evidence showing that the balance between r-Smad phosphorylation at the linker by region and at C-terminus could be of great importance. Linker phosphorylation is regarded to inhibit Smad2 and Smad3 signaling (Sapkota et al. 2006). Notably, decreased Smad3 activity by increased linker phosphorylation and decreased C-terminal phosphorylation is linked to tumor progression (Sekimoto et al. 2007; Wrighton et al. 2008; Matsuzaki et al. 2009).

2.7 The interaction of HIF and TGF-β pathways

TGF-β and HIF pathways partially regulate the same genes. Interestingly, co-regulated genes have been identified from erythropoiesis and angiogenesis pathways such as the vascular endothelial growth factor, VEGF. Indeed it has been shown to be positively regulated by HIF-1α and TGF-β both separately and together. Similarly to VEGF, erythropoietin and endoglin are activated by both HIF and TGF-β (Ema et al. 1997; Sanchez-Elsner et al. 2001; Breier et al. 2002; Sanchez-Elsner et al. 2002; Sanchez-Elsner et al. 2004).

TGF-β has been shown to activate HIF-1α *via* inhibition of PHD2 (McMahon et al. 2006). HIF-1α can also drive TGF-β transcription (Saed et al. 2002; Jiang et al. 2007). The ability of HIF-1α to activate TGF-β transcription and the ability of TGF-β to enhance HIF activity underlines the importance of these pathways in development and their co-operative potential in tumor progression.

2.8 Protein phosphatases regulating TGF-β signaling

The activation of TGF-β signaling is the work of protein phosphorylating kinases (Attisano et al. 2002). The counter forces for the kinases are phosphatases, which function to dephosphorylate proteins. There is a regulated equilibrium between the kinase targets and phosphatase targets as every phosphorylation creates a possible site for dephosphorylation. As there are many levels of kinase regulation for TGF-β signaling, also the phosphatases seem to regulate several levels of TGF-β signaling (Liu et al. 2010).

The activated receptors can be dephosphorylated by protein phosphatases PP1C and PP2A to attenuate or to shut down the signal (Shi et al. 2004; Batut et al. 2008). Downstream, several protein phosphatases have been identified to regulate TGF-β signaling. The small C-terminal phosphatases (SCPs) dephosphorylate Smad2 and Smad3 at the linker region and Smad1 at the C-terminus (Figure 6) (Sapkota et al. 2006). Linker region phosphorylation status affects the r-Smad/co-Smad complex formation and therefore it changes r-Smad transcriptional activity (Wrighton et al. 2006). The known phosphatases acting on TGF-β cascade and the target for dephosphorylation are summarized in Table 1.

Table 1 Summary of phosphatases acting on r-Smads or TGF-β receptors

Phosphatase	**Target**	**Co/regulatory factors**	**Effect**	**Ref**
PP2A	Receptor type 1 ALK4 and ALK5	B*alpha*	Receptor inhibition	(Batut et al. 2008)
PP2A	Receptor type 1 ALK4 and ALK5	B*gamma*	Receptor stabilization	(Batut et al. 2008)
PP2A	Smad1 C-terminus	To be identified	Smad1 signal attenuation	(Bengtsson et al. 2009)
PPM1A	r-Smad c-terminus	None	r-Smad signal switch off	(Duan et al. 2006; Lin et al. 2006)
PP1C	Receptor	Smad7, GADD34	Receptor inactivation	(Shi et al. 2004)
SCP1-3	Linker phosphorylation/Smad1 c-terminal phosphorylation	None	Smad-Smad interaction regulation. Smad degradation regulation	(Knockaert et al. 2006; Sapkota et al. 2006; Wrighton et al. 2006)

PP2A is a ubiquitous phosphatase with a vast variety of targets. In general PP2A is regarded as a tumor suppressor and inhibition of PP2A activity has been linked to malignancy (Junttila et al. 2007). PP2A is a multimeric

complex and the active enzyme is built around a 65 kD scaffolding (PR65) subunit that serves as a docking site for the catalytic and regulatory subunits (Figure 7). One catalytic and one regulatory subunit at the time can be bound to the scaffold subunit (Mumby 2007).

PP2A can specfically target a number of phosphorylated proteins because two variants of both scaffold and catalytic subunits and multiple different regulatory subunits exist. All in all the PP2A holoenzyme can be assembled in at least 30 or more different ways (Mumby 2007; Eichhorn et al. 2009). In Table 2 the PP2A subunits with their aliases and the known functions are listed.

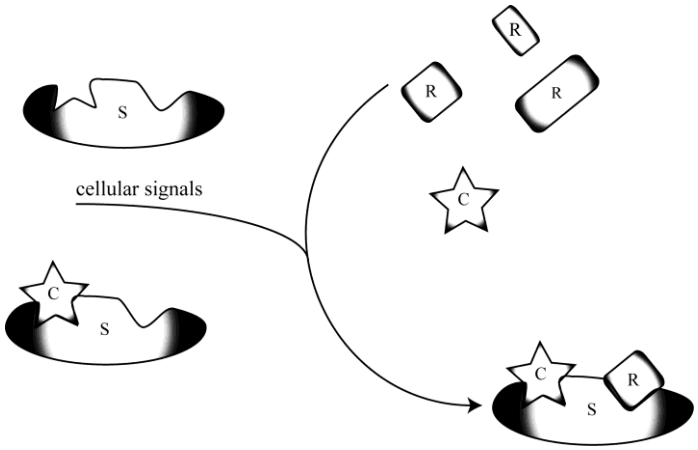

Figure 7 Assembly of the PP2A holoenzyme. PP2A consist of three main subunits: Scaffold (S), Catalytic (C), and Regulatory (R) subunits that together form a specific and active holoenzyme. The final functionality of PP2A is determined by one of the several different R subunits.

The phosphorylated TGF-β receptor is targeted by two phosphatases, PP1C and PP2A (Shi et al. 2004; Batut et al. 2008). PP1C uses Smad7 and GADD34 to specifically target and dephosphorylate TGF-β receptor (Figure 8) (Shi et al. 2004). Unlike the PP1C holoenzyme, PP2A is able to directly bind to the receptor *via* its own subunits (Batut et al. 2008). The multiplicity of different forms of PP2A enables it to either enhance or attenuate the TGF-β receptor activity depending on the regulatory subunit present on the scaffold. The PPP2R2A regulatory subunit leads to inhibition of Smad2 phosphorylation and degradation of the TGF-β receptor (Batut et al. 2008).

On the other hand, the PPP2R2D subunit causes receptor stabilization and enhances Smad2 phosphorylation (Batut et al. 2008).

The third phosphatase targeting the receptor regulated phosphorylation of r-Smads is PPM1A. Instead of targeting the receptors, PPM1A directly dephosphorylates the activated r-Smads (Figure 8). Similarly to Smad7, PPM1A inhibits r-Smad signaling as it is able to dephosphorylate all r-Smads at their C-terminus. Hence, PPM1A might work as an autoinhibitor or at least as an attenuator of TGF-β signal (Lin et al. 2006). Unlike PP1C or PP2A, PPM1A is monomeric and hence it does not need any co-proteins to be able to bind to its targets (Duan et al. 2006; Lin et al. 2006). There is also evidence that PPM1A inhibitory function, at least partially works *via* activating proteasomal degradation of r-Smads in the BMP activated signaling (Smad1, 5, and 8) (Kokabu et al. 2010). Whether this mode of action is also true for Smad2 and Smad3 is uncertain. One proposed Smad2 and Smad3 inhibition mechanism for PPM1A is the enhancement of the nuclear export of Smad2 and Smad3 proteins (Dai et al. 2011).

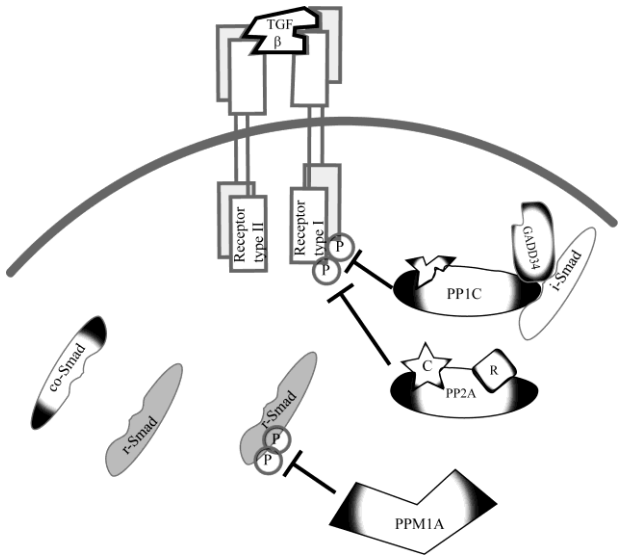

Figure 8. **PP2A, PP1C and PPM1A deactivate the TGF-β signaling.** PP2A and PP1C phosphatases are capable of inactivating type 1 receptor by dephosphorylating the catalytic domain of the receptor. PP2A holoenzyme can target the TGF-β-receptor *via* its own regulatory subunits. PP1C on the other hand uses Smad7 and GADD34 proteins to target the TGF-β receptor. PPM1A dephosphorylates activated rSmads and controls the duration of r-Smad signaling and hence response to TGF-β.

2.8.1 PP2A in cancer

Many oncogenes are kinases that have been studied vigorously and they have proven to be essential for cancer development. Therefore kinases are also targets for drug development (Hanahan et al. 2011; Baumann 2014). Protein phosphorylation is a rapid and reversible way to regulate protein functions. Kinases balance the actions of phosphatases. Hence, many of the phosphatases are regarded as tumor suppressors with some supporting evidence (Eichhorn et al. 2009; Kurimchak et al. 2012). The tumor suppressor role of PP2A was discovered by analyzing the mode of action of okadaic acid, which was shown to have tumor promoting capabilities (Bialojan et al. 1988). Okadaic acid inhibits a relatively wide variety of phosphatases if the concentration is increased but already at nanomolar concentrations it is a strong inhibitor of PP2A (Fernandez et al. 2002). *In vitro* studies of IC50 values show that PP2A is inhibited at a tenfold lower concentration than PP1C by okadaic acid (Bialojan et al. 1988; Fernandez et al. 2002).

Chemical inhibition of the PP2A can target more than 30 forms of active PP2A as the PP2A holoenzyme consists of three subunits, all coded by multiple genes and splice variants. Such an extensive inhibition is likely to be toxic to the cells (Eichhorn et al. 2009). Although, the multiple active PP2A compositions are still not fully characterized. One example of the versatile role of PP2A in the regulation of cellular signaling is its effect on TGF-β signaling. Depending on the regulatory subunit PP2A activates or inactivates TGF-β signaling. In other words even subtle changes in PP2A holoenzyme may have different or even totally opposite effects on cellular signaling and tumor progresion. (Batut et al. 2008; Kiely et al. 2015).

Changes in PP2A regulatory subunit expression or mutations are linked to malignancies. To date, the alterations that have been reported in PP2A subunits have been inactivating, underlining the tumor suppressor role of PP2A (Kurimchak et al. 2012; Kiely et al. 2015). However, besides the fact that all the functions of PP2A have not been characterized, there is only limited number of reports on the role of PP2A under tumor hypoxia. Hypoxia has been shown to correlate with PP2A activity in Glioblastoma multiforme and high PP2A activity has been linked to poor prognosis (Hofstetter et al. 2012).

3 Specific aims of the study

In this study we explored the gene expression pattern activated in hypoxia using gene expression array technologies. The results showed that Smad7, an inhibitor of Smad signaling was upregulated in hypoxia independently of TGF-β exposure. Because hypoxia and TGF-β are both known effectors in cancer progression and literature revealed that hypoxia and TGF-β pathways have overlapping functions, we investigated the hypoxia activated Smad7 efficacy on Smad2 and Smad3 inhibition. The results showed that only Smad3 would be inhibited under hypoxia. This finding raised the hypothesis of active dephosphorylation of Smad3 under hypoxia. The aim of this thesis was to study more closely the effect of hypoxia on TGF-β signaling.

The specific aims were:

1. To investigate the effect of hypoxia on the function of two TGF-β effectors: r-Smad2 and r-Smad3.

2. To study the effect of hypoxia on i-Smad7, a TGF-β inhibitor, function.

4 Materials and methods

More detailed information about the reagents and methods used are found in the original publications I and II.

Cell lines	Used in
HaCaT	I, II
HeLa	I, II
HepG2	II
HUVEC	II
NMuMG	Results
RCC4-	II
RCC4+	II

Antibodies	Used in
Actin	I
Anti-mouse Alexa488	I
anti-mouse Cy3	I
HA	I
HIF-1a	I
HIF-2a	I
PP2A catalytic	I
PPM1A	I
PR65	I
p-Smad2	I
p-Smad3	I
Smad2	I
Smad3	I
Strep	I

siRNA	Used in
siHIF1-a	II
siNon-Target	I, II
siPPM1A	I
siPR65-1	I
siPR65-2	I
siSmad7-1	II
siSmad7-2	II

Plasmids	Used in
ARE3-Luciferase	I, II
CAGA12-firefly-lusiferase	I, II
HIF-1a	II
HIF-2a	II
Strep-PR65	I
Strep-Smad3	I

Chemicals	Used in
BMP2	I, II
CoCl2	II
Effectene	I, II
Fostriecin	I
Fugene	I
G-418	II
Growth factor poor serum	I, II
Heparin	II
Hoechst	I, II
Human serum	II
Matrigel	II
Okadaic acid	I
Oligofectamine	I
SB431542	I, II
TGF- β1	I, II
Trans-IT-TKO	II

Adenovirus infections	Used in
adLacZ	II
adSmad7	II

Kits and special techniques	Used in
cDNA microarray	II
Hypoxia treatments	I, II
Invasion assay	II
Luciferase assay	I, II
Patient samples	II
Phosphatase assay	I
Quantitative reverse transcription polymerase chain reaction (TaqMan)	I, II
RNA-purification	I, II
Strep-purification	I
Western blotting	I, II

cDNA microarray

Cellular mRNA was extracted from the control and the treated cell cultures and transcribed to cDNA using reverse transcription reaction. The cDNA was then amplified and labelled using nucleotides conjugated with fluorescent labels. Control samples and treated samples were labelled with different fluorescent labels. Then the control and the treated samples were mixed and hybridized on cDNA oligo chip containing 3000 genes in triplicates. The chips were then scanned and the ratio between control and treated sample label fluorescence intensities were analyzed for treatment related changes in mRNA expression.

Invasion assay

Matrigel was cast on a semipermeable membrane at the bottom of a cell culture plate insert well, Thincert (Greiner bio-one). Normal cell culture media was applied outside the well and cells were seeded inside the insert well on top of the matrigel layer. After treatments the matrigel was removed and the inner surface of the semipermeable membrane was swiped using a cotton swap. The invaded cells which had migrated across the membrane were fixed using formaldehyde stained with fluorescent DNA stain (Hoecst) and scored under a fluorescent stereomicroscope (Lumar, Zeis)

Luciferase assay

The luciferase assays were performed using Dual luciferase kit from Promega. Shortly, cells were co-transfected with two different plasmids containing different luciferase enzymes. Control lusiferase was driven with constantly active minimal actin promoter and the other one by TGF-β responsive promoter. The control lusiferase signal was used to normalize transfection efficiencies. The corrected TGF-β responsive lusiferase signal values were used to study the effects on TGF-β activated protein production.

Phosphatase assay

For phosphatase activity assays HeLa cells were plated and let to adhere overnight. Then the cells were transfected with the bait protein fused with the Strep-tag. The control samples were transfected with PP2A structural subunit (PR65) fused with the Strep-tag. On the third day growth factor stripped media was changed to the cells and the cells were placed into hypoxia incubator under 1% O2 for 16 hours. On the fourth day the cells were treated with TGF-β for 30 minutes and lysed in ice cold lysis buffer (Junttila et al. 2005). Cell suspensions were homogenized by drawing the lysates through 18G needle 10 times on ice. The homogenized samples were centrifuged in four degrees of Celsius for ten minutes at 14000 RCF. The supernatants were first sampled for 50 µl input sample and applied to the Strep purification columns (Iba-Lifesciences) (see strep-purification below). After the sample had gone through the columns were washed five times with one column volume (200 µl) using Strep washing buffer. The samples were collected by eluting six times using 100 µl of elution buffer. Two fractions were collected in the same Eppendorf tube resulting as three 200 µl samples. The eluted samples were concentrated using Microcon YM-30 (Milllippore) columns until total volume was less than 100 µl. Then the samples were washed with 500 µl of phosphatase washing buffer and second time with 500 µl of phosphatase buffer containing BSA. All centrifugation steps were performed at four degrees of Celsius and 8000 RCF until the volume in the column was less than 100 µl. The concentrated samples were recovered by placing the Microcon column upside down into Eppendorf tube and centrifuged for four minutes at 8000 RCF in four degrees of Celcius. The recovered samples were adjusted to 110 µl volume and divided into two equal volumes into test tubes. One tube was then treated with a phosphatase inhibitor such as Okadaic acid and incubated for 30 minutes. The samples were combined with 50 µl of phosphatase reaction buffer and measured using Envision (PerkinElmer) plate

reader at 37 degrees of Celcius. The analysis was performed using RediPlateTM 96 EnzChek_ serine-threonine phosphatase assay kit (R33700, Invitrogen).

Buffers for phosphatase assay:

Strep-lysis buffer (2x)

50 mM Tris-HCl pH 7.4

1 mM EDTA

150 mM $NaCl_2$

0,5 % Triton-X100

Before use add to 1x Strep-lysis buffer

1x complete protease inhibitor

1 mM Na_3VO_4

Self made reaction buffer (2x)

100 mM Tris-HCl pH 7.0

0,2 mM $CaCl_2$

0,1 % Tween20

2 mM $NiCl_2$

Phosphatase washing buffer for concentrating the samples

1x self made reaction buffer

Washing buffer with BSA for concentration

1x self made reaction buffer with 125 µg/ml BSA

Reaction buffer

1x reaction buffer from the kit

1 mM $NiCl_2$ (40 mM stock)

Strep-purification

Strep-avidin column purification method was used for protein protein interaction studies. The protein of interest was cloned into a Strep-tag containing plasmid. This construct was then used for transient transfections. The negative controls were transfected with a plasmid expressing the Strep-

tag only. The transfected cells were lysed after the treatments. The cell lysates were applied into purification columns filled beads that bind to Strep-tag (Iba-Lifesciences). The columns were washed to remove all the remaining weakly bound proteins. The last step was to apply elution buffer to the column to release the Strep-taged proteins. The eluates were analyzed by western blotting to detect the bait and the interacting proteins. (Junttila et al. 2005)

5 Results

5.1 Hypoxic regulation of Smad family genes

The cDNA microarray screens were conducted to find novel genes activated by hypoxia in cancer cells. Well characterized hypoxia activated genes present on chip such as *Carbonic anhydrase 9* and *Hemeoxygenase I* were found to be upregulated under hypoxia in cDNA array analysis (Lee et al. 1997; Wykoff et al. 2000). In addition to the known hypoxia activated genes *Smad7* gene was upregulated under hypoxia (II Figure 1). Smad7 is an inhibitor of the TGF-β pathway. Smad1, Smad2, Smad3 and Smad4 genes were present on chip but their expression levels were not affected by hypoxia (II Figure 1A). Results obtained from the cDNA microarrays were verified by analyzing biological replicates from hypoxia or hypoxia mimetic CoCl2 treated samples using quantitative reverse transcription PCR (Q-RT-PCR) (II Figure 1 B and C).

Q-RT-PCR was used to study the dependencey of the hypoxic activation of *Smad7* gene on HIF-1α and pVHL (II Figure 2 and Supplemental Figure 2 and 3). We used a Renal Clear Cell Carcinoma (RCC) derived cell line that is pVHL deficient (RCC4-) and a RCC cell line where pVHL was stably transfected (RCC4+) to reactivate normal HIF-regulation. In RCC- the lack of pVHL function leads to constant HIF-transcriptional activity even in normoxia and hence the HIF target genes are constantly upregulated (Maxwell et al. 1999). The results from RCC cell lines showed that in RCC4+ cells the *Smad7* gene was upregulated under hypoxia. However, in RCC4- cell line the Smad7 mRNA levels were both in normoxia and hypoxia at the same level than in RCC4+ cells under hypoxia. This indicates that hypoxic regulation of *Smad7* gene was dependent on functional pVHL.

To investigate the HIF dependency of Smad7 gene activation under hypoxia we used HIF1-α and HIF2-α transfections and HIF1-α depletion by siRNA in HeLa cells (II Figure 2 and Supplemental Figure 2 and 3). Smad7 gene was activated both with HIF1-α and HIF2-α transfections whereas siRNA depletion of HIF1-α prevented Smad7 gene activation under hypoxia.

Hypoxic activation of Smad7 gene was seen even when we treated the cells with an inhibitor of TGF-β receptor. These results showed that Smad7 gene was activated in hypoxia independently of TGF-β and in a HIF-signaling dependent manner (II Suplemental Figure 2).

5.2 Smad3 phosphorylation is inhibited during hypoxia

Activation of Smad7, a known inhibitor of Smad2 and Smad3, under hypoxia (II Figure 1) provoked a hypothesis of inhibition of r-Smad C-terminal phosphorylation during hypoxia. This hypothesis was tested by assessing the phosphorylation status of Smad2 and Smad3 by westernblotting. A hypoxic inhibitory effect on r-Smad activity by dephosphorylation regulation was found to be Smad3 specific and TGF-β1 signaling-specific. Cells treated with BMP under hypoxia did not show any difference in Smad phosphorylation between normoxia and hypoxia. BMP is activating phosphorylation of Smad1 whereas TGF-β1 activates Smad2 and Smad3 phosphorylation. The antibody used for westernblotting binds to both Smad1 and Smad3 phosphorylated C-terminus (I Figure 1). Furthermore, we investigated in several cell lines the r-Smad response to TGF-β stimulus during normoxia and hypoxia. In addition to the results shown in HeLa and HaCaTs cell lines described earlier, the hypoxic inhibition of Smad3 but not Smad2 was seen in SCC cell lines and mouse NMuMG cells (Figure 9). The effect was similar from cell line to cell line.

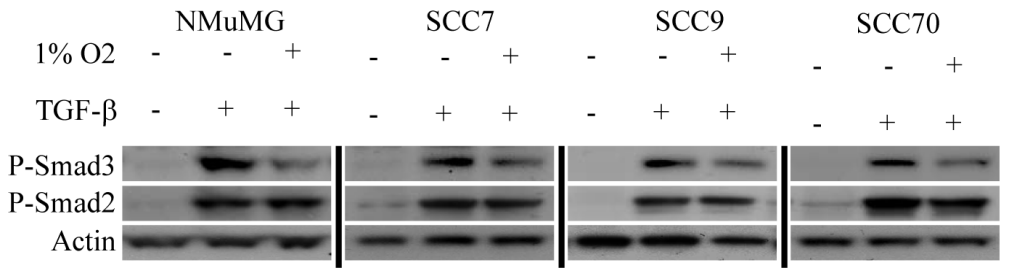

Figure 9 Smad3 but not Smad2 phosphorylation is inhibited during hypoxia. Mouse NMuMG and three SCC cell lines gave the same result when exposed to hypoxia before TGF-β exposure.

R-Smads are known to be regulated by ubiquitination-mediated proteosomal degradation. This was not the case during hypoxia as the total levels of Smad2

and Smad3 remained constant regardless of the hypoxia treatment (I Figure 1). The fact that Smad2 phosphorylation responded normally to TGF-β stimulus when Smad3 did not, suggested a receptor-independent mechanism. The evidence for this was obtained by ectopically expressing constantly active mutated form of the ALK-5 receptor. Even with TGF-β independent activation of Smad3 phosphorylation the hypoxic effect prevailed and Smad3 C-terminus remained to be dephosphorylated in hypoxia (I Figure 2)

5.3 PP2A activity and Smad7 are needed for Smad3 inhibition in hypoxia

As the inhibition of Smad3 C-terminal phosphorylation was independent on receptor activity and Smad3 protein level did not decrease we investigated the possibility of dephosphorylation. We first tested Okadaic acid (OA) as it inhibits many phosphatases (Fernandez et al. 2002). Already low concentration of OA rescued the Smad3 phosphorylation (I Figure 3A) and transcription activation function (I Figure 3B) in hypoxia. Smad3 inhibition in hypoxia led to failed induction of TGF-β-Smad3 responsive gene P15 and downregulation of ID1 gene (I Figure 8 B and C). The inhibition of PP2A activity or Smad7 depletion by siRNA treatment during hypoxia rescued the target gene expression regulation (I Figure 8 B and C and II Supplemental Figure 5). These findings suggested that both Smad7 and PP2A are needed for hypoxic alteration of TGF-signaling. The low OA concentration suggested that PP2A could be the phosphatase responsible for Smad3 dephosphorylation. Inhibiting PP2A activity with Fostriecin (I Figure 8A) or siRNA against PR65, the structural subunit of PP2A, effectively rescued Smad3 phosphorylation (I Figure 3C-E) supporting the importance of the PP2A in the hypoxic dephosphorylation of Smad3. We also showed that depleting PPM1A using siRNA did not rescue Smad3 phosphorylation in hypoxia (I Figure 3F). In addition we analyzed the effect of PR65 siRNA depletion on Smad3 phosphorylation under hypoxia in several cell lines. All the cell lines show inhibition of Smad3 phosphorylation under hypoxia. Importantly siRNA depletion of PR65 in human derived cell lines (HaCaT, SCC7, SCC9 and SCC70) rescued the Smad3 phosphorylation under hypoxia (Figure 10).

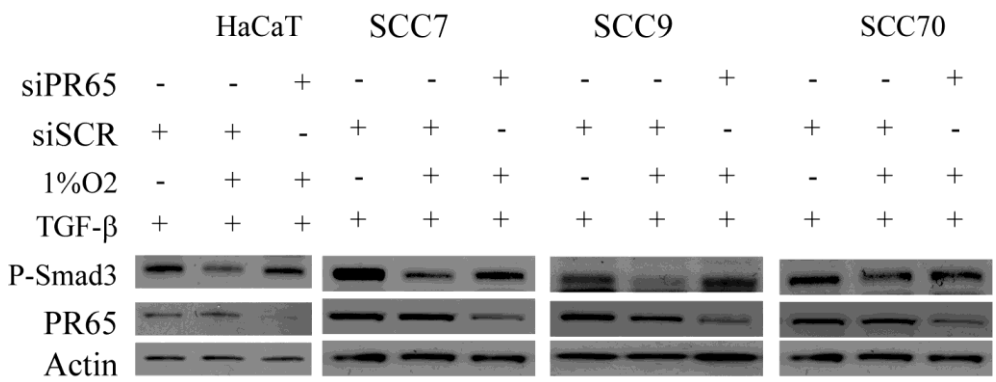

Figure 10 PR65 depletion rescues Smad3 hypoxic phosphorylation in several cell lines. All the cell lines show hypoxic inhibition of Smad3 phosphorylation. Deletion of PR65 by siRNA reinstates the Smad3 phosphorylation.

PP2A specificity is defined by the regulatory subunit which binds to PR65-scaffold in addition to catalytic subunit which is also bound to PR65. Knocking down different regulatory subunits using siRNA transfections gives detailed information about the dephosphorylation targets for different PP2A complexes. Interestingly unpublished from a master's thesis suggests that PPP2R5E subunit might be targeting PP2A to Smad3 (Tähtinen 2011). (The master's thesis was done parallel to this thesis research.) Depleting PPP2R5E by siRNA not only rescued the Smad3 hypoxic phosphorylation but also prevented PR65 to co-purify with Smad3 in Strep purification assay (Tähtinen 2011). Below Table 2 summarizes the previously published results from PP2A siRNA knockdown experiments and the unpublished data.

Table 2 PP2A subunits and their function.

Subunit	Alias1	Alias 2	Function	Effect of siRNA depletion in TGF-*beta* signaling	Ref
PPP2R1A	PR65 *alpha*	A alpha	Scaffold	Rescue of Smad3 C-terminal phosphorylation in hypoxia.	(I Figure 3, 7 and Suplemental Figure 2)
PPP2R1B	PPR65 beta	A beta	Scaffold	Unknown	
PPP2CA	Catalytic *alpha*		Catalytic	Unknown	
PPP2CB	Catalytic *beta*		Catalytic	Rescue of Smad3 C-terminal phosphorylation in hypoxia	(Tähtinen 2011)

Table 2 (continued) PP2A subunits and their function.

Subunit	Alias1	Alias 2	Function	Effect of siRNA depletion in TGF-*beta* signaling	Ref
PPP2R5E	PR61 *epsilon*	B' *epsilon*	Regulation	Rescue of Smad3 C-terminal phosphorylation in hypoxia. Inhibition of Smad3 PR65 interaction. Increased cancer risk in *in vivo* deletions	(Grochola et al. 2009; Dupont et al. 2010; Tähtinen 2011)
PPP2R4	PR53		Regulation	minor inhibition of Smad2 and Smad3 phosphorylation	(Tähtinen 2011)
PPP2R2B	PR55 *beta*	B *beta*	Regulation	Inhibition Smad2 and Smad3 phosphorylation	(Tähtinen 2011)
PPP2R5D	PR61 *delta*	B' *delta*	Regulation	Inhibition of Smad2 and Smad3 phosphorylation	(Tähtinen 2011)
PPP2R5B	PR61 *beta*	B' *beta*	Regulation	Inhibition of Smad2 and Smad3 phosphorylation	(Tähtinen 2011)
PPP2R5A	PR61 *alpha*	B' *alpha*	Regulation	Rescue of Smad3 C-terminal phosphorylation in hypoxia. Inhibition of Smad3 PR65 interaction	(Tähtinen 2011)
PPP2R2A	PR55 *alpha*	B *alpha*	Regulation	Inhibition of Smad2 and Smad3 C-terminal phosphorylation. Degradation of ALK5.	(Batut et al. 2008; Tähtinen 2011)
PPP2R3B	PR48	B" *beta*	Regulation	Rescue of Smad3 C-terminal phosphorylation under hypoxia	(Tähtinen 2011)
PPP2R5C	PR61 *gamma*	B' *gamma*	Regulation	No effect	(Tähtinen 2011)
PPP2R2D	PR55 *delta*	B *delta*	Regulation	Enhancement of Smad2 C-terminal phosphorylation	(Batut et al. 2008)
PPP2R2C	PR55 *gamma*	B *gamma*	Regulation	Rescue of Smad3 C-terminal phosphorylation in hypoxia	(Tähtinen 2011)
PPP2R3A	PR72	B" *alpha*	Regulation	Unknown	
PPP2R3A	PR130	B" *alpha*	Regulation	Unknown	

5.4 Under hypoxia, Smad7 switches from an invasion inhibitor to invasion promoter in a PP2A dependent manner

Smad7 overexpression has been reported to inhibit malignant cell invasion *in vitro* (Leivonen et al. 2006). This finding was repeated during normoxia when malignant HeLa and UTSCC cells were studied in matrigel invasion assay. Notably, during hypoxia overexpression of Smad7 did not inhibit cell invasion through matrigel (II Figure 5 and Supplemental Figures 6 and 8). The non-invasive HaCaT cells were able to travel through matrigel only when exposed to simultaneous hypoxia and TGF-β exposure (II Figure 4A). During these conditions, depleting Smad7 or HIF1-α by siRNA reduced the number of invaded cells to a normoxic control level (II Figure 4B and C). Smad7 involvement during malignancy is evident in clinical cancer samples where Smad7 upregulation was clearly present in tumor tissues (II Figure 6). Importantly, Smad7 upregulation was tightly correlated with tumor hypoxia in clinical samples (II Figure 6C and Supplemental Figure 7).

6 Discussion

6.1 Hypoxia enhances the tumorigenic potential of TGF-β

Both hypoxia and TGF-β have long been known to be key elements in cancer progression. Likewise, both factors and their functional response mechanisms, including HIF1-α signaling, are needed for development. Or to be more accurate, a functional response mechanism for hypoxia is needed for embryonic development and cell survival. Also both HIF1-α and TGF-β are essential parts of wound and trauma repair and physiological responses to exercise (Mason et al. 2004; Kjaer et al. 2006). TGF-β and HIF1-α signaling pathways are needed for activation of angiogenesis, cell migration, and differentiation as well as for regulating immune responses. Gaining control of these mechanisms that normally operate during embryonic development and wound healing can make a cancer cell progress successfully.

The importance of individual TGF-β-[SF] members in development is not yet fully understood. It is likely that complete blocking of TGF-β-superfamily members would cause embryonic lethality. A Smad2 knockout is embryonically lethal but Smad3 knockouts are viable (Brown et al. 2007). Interestingly Smad3 knockout mice show more rapid wound closure in the skin than wild type mice. The accelerated process of healing was reported to result from increased keratinocyte proliferation. *In vitro* the Smad3 knockout keratinocytes showed increased proliferation, decreased matrix adhesion and impaired directional migration (Ashcroft et al. 1999). Smad3 knockouts also show reduced inflammation at trauma site due to decreased monocyte infiltration into the wound area (Ashcroft et al. 1999). The data in this thesis shows that Smad3 is C-terminally dephosphorylated by PP2A in hypoxia but Smad2 remains fully activated. In theory this is analogous to a Smad3 knockout where Smad2 is still present. Smad3 inactivation during hypoxia by PP2A and Smad7 was found to increase cell invasiveness *in vitro*. The role that C-terminally non-phosphorylated Smad3 plays in TGF-β signaling is unknown. There is evidence that Smad3 linker phosphorylation coupled with

C-terminal non-phosphorylation results in increased invasive capacity (Matsuzaki et al. 2009). Therefore, it could be argued that Smad2 is likely to be crucial for cellular motility during gastrulation while Smad3 seems to be required for transducing the proper signals or maintaining homeostasis of TGF-β in fully developed organisms.

Hypoxia is present in most, if not all, tumors because the cancer tissue is rapidly outgrowing angiogenesis. In small tumors, hypoxia probably is not present but as the tumor grows hypoxia is inevitably generated. Hypoxia is one of the main factors contributing to selection pressure that eventually gives rise to metastatic cells (Vaupel 2004). An analogy can be seen between hypoxia and TGF-β as in small benign tumors, TGF-β functions as a growth suppressor but in more advanced malignant tumors TGF-β supports tumor growth. TGF-β inhibits the formation of primary tumors, as shown in an elegant mouse study by Cui et al. (Cui et al. 1996). However, in the same study the authors show that a TGF-β stimulus is beneficial for metastasis. To become a cancer cell, a cell needs to escape its normal regulatory cycles and ensure a sufficient energy supply. To fulfill the latter, the utilization of the HIF pathway might be a good strategy to sustain angiogenesis and energy supply. However, to be able to do that the cells need hypoxia or some other means to inactivate PHD enzymes. It is here where the interaction of TGF-β with hypoxic response mechanisms becomes useful. For example, the expression of PHD2, the main HIF1-α regulator, might be downregulated in response to TGF-β (McMahon et al. 2006). Evidence from this thesis shows that the interplay between hypoxia and TGF-β is dependent on Smad7 and PP2A and the above mentioned mechanism might enhance the phosphatase dependent regulation of TGF-β signaling. It could be argued that TGF-β creates a similar selection pressure for metastasis formation compared to hypoxia. Once the primary tumor has emerged, the tumor microenvironment causes a selection pressure where hypoxic signaling, TGF-β-pathway and a possibly altered PP2A activity play their parts to ultimately drive cancer progression.

In addition to hypoxia, pH is another crucial microenvironmental factor that regulates cancer cell fate. The level of pH is also essential for enzyme activity and stability. The purified catalytic subunit of PP2A has pH optimum at pH 8.7. At physiological pH, the activity of the catalytic subunit is only around 20 percent of the maximum. The catalytic subunit showed no activity at a pH

below five and above 10 (Rivas et al. 2000). The effect of pH on the activity of different PP2A holoenzymes is not known. It is probable that the holoenzyme pH optimum is dependent on the subunits present on the scaffold. Interestingly, a low pH seems to inhibit TGF-β release from platelets *in vitro* (Liu et al. 2003) but a low pH, below five, can activate latent TGF-β (Annes et al. 2003).This finding could explain why aerobic glycolysis (see the Warburg effect in Section 2.2) is beneficial for cancer progression as high lactate production creates a low pH microenvironment, *in vivo*. Extracellular pH in tumors is reported to drop as low as a pH 5.8 (Tannock et al. 1989; Gerweck et al. 1996). However, during *in vitro* cell culture experiments, the culture media is buffered, which prevents this phenomenon. Therefore, *in vitro* pH is maintained closer to the intracellular pH during the *in vivo* situation. Even in tumors the intracellular pH is maintained close to a pH 7 regardless of the extracellular pH (Zhang et al. 2010). Low pH might enhance latent TGF-β activation and therefor potentiate TGF-β signaling in tumor microenvironment. However pH is not likely to have an effect on PP2A activity because of tight control of intracellular pH.

6.2 PP2A functions in hypoxic tumors

In addition to HIF1-α and TGF-β signaling also PP2A is essential for development. HIF1-α knockout mice die *in utero* (Kotch et al. 1999). Also, PP2A activity is needed for embryo survival: knockout of the catalytic subunit results embryonic lethality (Gotz et al. 1998). During cancer, the tumor suppressor function of PP2A is well-documented. The evidence for this first emerged from the carcinogenic effect of okadaic acid. Okadaic acid is a potent PP2A inhibitor that can totally block the PP2A activity and hence it targets some 30 or more different forms of PP2A (Fernandez et al. 2002; Eichhorn et al. 2009). More specific evidence has been achieved by siRNA studies that provide a more detailed picture about PP2A. Unlike okadaic acid, siRNA targets only one form of PP2A at a time. These studies show an interesting duality in PP2A function. By knocking down one regulatory subunit of PP2A, TGF-β signaling gets boosted. However, when another regulatory subunit is knocked down, this results in attenuation of TGF-β signaling (Table 2 for details).

Total block of PP2A activity seems drive tumor formation, however, it cannot be ruled out that an active form of the PP2A holoenzyme would be utilized by malignant cells to drive tumor progression. The dual role of PP2A in TGF-β signaling regulation offers one possible example of a delicate balance that when disturbed could potentially be harmful. The work in this thesis provides evidence for this hypothesis and for a possibly malignant function for PP2A. During hypoxia as the PP2A catalyzed Smad3 dephosphorylation led to more aggressive features of immortalized cells.

Preliminary unpublished data acquired during the thesis research raised a hypothesis that PPP2R5E regulatory subunit could be a potential candidate for targeting PP2A to specifically dephosphorylate Smad3 under hypoxia. In genetic screening PPP2R5E snip variants have been linked to malignancies (Grochola et al. 2009). Noticeably, PPP2R5E has been shown to be of high importance during embryos dorsal development (Yang et al. 2003).

Given the apparent developmental importance of PP2A/PPP2R5E complex and the fact that Smad2 knockouts are not viable but Smad3 knockouts are born healthy, it is very tempting to suggest that during embryo development PP2A/PPP2R5E inhibits Smad3 activity by dephosphorylation while leaving Smad2 still fully active. This might be driven by hypoxia, thus benefiting tumors and converting TGF-β and PP2A from tumor suppressors to tumor promoters. One useful tool for addressing the causality of PP2A function during TGF-β signaling and development could be Thalidomide and its analogs. Lenalidomide, a Thalidomide analog, is known to cause birth defects and it has also been shown to inhibit PP2A (Wei et al. 2009) This inhibition of PP2A is hypothesized to be partially behind the cancer drug properties of Thalidomide analogs (Shortt et al. 2013). In the light of the results in this thesis, it would be interesting to see if Thalidomide or its analogs would have any effect on Smad3 phosphorylation during hypoxia.

6.3 Effect of hypoxia on Smad7 function

Regulating TGF-β pathway has long been a target for drug development. Inhibiting TGF-β signaling is a tempting strategy for treating a metastasizing cancer. However, this approach runs a risk of losing TGF-β's growth regulatory and tumors suppressive functions. Endogenous TGF-β inhibitor Smad7 offers a good example of potential inhibition of TGF-β being beneficial for cancer. Smad7 is an inhibitor of the TGF-β pathway and it has been shown to be upregulated in many cancers. Smad7 is also effectively upregulated by TGF-β and hypoxia s demonstrated in this thesis.

Smad7 expression status could be used as a prognostic marker in colorectal cancer (Boulay et al. 2003). Boulay *et al.* show that deletion of Smad7 gene promotes better patient survival than when Smad7 is normally expressed. Moreover, duplication of the Smad7 gene makes the patient survival even worse (Boulay et al. 2003). The explanation for cancer progression and Smad7 overexpression could be found from knockout studies. Smad7 knockout mice develop a malfunctioning vasculature, heart failure and die soon after birth (Vargesson et al. 2001; Chen et al. 2009). Smad7 partial knockouts are viable but they show reduced growth (Ueno 2000). Another study by Halder et al. shows that Smad7 overexpression induces metastasis formation in liver originating from colorectal cancer. The study shows that the metastasis in liver have increased expression of cellular tight junction proteins, E-cadherin and Claudins (Halder et al. 2008). This finding is supported by the study by Azuma et al on mammary cancer in mice, where forced Smad7 expression increased the expression of tight junction proteins. This study also shows that strong overexpression of Smad7 inhibits metastasis formation (Azuma et al. 2005). These partially contradictory findings emphasize the complexity of TGF-β signaling. However, one explanation could be found from the results of this thesis.

Hypoxia increases Smad7 expression and targets PP2A to shut off Smad3 mediated signaling. Smad2 signaling remains intact and might activate malignant transformation of differentiated cells. Taken together, it could be suggested that hypoxia, TGF-β, and the PP2A with PPP2R5E-regulatory

subunit are of high importance during cell differentiation and migration during gastrulation phase of the embryonic development. Unfortunately, an efficient cancer cell benefits from recruiting these similar players that the cells need during gastrulation. From the literature and the results of this study it is tempting to conclude that Smad3 is needed for the “good” response of TGF-β in a fully differentiated cell. A developing organism and probably stem cells in turn might want to keep Smad3 inactive (James et al. 2005). Overactive or dysregulated PP2A/PPP2R5E-complex together ith Smad7 and Smad2 may drive the malignant transformation of cells. Smad3 is part of the referee committee and if it is taken out then the “bad” and the “ugly” can play.

7 Summary

TGF-β is an important regulator of cell proliferation and growth, and it is also a well-acknowledged tumor suppressor. PP2A function is also associated with tumor suppression. Hypoxia, on the other hand, drives tumor progression, during which also TGF-β´s function can switch from tumor suppressive to tumor promoting. However, the reasons for TGF-β switching teams is not fully understood.

In this study, we demonstrate that hypoxia upregulates the TGF-β inhibitor protein, Smad7, independently of TGF-β. Under hypoxia. Smad7 activation together with PP2A activity leads to inhibition of TGF-β effector protein Smad3 C-terminal phosphorylation. This inhibition is a result of Smad3 dephosphorylation by PP2A and it is independent of TGF-β receptor activity. Dephosphorylation in hypoxia is highly specific to Smad3 because Smad2 a near homolog of Smad3, responds normally to TGF-β stimulus during hypoxia.

These findings reported here suggest that hypoxia leads to the formation of a Smad3-specific PP2A-complex in a Smad7-dependent manner. Under hypoxia, dephosphorylation leads to Smad3 inactivation. As a result, the hypoxic altering of TGF-β signaling leads to malignant changes in cell behavior *in vitro*, that migth result in tumor growth or metastasis formation *in vivo*.

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