Cross-talk between cyclic AMP and TGFβ signaling in breast cancer cells

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LIST OF ABBREVIATIONS

α	anti
Akt	cellular homolog of murine thymoma virus Akt8 oncoprotein, also known as PKB
ALK	activin receptor-like kinase
AP1	activator protein 1
APS	ammonium persulfate
ATF-1	activating transcription factor 1
ATG gene	autophagy gene
ATP	adenosine triphosphate
BMP	bone-morphogenetic protein (member of the TGFβ family of ligands)
cAMP	cyclic adenosine 3′, 5′-monophosphate
CAF	carcinoma-associated fibroblast
CBP	co-activator of CREB-binding protein
Cdc42	cell division control protein 42
CDK	cyclin-dependent kinase inhibitor
CFTR	cystic fibrosis transmembrane conductance regulator
CM	conditioned cell culture medium
CRE	cAMP-responsive element
CREB	cAMP-responsive element binding protein
CREM	cAMP-response element modulator
CTGF	connective tissue growth factor
Cyr61	cysteine-rich angiogenic inducer 61
DAPK	death-associated protein kinase
DCIS	ductal carcinoma *in situ*
E2	17β-estradiol
ECM	extracellular matrix
EIA	enzyme immuno assay
EMT	epithelial-mesenchymal transition
EndMT	endothelial-mesenchymal transition
Epac	exchange proteins directly activated by cAMP
ER	estrogen receptor
c-Erb	cellular homolog of avian erythroblastic leukemia virus oncogene
ErbB1/Her1/EGFR	viral erythroblastosis virus oncogene homolog 1/ human epidermal growth factor receptor 1, epidermal growth factor receptor
ErbB2/Her2	viral erythroblastosis virus oncogene homolog 2/ human epidermal growth factor receptor 2
ERK	extracellular signal-regulated kinase
Ets	E26 transformation-specific transcription factors
FSK	forskolin
GADD45β	growth arrest and DNA damage-inducible 45β
Grb2	growth factor receptor-bound protein 2
GSK3β	glycogen-synthase kinase 3 β
HAT	histone acetyltransferase
HDAC	histone deacetylase
IBC	invasive breast cancer
ICER	inducible cAMP response element repressor
ID1	inhibitor of DNA-binding 1
JNK	c-Jun N-terminal kinase
LB medium	Luria-Bertani medium
MAPK	mitogen-activated protein kinase
MCF-7	name of a breast cancer cell line established by the Michigan cancer foundation
MMP	matrix metalloproteinase

Myc	oncogene, discovered in avian myelocytomatosis virus
OPG	osteoprotegerin
PAI-1	plasminogen activator inhibitor-1
Par6	partition-defective complex protein 6
PI3K	phosphoinositide 3-kinase
PKA	protein kinase A
PKB	protein kinase B, also known as Akt
PM	plasma membrane extract
PTHrP	parathyroid hormone-related peptide
Raf	small GTPase, discovered in and therefore named after rat fibrosarcoma
RANKL	receptor activator for nuclear factor λB ligand
Ras	small GTPase, discovered in and therefore named after rat sarcoma
RhoA	Ras homolog gene family, member A
RPMI	cell culture medium designed at the Roswell Park Memorial Institute
SARA	Smad anchor for receptor activation
SBE	smad binding element
SDF-1	stromal cell-derived factor 1
SDS	sodium dodecyl sulfate
SHIP	Src homology 2 (SH2) domain-containing inositol phosphatase
α-SMA	alpha-smooth muscle actin
Smad	derived from the Sma and MAD gene homologues in *Caenorhabditis elegans* and *Drosophila melanogaster*
SOS	son of sevenless
SP	side population
Sp1	specificity protein 1
SRC-1	steroid receptor co-activator-1
uPA	urokinase-type plasminogen activator
TAK1	TGFβ-activated kinase 1
TAZ	transcriptional co-activator with PDZ-binding motif; also known as WWTR1, for WW-domain containing transcription regulator 1
TβRI	type I TGFβ receptor
TβRII	type II TGFβ receptor
TEMED	N,N,N',N'-tetramethylethylenediamine
TGFα	transforming growth factor α
TGFβ	transforming growth factor β
TIMP	tissue inhibitor of metalloproteinase
TRAF6	TNF receptor-associated factor 6
PTEN	phosphatase and TENsin homolog deleted on chromosome 10
VEGF	vascular endothelial growth factor
YAP	Yes-associated protein
ZEB	Zinc finger E-box-binding homeobox

INTRODUCTION

1. An introduction to breast cancer

Breast cancer is one of the most frequent malignancies and major cause of cancer death of women. It is not the primary tumor that is the most dangerous but the formation of metastases which often happens very early in tumor development, when the tumor is still small or may not even be detectable. Once the metastatic disease has established it is incurable in almost all cases (Mego *et al.* 2010).

In order to understand the origin of breast cancer a detailed knowledge of the structure of the breast tissue is required. Adult breast tissue basically consists of 15-20 lobes of glandular (epithelial) tissue that are supported by fibrous connective tissue and embedded in fat pads (Fig. 1). The non-epithelial tissue is collectively named stroma and contains fibroblasts, immune cells, endothelial cells, adipocytes and extracellular matrix (Tiede & Kang 2011, Visvader 2009). The mammary lobes contain alveolar units as central structures that give way to ducts that merge into larger lactiferous ducts which opens onto the nipple. The alveolar and ductal lumina are lined by an inner luminal epithelial and an outer myoepithelial (also called basal) cell layer which is surrounded by the basement membrane (Offiah *et al.* 2011).

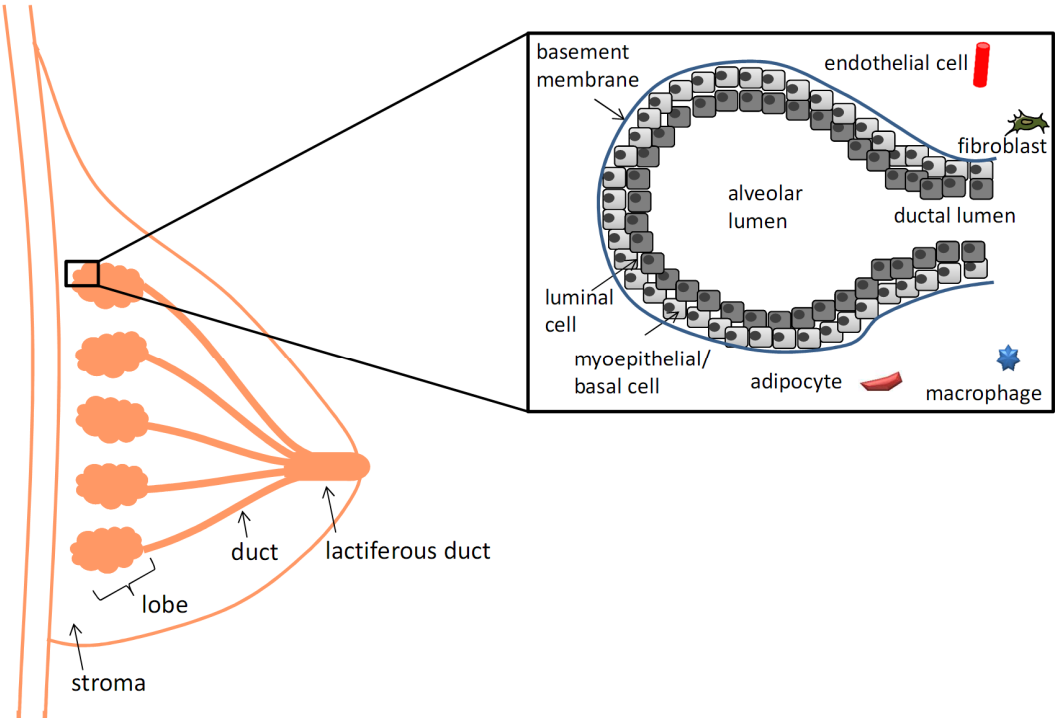

Figure 1. Schematic representation of the human mammary gland; inlet: alveolar unit.

The human mammary gland consists of lobes which contain alveolar units as central structues embedded in stroma. The alveolar units give way to ducts that merge into larger lactiferous ducts that opens onto the nipple. Inlet: The alveolar and ductal lumen are lined by an inner layer of epithelial cells and an outer layer of myoepithelial cells surrounded by the basement membrane. The surrounding stroma contains adipocytes, fibroblasts, macrophages and endothelial cells.

The morphology of the mammary tissue differs between developmental stages. During puberty the ducts multiply and elongate. In the course of a pregnancy, the alveolar subunits expand and alveolar luminal cells transform into milk-producing cells while the myoepithelial cells serve as contractile elements. The ability of an organ to undergo these massive changes requires the presence of specialized cells. A variety of adult mammary gland stem cells within the breast tissue has been detected that drives these processes (Tiede & Kang 2011).

Most breast tumors arise from the luminal epithelial cells. The proliferation of epithelial cells is considered the first step in mammary tumorigenesis (Lee *et al.* 2006). Changes in cell adhesive properties and loss of apical-basal polarity are secondary events. As the cell mass fills the lumen and diversifies, a so-called ductal carcinoma *in situ* (DCIS) develops. Further changes allowing tumor cells to penetrate the myoepithelial cell layer and the basement membrane lead to an invasive breast cancer (IBC) and are the basis for a metastatic disease. Breast cancer preferentially spreads into lung, liver, brain and bone (Weigelt *et al.* 2005).

Breast cancer is a heterogeneous disease. It has been postulated that different phenotypes arise from the diversity in stem and progenitor cell populations present in the mammary gland (Visvader 2009). Several subtypes could be identified by different methods. Using immunohistochemical staining the expression levels of estrogen receptor alpha (ERalpha), progesterone receptor (PR) and ErbB2/Her2 receptor are determined to allow classification as ER+, PR+ or Her2+ tumors. About 70 % of the tumors are estrogen receptor-positive tumors. The ER-positive tumor uses the ER as the molecular basis for its proliferative activity. The estrogen receptor is an intracellular receptor that serves as a transcription factor after ligand binding. Endocrine therapy interrupts this pathway by either applying estrogen antagonists (tamoxifen) which bind the receptor but prevent the recruitment of co-factors and activation of target genes (Michalides *et al.* 2004) or inhibit aromatase, an enzyme involved in estrogen production. In ~25 % of the patients, the Her2 oncogene is overexpressed which is mostly caused by amplification of the corresponding erbb2 gene (Murphy & Morris 2012). Patients with this type of tumor benefit from drugs affecting the receptor`s dimerization or its kinase activity. Unfortunately, many tumor cells develop a resistance against these inhibitors and an altered therapeutic intervention is then needed. Moreover, a subgroup of tumors does not express ER, PR or Her2 receptors and has to be treated differently. Because of the absence of biomarkers of these so-called triple-negative tumors no targeted therapy can be applied. Many of these tumors show elevated expression of the ErbB1/epidermal growth factor (EGF) receptor. Targeting this receptor might serve as a novel therapeutic strategy in the future (Tischkowitz *et al.* 2007). Since the immunohistochemical determination of breast cancer markers does not seem to be sufficient to identify all different breast cancer subtypes and because of the lack of targeted therapies for triple-negative tumors, alternative classification methods have been developed. For this purpose, tumor samples were examined by cDNA

microarrays. In an initial screening, the expression of over 8000 genes was measured. Genes were clustered according to similar expression patterns found among the samples. Each of the gene clusters included functionally related genes, with the proliferation cluster containing most of the clustered genes. The group that performed this initial study suggested four breast cancer subtypes, namely luminal-like, basal-like, Her2-enriched and a normal breast tissue-like subgroup (Perou *et al.* 2000). Further examination and a larger sample recruitment confirmed these subtypes and revealed different luminal types (Sorlie *et al.* 2001, Sorlie *et al.* 2003). Later, a sixth claudin-low subtype has been identified named after a low expression of claudins, a class of cell-cell adhesion molecules (Herschkowitz *et al.* 2007). Most recently, a novel subclassification is in discussion. Based on this new study six subtypes were distinguished, including four luminal types and a novel 'molecular-apocrine' subtype, while the Her2-group was no longer acknowledged as a separate group (Guedj *et al.* 2012).

Despite the advances in understanding the molecular biology of mammary tumors, subtyping by gene profiling is rarely applied in the clinic and used for treatment decisions. Still most therapy decisions are based on conventional subtyping by immunohistochemistry. In more advanced stages of ER-positive tumors as well as in cases of Her2-positive and triple negative tumors chemotherapy is routinely given. Chemotherapy is often accompanied by severe side effects. Since some breast cancer types have a very low risk of recurrence, it would not be necessary to let the patients with these tumors suffer from toxic side effects. For assessment of the risk of recurrence, new biomarkers are now available allowing to separate patients who will benefit from chemotherapy from those who will not. For breast cancer patients with lymph node-negative tumors, urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1) are such markers (Schmitt *et al.* 2011). Recently, DNA microarrays and RT-PCR-based tests measuring expression levels of only a few genes became also available to determine the molecular subtype and to predict the patient`s outcome and response to chemotherapy. Among those, the PAM50 assay and the three-gene model for identifying the major and clinically relevant molecular subtypes of breast cancer are promising (Prat *et al.* 2012).

Taken together, it is now clear that breast cancer is a heterogeneous disease. The subtypes differ in the type of cell they originate from and in gene expression patterns. Tests that determine gene expression signatures for individual tumors are currently being developed for clinical use. In the future, new predictive biomarkers have to be identified for the individual breast cancer subtypes to allow the development of targeted therapies. Moreover, therapies to specifically attack resistant tumor cells as well as metastasized cells need to be identified.

2. Cyclic AMP signaling

The cyclic adenosine 3′, 5′-monophosphate (cAMP) pathway serves as an effector mechanism for hormones, growth factors and neurotransmitters and is involved in cellular functions ranging from T cell development and spermatogenesis to long-term memory or blood pressure regulation (Haus-Seuffert & Meisterernst 2000). Processes, like cellular differentiation, cell cycle regulation or cytoskeletal rearrangements, are controlled by cAMP and are relevant with regard to carcinogenesis (Sands & Palmer 2008). Responses downstream of cAMP are both dependent and independent of transcription.

2.1. The cAMP/PKA/CREB signaling cascade

The intracellular second messenger cAMP is generated from ATP by adenylyl cyclases. The activities of these enzymes are controlled by stimulatory and inhibitory G-proteins. These are connected to transmembrane G-protein-coupled receptors (GPCRs) that can be activated by a broad range of extracellular signaling molecules. Degradation of cAMP is performed by phosphodiesterases. Thus, the cellular cAMP level results from the activities of adenylyl cyclases and phosphodiesterases.

Elevation of the intracellular cAMP level affects cyclic nucleotide-gated ion channels, exchange proteins directly activated by cAMP (Epacs) and cAMP-dependent protein kinase A (PKA). PKA is an autoinhibited tetramer consisting of two regulatory and two catalytic subunits. Upon activation cAMP binds to the regulatory subunits in a 2:1 ratio and induces a conformational change of the complex (Krauss 2008). The subsequent release of the two catalytic subunits allows the catalytic subunit to become active. The activity is further increased by autophosphorylation of a threonine residue at position 197 in the activation loop. Passive diffusion leads to translocation of the activated PKA catalytic subunits into the nucleus where they phosphorylate the transcription factor cAMP-responsive element binding protein (CREB) at serine residue 133 (Fig. 2). Once activated, dimerized CREB binds to conserved cAMP-responsive elements (CRE; TGACGTCA or less active as half-site motif CGTCA) and recruits the co-activator CREB-binding protein (CBP) to the promoter (Mayr & Montminy 2001). CBP associates with the RNA polymerase II transcriptional initiation complex. Together, these functions contribute to transcriptional activation.

Serine/threonine phosphatase PP-1-mediated or protein phosphatase 2A-mediated dephosphorylation of CREB causes its inactivation. Whether dephosphorylated CREB binds DNA is a matter of debate. However, the majority of reports support the view that unphosphorylated CREB, at least as a monomer, is already attached to the DNA and that phosphorylation leads to dimerization of the DNA-bound CREB and subsequent CBP

recruitment (Mayr & Montminy 2001). Importantly, this recruitment is a rare event and strictly cell-type and context-dependent for a given gene (Zhang *et al.* 2005). Multiple additional factors are required for a CREB-CBP interaction.

The cAMP-regulated CREB family of transcription factors also includes cAMP response element modulator (CREM) and activating transcription factor 1 (ATF-1). A truncated form of CREM, referred to as ICER (inducible cAMP response element repressor) lacks the DNA-binding domain and serves as a negative regulator of cAMP signaling (Mayr & Montminy 2001). Additionally, a number of structurally different proteins with the common prefix CRE or ATF (activating transcription factor) have been identified that also bind to CRE sites. Only some of them are inducible by the cAMP pathway (Hai & Hartman 2001).

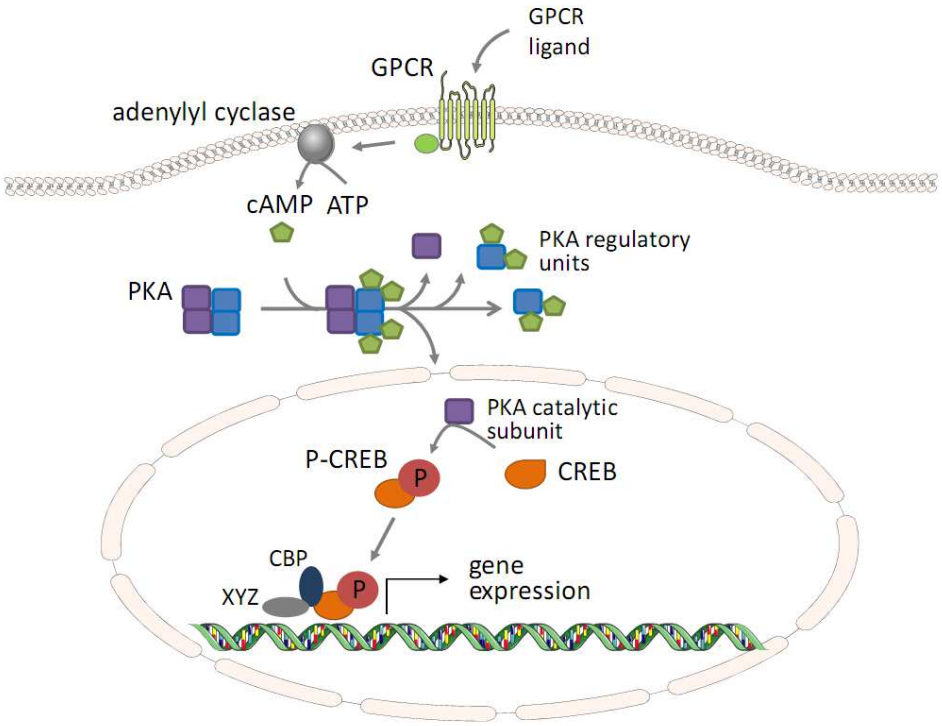

Figure 2. Schematic representation of the cAMP/PKA/CREB signaling cascade.

Upon stimulation of G_S-protein-coupled receptors the adenylyl cyclase is activated which generates cAMP. In response, cAMP binds to PKA which leads to the release of its catalytic subunits that enter the nucleus and activate the transcription factor CREB by phosphorylation and therefore initiates CREB-mediated transcription.

2.2. The role of the cAMP/PKA/CREB signaling pathway in breast cancer

Initially, the cAMP/PKA/CREB signaling pathway was found to inhibit the proliferation of breast cells. This finding has been obtained for non-tumorigenic and tumorigenic mammary cell lines after exposure to cAMP-elevating agents (Starzec *et al.* 1994). Also Zivandinovic and co-workers described an anti-proliferative effect of cAMP elevation in estrogen receptor-positive MCF-7 cells (Zivadinovic *et al.* 2005). In this cell line, adiponectin has been

proposed as an extracellular signal capable of inducing a rise in the cAMP level and in PKA activity (Li *et al.*) Another study reports that leptin potentiates the growth-suppressive effect of cAMP-elevating factors. This was dependent on PKA and involved a downregulation of cyclin D and cyclin A and an upregulation of the $p27^{Kip1}$ protein level (Naviglio *et al.* 2009). Since adiponectin and leptin are factors produced in adipocytes, these findings suggest an anti-cancer effect on epithelial cells by the surrounding adipose tissue via cAMP elevation.

In breast cancer, cAMP/PKA signaling also plays a role in establishing and maintaining resistance to tamoxifen, a common drug delivered to patients with ER-positive breast cancer. PKA-dependent phosphorylation of ERα at a serine residue at position 305 (S305) is involved in the development of this resistance. Following this phosphorylation, tamoxifen could still bind to the receptor, but failed to induce an inactive conformation. Instead, the receptor was activated (Michalides *et al.* 2004). This activation was due to an altered interaction of ERα with the steroid receptor co-activator-1 (SRC-1) allowing SRC-1 to facilitate RNA polymerase II recruitment and promote ER-dependent transcription (Zwart *et al.* 2007). Clinical studies supplied evidence for the notion that co-expression of PKA and ERαS305-P support tumor progression in breast cancer patients (Kok *et al.* 2011). Another study could show that, in tamoxifen-resistant MCF-7 cells, 17β-estradiol (E2) and the Her4 ligand heregulin promote CRE activity presumably via phosphorylation of glycogen-synthase kinase 3 β (GSK3β) (Oyama *et al.* 2011).

Recently, it was reported that PKA confers resistance to another breast cancer drug, trastuzumab, which targets the Her2 protein. In a trastuzumab-resistant subclone of BT474 cells, a number of genes known to regulate PKA activity were found to be de-regulated relative to parental cells (Gu *et al.* 2009). Experimental downregulation of the PKA regulatory subunit IIα in parental Her2-positive BT474 and SK-Br-3 cell lines also resulted in partially resistant phenotypes. Besides, protein kinase B (PKB, Akt) dephosphorylation by trastuzumab was successfully inhibited in the presence of forskolin suggesting that cAMP counteracts the inhibitory activity of trastuzumab.

In addition to tumor cells, tumor-associated adipose tissue was reported to show aberrant cAMP signaling. In these adipocytes, the expression of the enzyme aromatase, which is responsible for estrogen production, relies on CREs in an alternative aromatase gene promoter which could be induced by cAMP via CREB (Sofi *et al.* 2003). Estrogen is an important factor that stimulates proliferation of breast cancer cells. It should be noted that aromatase expression is significantly higher in adipose tissue of tumor-bearing breasts than in normal breasts suggesting a contribution of adipose tissue to cancer development.

Signaling events involving PKA and CREB also seem to be important in advanced breast cancer. Significantly higher CREB1 mRNA levels were measured in cancerous compared to normal breast tissues (Chhabra *et al.* 2007). Patients suffering from advanced breast cancer showed higher RNA levels of CREB1. Furthermore, elevated CREB1 protein expression correlated with a bad prognosis and was also more abundant in high grade as well as in lymph node-positive tumors. Additional evidence for a possible involvement of cAMP signaling in breast cancer progression was presented by Chioni and co-workers. This group could clearly demonstrate that cAMP and PKA regulate the voltage-gated $Na^{(+)}$ channel Nav1.5-dependent migration of MDA-MB-231 cells (Chioni *et al.* 2010). Furthermore, CREB signaling was also associated with an increase in proliferation, migration and invasion of triple negative MDA-MB-231 cells (Son *et al.* 2010).

cAMP/PKA signaling also participates in the formation and establishment of bone metastases. The bone sialoprotein (BSP), whose high expression is an indicator of bone metastatic potential of cancer cells, is regulated by CRE-containing promoter elements (Detry *et al.* 2008). However, its activation may be independent of an activated PKA, since CREB is already bound to CRE and promoter activity could not be increased by cAMP. Hence, in this case, a constitutively active CRE exists in the BSP promoter as shown for MCF-7 and MDA-MB-231 cells. Other genes, such as parathyroid hormone-related peptide (PTHrP), involved in bone metastasis are regulated by CREB in MDA-MB-231 cells that *per se* exhibit higher CREB expression and CREB phosphorylation than non-metastatic MCF-7 cells. Ectopic expression of CREB resulted in enhanced expression of PTHrP, MMP2 and MMP9 in MDA-MB-231. *In vivo* studies with mice showed massive bone destruction after injection of the CREB-transformed breast cancer cells (Son *et al.* 2010). In an attempt to investigate the bone metastatic properties of stem-like breast cancer cells, side population (SP) cells were isolated from MDA-MB-231 cells. This subpopulation of cells is defined by their ability to exclude Hoechst 33342 dye, which is a feature of stem-like cancer cells (Hiraga *et al.* 2006). However, the study did not reveal a difference in the bone metastatic potential between SP and non-SP cells despite the fact that MCF-7 SP cells exposed increased CREB and P-CREB levels (Wang *et al.*).

In conclusion, cAMP signaling induces anti-proliferative effects in early tumor stages but is also involved in breast cancer progression of advanced stages and contributes to the development of resistance to pharmaceutical intervention.

3. The TGFβ signaling pathway in breast cancer

The transforming growth factor-β (TGFβ) signaling pathway plays a role in almost all cellular processes, including cell cycle regulation, differentiation, adhesion and invasion. Components of this pathway are ubiquitiously expressed throughout the body at various developmental stages as well as in health and disease. The canonical TGFβ pathway is characterized by three major steps. The ligand binds to the extracellular domains of a membrane-bound receptor which then phosphorylates a transcription factor and induces its translocation into the nucleus where it activates or represses transcription.

3.1. The TGFβ signaling cascade

3.1.1. The canonical TGFβ signaling

TGFβ-like proteins form a family of cytokines comprising more than 40 members. Besides TGFβ 1-3 it includes bone morphogenetic proteins (BMPs) 1-8, activins 1-5 and inhibins. The different ligand classes require different receptor types. Signals provided by the TGFβ ligands are transduced into the cell by a type I TGFβ receptor (ALK1-7) and a type II TGFβ receptor [TβRII; bone morphogenetic protein receptor type II (BMPRII), ACTRII, ACTRIIB and anti-Mullerian hormone receptor, type II (AMHRII)]. The receptors contain intracellular serine/threonine kinase domains (Bierie & Moses 2006). Upon binding of TGFβ to TβRII a heterotetrameric complex consisting of two receptors of each type is generated. This constellation allows the phosphorylation of type I TGFβ receptor by type II TGFβ receptor (Fig. 3). Activated by phosphorylation, the type I TGFβ receptor then phosphorylates receptor-activated Smads (referred to as R-Smads) which, in response, are released from the plasma membrane. In most cell types, activin receptor-like kinase 5 (ALK5) is the predominant type I receptor. Therefore, the term TβRI is often used equivalent to ALK5 (also in this study). Five R-Smads have been found in mammalian cells, of which Smad2 and 3 are activated by TβRII-ALK5 and Smad1, 5 and 8 by TβRII-ALK1 (Ikushima & Miyazono 2010) Two phosphorylated R-Smads Smad2 and/or Smad3 (depending on spatial and temporal expression) oligomerize with one Smad4 molecule (Co-Smad), which does not require phosphorylation, and translocate into the nucleus. After binding to the DNA at specific Smad binding elements (SBEs = AGAC boxes) Smads serve as transcriptional activators or repressors. They usually act in concert with other transcription factors. Among those, E26 transformation-specific transcription factors (Ets), activator protein 1 (AP1), specificity protein 1 (Sp1), p300, co-activator of CREB-binding protein (CBP) and retinoblastoma-like 1 (RBL1) are known so far (Ikushima & Miyazono 2010, Lindemann *et al.* 2001). In order to terminate Smad-dependent transcription R-Smads are de-phosphorylated and exported out of the nucleus. (Inman *et al.*, 2002;Lin *et al.*, 2006;Reguly & Wrana 2003, Tang *et al.* 2011).

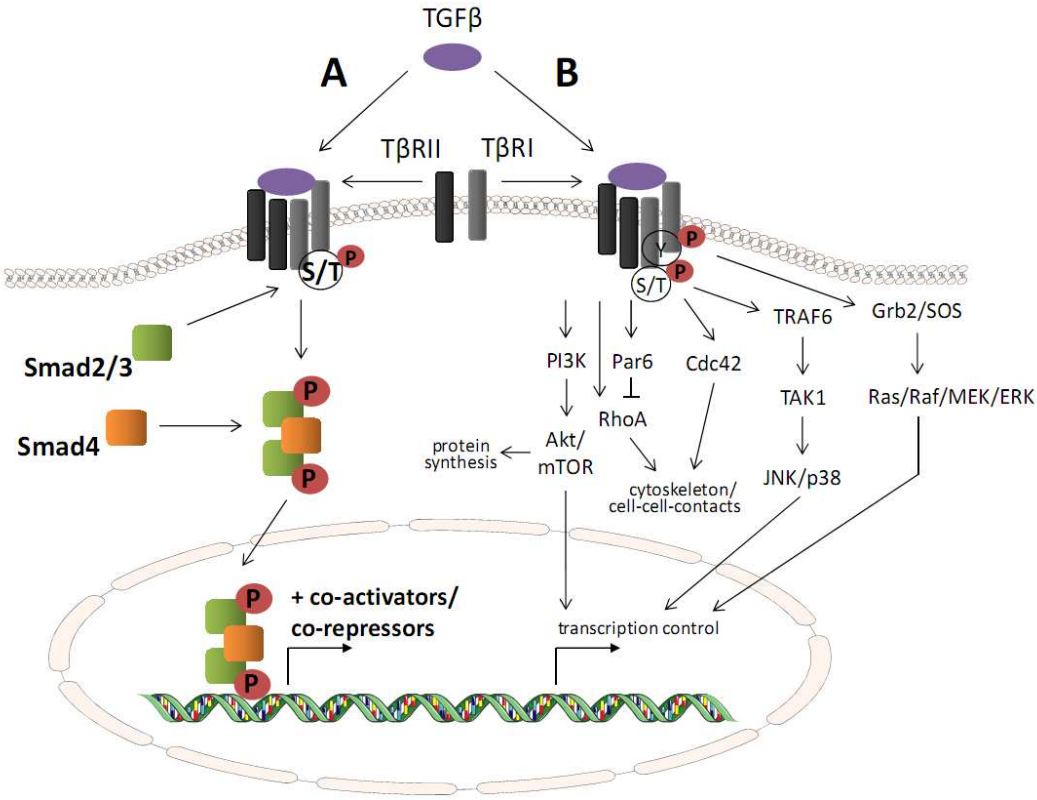

Figure 3. Schematic representation of canonical (A) and non-canonical (B) TGFβ signaling.

In the presence of TGFβ, a heterotetrameric receptor complex consisting of TβRI and TβRII receptors is formed. (A) In this constellation, TβRII phosphorylates TβRI, which then recruits and phosphorylates Smad2/3. Smad2/3 associates with Smad4 and enters the nucleus where it regulates transcription in conjunction with co-activators or co-repressors. (B) Besides Smads, other proteins may be regulated by the TGFβ receptor complex. Non-canonical TGFβ signaling via the PI3K/Akt, TRAF6/TAK1/JNK/p38 or the Ras/Raf/MEK/ERK pathways regulates transcription and alterations of the cytoskeletal organization and cell-cell-junctions are mediated by Par6/RhoA or Cdc42.

3.1.2. Non-canonical TGFβ signaling

Apart from the Smad-dependent, also called canonical TGFβ signaling, TGFβ is able to modulate the activities of multiple other signaling pathways. Among these non-canonical actions, TGFβ affects the mitogen-activated protein kinase (MAPK) cascades. Their activation usually results from the ligand-induced stimulation of receptor tyrosine kinases (RTKs). It has been found that the TGFβ receptors also harbor tyrosine residues additional to their serine/threonine residues whose phosphorylation is required for Smad phosphorylation. Phosphorylation of these tyrosine residues allows the recruitment of the adaptor proteins growth factor receptor-bound protein 2 (Grb2) and son of sevenless (SOS) that initiate the Ras/Raf/MEK/ERK pathway and may thus mimic RTK activation leading to the activation of this pathway. The other MAPK pathways which induce c-Jun N-terminal kinase (JNK) and p38 kinase activation may also be switched on by TGFβ but not as effectively. In this case, TGFβ activates the MAP3K TGFβ-activated kinase 1 (TAK1), which initiates a phosphorylation cascade leading to activation of JNK and p38. The interaction between the

TGFβ receptor complex and TAK1 is mediated by TNF receptor-associated factor 6 (TRAF6).

Additionally, TGFβ may also interfere with pathways controlling the cytoskeletal organization that is important for cell morphology and migration. To regulate migration, TGFβ acts on the Rho-like GTPase RhoA. One the one hand, RhoA is rapidly activated by TGFβ which enables stress fiber formation. On the other hand, TGFβ also initiates RhoA protein degradation by activating partition-defective complex protein 6 (Par6) (Zhang 2009). This process starts delayed and initiates tight junction disassembly. Both reactions are required for TGFβ-dependent EMT (for detail see section 3.2.2). Moreover, TGFβ activates the Cdc42 GTPase that leads to complex formation with the tight junction protein occludin and therefore attraction of TβRI to tight junctions where it contributes to TGFβ`s EMT-initiating function.

TGFβ has also been described as an activator of the phosphatidylinositol 3-kinase (PI3K) pathway. The phosphorylation of Akt (also known as protein kinase B; PKB) and subsequent activation of the mammalian target of rapamycin (mTOR) are involved in TGFβ-induced EMT, protein synthesis activity, cell migration, apoptosis and growth inhibition (Zhang 2009).

Additionally, TGFβ interacts with other pathways. Since TGFβ regulates the expression of Wnt5a, it has been shown to suppress Wnt/β-catenin signaling (Serra *et al.* 2011). Furthermore, evidence has been collected for an antagonistic effect of ER signaling on TGFβ-induced transcription and functions (Band & Laiho 2011). Interaction between TGFβ and cAMP pathway has also been described and are summarized in section 4.

3.1.3. Regulation of TGFβ signaling

The TGFβ signaling pathway appears simple but, since its impact on cellular functions is immense, every component is tightly regulated. The TGFβ protein is produced as a latent high molecular weight complex, which is processed several times. First of all, a propeptide is cleaved but stays attached to the complex non-covalently in the course of secretion. TGFβ still keeps its inactive state as it is embedded in the extracellular matrix and bound by latent TGFβ-binding proteins. Factors like plasmin or thrombospondin-1 mediate the release of TGFβ in its active form (Blobe *et al.* 2000).

At the receptor level the abundance and ratio of type I and II receptors might determine the rate of signal transduction into the cell (Pannu *et al.* 2004). Moreover, other receptors, like the type III receptors, influence ligand binding efficiency. They bind TGFβ and pass it on to the

signaling receptors without being able to promote signal transduction by itself (Blobe *et al.* 2000).

Several factors define the level of activated Smads in the nucleus and therefore TGFβ-dependent gene expression. Among them are a number of nuclear import or export factors and retention proteins in the cytoplasm. As an example, Smad anchor for receptor activation (SARA) which has been described as indispensable for optimal positioning of Smads for receptor-mediated phosphorylation also inhibits the nuclear import of unphosphorylated Smad2 under certain conditions (Xu *et al.* 2000). Non-phosphorylated Smads constantly shuttle between cytoplasm and nucleus, but once phosphorylated, the nuclear location is favored (Chen & Xu 2011, Varelas *et al.* 2011). As a major player in controlling the phosphorylation status of TGFβ-activated Smad2/3 the phosphatase PPM1/PP2C has been claimed (Lin *et al.* 2006). The function of activated R-Smads can also be impaired by subjecting Smad4 to reversible monoubiquitination which alters its binding capabilities (Dupont *et al.* 2009). Recently, two other functionally redundant proteins, Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ), were shown to interact with TGFβ-induced Smad complexes and thereby determine their subcellular localization (Varelas *et al.* 2008, Varelas *et al.* 2011). Usually, a nuclear retention is favored, whereas, at high cell density, YAP is predominantly located in the cytoplasm also relocating the Smad complexes. As a consequence, TGFβ-induced gene expression is lower in high-density cells.

The total Smad3 protein content is regulated on the RNA and on the protein level (Daly *et al.* 2010). The major ubiquitin E3 ligases responsible for proteasomic degradation of Smad proteins are Smad ubiquitin regulatory factors (Smurfs) 1 and 2 as well as SCF/Roc (Izzi & Attisano 2004, Lin *et al.* 2000, Lo & Massague 1999, Tang *et al.* 2011). Smurf ubiquitin ligases are recruited upon binding of inhibitory Smads (I-Smads; Smad6 and 7) (Kavsak *et al.* 2000) which are TGFβ transcriptional targets and generate a negative feedback loop for TGFβ signaling (Imamura *et al.* 2012).

3.2. Implications of TGFβ in breast cancer

The ubiquitious expression of TGFβ pathway components and the tight regulation of the TGFβ pathway suggest an important function in balancing tissue homeostasis. The finding that this factor is capable of subjecting fibroblasts to malignant transformation had led to its naming and started investigations about TGFβ`s role in tumorigenesis (Moses *et al.* 1981, Roberts & Sporn 1985).

Paradoxically, soon studies showed a clear growth-suppressive effect of TGFβ on epithelial cells (Fig. 4) (Zu *et al.*). *In vivo* experiments in mice confirmed opposing TGFβ functions. Less tumor formation was observed in TGFβ transgenic mice after chemical induced carcinogenesis compared to wildtype mice. Her2-expressing mice also showed less tumors, when TβRI was expressed in the mammary epithelium. In addition, mammary epithelium-specific expression of a dominant negative TβRII resulted in a higher occurrence of spontaneous tumors (Gorska *et al.* 2003). In contrast to these findings, human DCIS and IBC often show a downregulation of TβRII. Along these lines, it was shown that a reduced level of nuclear phosphorylated Smad2/3 correlated with a higher tumor grade and size (Zu *et al.*). Moreover, the expression of extracellular TGFβ1, TβRII and phospho-Smad2 seems to be linked to tumorigenesis at an earlier age (Figueroa *et al.* 2010).

3.2.1. The role of TGFβ in regulating cell growth

TGFβ is an important regulator of cell growth, since it affects proliferation, apoptosis and autophagy. It leads to G1 phase cell cycle arrest by inducing the expression of cyclin-dependent kinase inhibitors (CDKs) $p15^{INK4b}$, p21, p27 and p57 and downregulation of the pro-proliferative transcription factor c-Myc. The growth-inhibitory effect of TGFβ is most prominent in epithelial and hematopoietic cells (Ikushima & Miyazono 2010, Lin *et al.* 2007, Ravitz *et al.* 1996).

On the other hand, TGFβ promotes proliferation of mesenchymal cells, such as smooth muscle cells, by upregulation of platelet-derived growth factor (PDGF). Induction of PDGFA and PDGFB by TGFβ has also been shown in glioma and osteosarcoma (Ikushima & Miyazono 2010). These ligands activate mitogenic pathways that counteract the anti-proliferative TGFβ effect. Moreover, in advanced breast cancer, cells often overcome growth depression by TGFβ by disrupting the Smad complex responsible for binding to an inhibitory element in the *c-myc* promoter (Chen *et al.* 2001). Additionally, another common observation that explains suppression of TGFβ-mediated growth inhibition in cancer is the TGFβ-dependent activation of NFAT. Induction of this pathway counteracts c-Myc downregulation (Singh *et al.*).

An activating as well as repressing function of TGFβ concerning apoptosis and autophagy has also been described. TGFβ induces the expression of the pro-apoptotic death-associated protein kinase (DAPK), growth arrest- and DNA damage-inducible 45β (GADD45β), phospholipid phosphatase Src homology 2 domain-containing inositol phosphatase (SHIP), TGFβ-inducible early response gene 1 and Bcl-2 family member BIM. In contrast, differentially expressed in chondrocytes 1 (DEC1) is an anti-apoptotic TGFβ target. As for the regulation of autophagy, which is a survival mechanism that involves self-digestion of cellular

proteins, TGFβ increases transcription of autophagy (ATG) genes. Whether this event enhances or abrogates tumor cell survival is currently under investigation (Ikushima & Miyazono 2010, Imamura *et al.* 2012).

3.2.2. The role of TGFβ in breast cancer progression & metastasis

TGFβ has been shown to induce migratory and invasive behavior of cancer cells. For that, cells have to adopt a more mesenchymal phenotype and lose their epithelial characteristics, a process known as epithelial-mesenchymal transition (EMT) (Fig. 4). This is accompanied by a change in cell morphology from a rounded or cobble stone-like shape to a spindle-like appearance. Loss of proteins involved in maintaining an apical-basal polarity, cytoskeletal rigidity or cell-cell adhesion, such as E-cadherin or the cytokeratins 8, 18 or 19 reduces cell-cell contacts. An upregulation of intermediate filaments and matrix components, like vimentin, fibronectin, vitronectin, collagen as well as extracellular matrix (ECM)-degrading enzymes like matrix metalloproteinases, allows cells to migrate and invade into the surrounding tissue (Al Saleh & Luqmani 2011). The transcription factors Snail, Zinc finger E-box-binding homeobox 2 (ZEB) and bHLB basic helix-loop-helix (Twist) are involved in these changes and are upregulated by TGFβ. It is thought that EMT is a milestone event in the progression from a primary localized tumor to a metastasized disease (Ikushima & Miyazono 2010). In this context, it is likely that EMT may also be important for the generation of circulating tumor cells (CTCs), cells found in the bloodstream after having evaded from the primary tumor (Fig. 4). In addition, EMT is also associated with resistance to apoptosis (Mego *et al.* 2010).

Recently, Mani *et al.* showed that EMT in response to TGFβ also allows breast cancer cells to acquire stem cell-like characteristics (Fig. 4) which is accompanied by a higher tumor-initiating potential. Hence, TGFβ may be linked to stemness (Mani *et al.* 2008). In the last years, a lot of evidence has been collected supporting a cancer stem cell theory in breast cancer. According to this model, the tumor contains a subpopulation of cancer stem cells (CSCs) that are able to self-renew, to differentiate in multiple lines and to initiate tumorigenesis. In breast cancer, $CD44^{+}/CD24^{-}$ cells seem to meet these criteria (Al-Hajj *et al.* 2003). The CSCs show higher activation of TGFβ signaling compared to the bulk tumor (Hardt *et al.* 2012, Shipitsin *et al.* 2007). Along these lines, it was shown that blocking the autocrine TGFβ signaling impaired the maintenance of stem cell properties in primary mammary epithelial cells (Scheel *et al.* 2011). Further evidence supports the view that TGFβ is linked to an increased cell population with CSC characteristics (Oliveras-Ferraros *et al.* 2011, Wang *et al.* 2011). These groups uncovered miR181 as the mediator for the CSC-promoting TGFβ effect.

The TGFβ pathway has been shown to be important for the formation of metastasis (Fig. 4). After transfection of mesenchymal MDA-MB-231 breast cancer cells with a dominant negative type II TGFβ receptor bone colonization by these cells was significantly reduced. Introduction of a constitutively active type I TGFβ receptor restored the ability to induce bone metastasis (Yin *et al.* 1999).

In contrast, breast cancer cells of the epithelial type behave differently. MCF-7 cells that ectopically overexpress TβRI showed an increased TGFβ pathway activity and less metastatic burden (Micalizzi *et al.* 2010). Consistent with this finding, in MCF-7 cells ectopically expressing a dominant negative type II TGFβ receptor more metastases were found (Micalizzi *et al.* 2009).

TGFβ has also been shown to enhance bone destruction after tumor cells have begun to colonize this organ. Bone-invading tumor cells activate osteoblasts which then initiate the osteoclast-dependent degradation of the bone mass. As a consequence, TGFβ is released from the bone matrix and triggers the release of osteolytic factors, such as parathyroid hormone-related protein (PTHrP), by tumor cells. In response, receptor activator for nuclear factor κB ligand (RANKL) is produced by osteoblasts leading to further stimulation of osteoclast differentiation and thus to more bone destruction (Maroni *et al.* 2010). In addition, hypoxic metastatic cells produce higher TGFβ levels. Hence, hypoxia may enhance TGFβ-mediated bone destruction (Dunn *et al.* 2009).

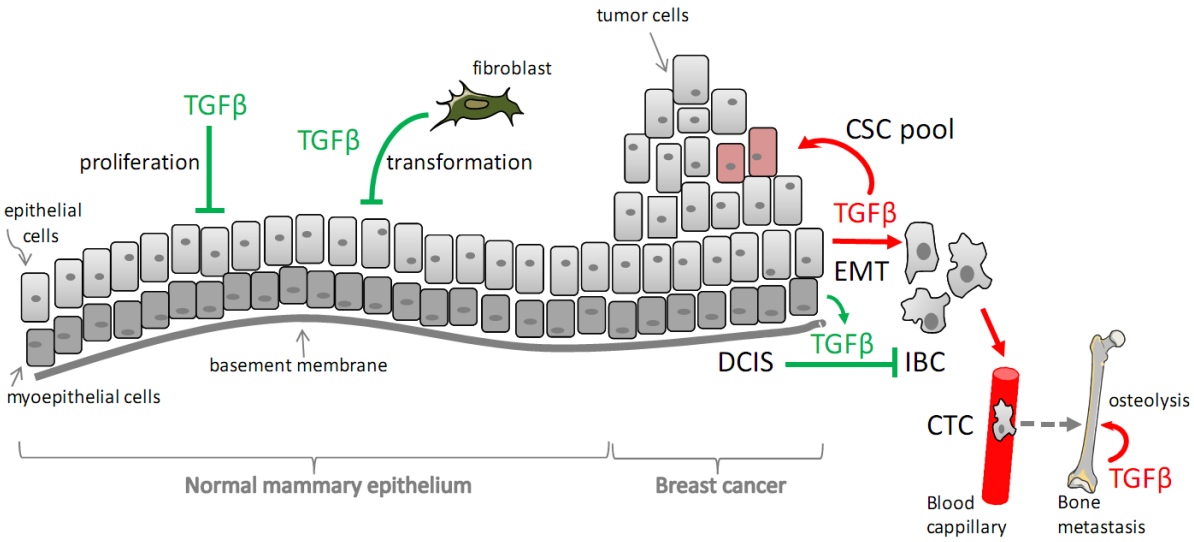

Figure 4. Dual role of TGFβ in tumor development and progression.

In early tumor stages, TGFβ is a tumor suppressor which inhibits the proliferation of epithelial cells, epithelial cell transformation and progression from a carcinoma *in situ* to an invasive carcinoma. Induction of EMT by TGFβ generates invasive tumor cells and increases the CSC pool. Release of TGFβ from the bone matrix supports osteolysis in bone-metastasized breast cancer. Red lines indicate tumor-promoting, green lines tumor-suppressing TGFβ functions.
DCIS = ductal carcinoma *in situ*; IBC = invasive breast cancer; CSC = cancer stem cell; EMT = epithelial-mesenchymal-transition; CTC = circulating tumor cell

Taken together, once cancerous cells have overcome growth inhibition by TGFβ they may profit from TGFβ-induced EMT in the course of advancing to a metastatic disease. Importantly, TGFβ may also be involved in the generation of the cancer stem cells in breast cancer. Today, the idea of a dual function of TGFβ in cancer development and progression, also known as the TGFβ paradox, has been widely accepted (Roberts & Wakefield 2003). The events that lead to this switch in TGFβ function are not clearly defined up to now. Both, changes within the tumors cells and alterations within the tumor microenvironment are likely to contribute to that switch (Ikushima & Miyazono 2010, Micalizzi *et al.* 2010, Parvani *et al.* 2011).

3.2.3. TGFβ and the tumor microenvironment

As discussed for metastasized bone, TGFβ signaling affects both the tumoral and stromal compartment of the cancer lesion. Hence, the effects of TGFβ on the tumor stroma which may account for more than 50 % of the tumor bulk and on myoepithelial cells have also been considered as driving forces in tumor progression (Zu *et al.*).

Gene expression studies revealed differences between myoepithelial cells isolated from normal breast tissue, DCIS and IBC. The most striking differences were found between normal breast cells and cells from DCIS. In DCIS-associated myoepithelial cells, genes involved in differentiation were found to be downregulated, whereas tumor-promoting genes were upregulated (Allinen *et al.* 2004, Place *et al.* 2011). Furthermore, in cell culture, normal myoepithelial cells prevented DCIS tumor cells from progression to an invasive state. This effect was dependent on TGFβ signaling (Fig. 4) suggesting a tumor-suppressive role for TGFβ in this experimental setting (Place *et al.* 2011).

Stromal fibroblasts have also been shown to block malignant transformation of neighboring epithelial cells. This effect again depends on TGFβ signaling (Fig. 4) which suppresses the release of tumor-supporting factors (Bhowmick *et al.* 2004). However, the response to TGFβ differs between different types of fibroblasts. Transcriptional profiling revealed that fibroblasts isolated from different organs show different gene expression patterns (Chang *et al.* 2002, Place *et al.* 2011). From breast cancer lesions, cells with an “activated fibroblast” signature have been isolated. A similar profile could be achieved by exposing fibroblasts to serum. Activated fibroblasts differ from normal fibroblasts in the transcript levels of genes related to wound healing (Chang *et al.* 2004). The fibroblasts with altered gene expression found in breast cancer are termed carcinoma-associated fibroblasts (CAFs). Some of them express alpha-smooth muscle actin (α-SMA), a typical marker of myofibroblast, and are therefore also called carcinoma-associated myofibroblasts. CAFs may arise from normal stromal fibroblasts and maintain their activated state in the presence of TGFβ (Fig. 5) and

stromal cell-derived factor 1 (SDF-1) (Kojima *et al.*). Isolated CAFs and normal stromal fibroblasts also showed different TGFβ responses. CAFs showed enhanced myofibroblast differentiation, elevated expression of fibronectin and laminin and, compared to normal stromal fibroblasts, a greater ability to enhance the invasive behavior of MDA-MB-231 breast cancer cells in response to TGFβ (Casey *et al.* 2008) (Fig. 5). In part, CAFs are generated by differentiation of mesenchymal stem cells (MSCs) which enter tumors presumably in an attempt to "repair" the tumor, which is mistaken by the MSC as a wounded tissue (Dittmer 2010, Mishra *et al.* 2008) (Fig. 5). A study conducted by Mishra and Banerjee found evidence for a participation of TGFβ in this process. (Mishra & Banerjee 2011). In addition, tumor exosomes stimulated the TGFβ pathway in stromal MSCs and promoted their differentiation into tumor-associated myofibroblasts (Cho *et al.* 2012).

The conversion of endothelial cells to mesenchymal cells, in a process referred to as endothelial-mesenchymal transition (EndMT), is thought to be another way to generate CAFs (Fig. 5). TGFβ is one of the known inducers of this conversion (van Meeteren & ten Dijke 2012); (Miyazono *et al.* 2011). CAFs support tumor development by different mechanisms (Xing *et al.* 2011).

Fibroblasts resident in the mammary tissue are the predominant cell type responsible for constituting and remodelling the ECM (Fig. 5). For this reason, it is not surprising that alterations in the fibroblast population in cancerous tissues contribute to ECM changes. TGFβ is a strong inducer of fibronectin and collagen expression. Fibronectin in connection with integrin receptors serves as a signaling molecule that may enhance proliferation and migration of epithelial cells. Elevated amounts of collagen were found to be associated with mammary tumors. Moreover, a higher collagen content and cross-linking enhanced matrix rigidity and increased tumor cell invasion. The remodelling of the matrix is dependent on MMPs released by fibroblasts. These enzymes do not only degrade matrix components, but also induce the release and therefore activate matrix-embedded growth factors which may promote tumor activities (Parvani *et al.* 2011, Place *et al.* 2011).

TGFβ has also been made responsible for tumor angiogenesis (Fig. 5). They support endothelial cell growth through induction of the pro-angiogenic factors connective tissue growth factor (CTGF) and vascular endothelial growth factor (VEGF). Moreover, in endothelial cells, TGFβ was shown to increase the expression of matrix metalloproteinases 2 and 9 (MMP-2, -9) and to downregulate tissue inhibitors of metalloproteinase (TIMPs) leading to enhanced migration and invasion of these cells (Miyazono *et al.* 2011). Additionally, in mammary tumor-induced bone lesions, TGFβ contributed to angiogenesis via the upregulation of vascular endothelial growth factor (VEGF) and monocyte chemotactic protein (MCP)-1 (Wilson *et al.* 2010).

Although TGFβ is a potent chemoattractant for T-lymphocytes and neutrophils it has been shown to act as an inhibitor of effector functions of T-cells, NK-cells and macrophages (Fig. 5). In tumor cells, TGFβ upregulated FAS ligand (FASL). Usually, FASL-expressing cells are recognized and lysed by neutrophils, but this effect is also suppressed by TGFβ. Moreover, this surface antigen leads to apoptosis of T-cells that produce the receptor counterpart FAS. Together, these mechanisms contribute to the inability of the immune system to conquer tumor cells (Bierie & Moses 2006).

In summary, TGFβ serves as a tumor suppressor in early tumor stages mainly by preventing proliferation, while in later stages it supports tumor progression via promotion of EMT and colonization of the bone. Besides its tumor cell-autonomous functions TGFβ acts as a microenvironment remodeller by upregulating secretory proteins in different cell types. TGFβ signaling is involved in the cross-talk of cancer cells and cancer-associated fibroblasts, reprogramming of immune cells and transdifferentiation of different other cell types. Hence, in advanced tumors with complex alterations also within the tumor microenvironment, TGFβ contributes further to tumor promotion.

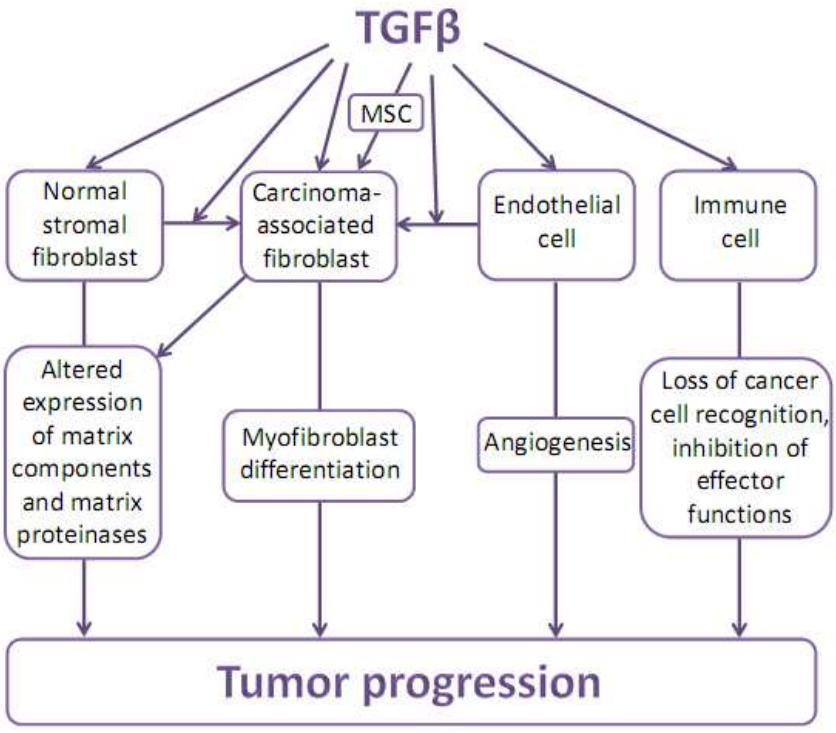

Figure 5. TGFβ`s effects on stromal cells promote tumor progression.

TGFβ contributes to the differentiation of normal stromal fibroblasts, mesenchymal stem cells (MSCs) and endothelial cells to carcinoma-associated fibroblasts (CAFs). Both, normal stromal fibroblasts and CAFs induce changes within the tumor matrix. CAFs, in part, differentiate into myofibroblasts in response to TGFβ. Under the influence of TGFβ, endothelial cells promote angiogenesis. TGFβ affects immune cells which may lose their effector functions and their ability to recognize tumor cells.

4. Interactions of cAMP and TGFβ signaling

An enormous number of reports describe the single effects of either cAMP or TGFβ in different cell types but only a few show effects in combination. Most investigations on combinatorial effects report an inhibitory action of cAMP on TGFβ stimulation of genes. Some show synergistic activation (Tab. 1).

Table 1. Studies that described cAMP/TGFβ cross-talk and proposed a mechanism.

authors	**cell type**	**cAMP effect on TGFβ-dependent gene expression**	**mechanism**
Schiller *et al.* 2010	Human dermal fibroblasts	inhibitory	↓ Smad3-CBP/p300 complex
Schiller *et al.* 2010	Human dermal fibroblasts	promoting	Close vicinity of CREB and Smad binding sites
Xing *et al.* 2009	Rabbit corneal keratinocytes	inhibitory	No change in Smad or MAPK pathway ↓ RhoA activation
Gressner *et al.* 2008	Rat hepatocytes	inhibitory	↓ Smad2 level ↓ P-Smad1/3 ↓ Smad2/3-CBP/p300 complex
Liang *et al.* 2008	Neuron-enriched cerebral cortical cells of rats	inhibitory	↓ Smad3/4-CBP/p300 complex
Liu *et al.* 2006	Rat cardiac fibroblasts	inhibitory	↓ Smad-CBP1 complex ↓ P-ERK
Ohta *et al.* 2008	Mouse myoblast cell line C2C12	promoting	Transcriptionally active P-Smad1/5/8-P-CREB-CBP complex formed

In cardiac fibroblasts, cAMP-elevating agents led to attenuation of the stimulating TGFβ effect on α-SMA and collagen I and III protein expression. The authors describe a reduced ERK1/2 phosphorylation, when cells were treated with cAMP-elevating agents plus TGFβ as compared to cAMP-elevating agents or TGFβ alone. Pharmaceutical inhibition of ERK1/2 phosphorylation also resulted in an abrogation of the TGFβ effect. Additionally, cAMP was also found to disrupt the interaction of Smads with the co-activator CBP1 which contributed to the negative effect of cAMP on TGFβ signaling (Liu *et al.* 2006). In another report, a

decrease in TGFβ-induced α-SMA expression by cAMP was shown by using keratinocytes. In this case, no changes in phosphorylation of ERK or other MAPKs nor in the level of phospho-Smad3 or in phospho-Smad3-CBP complex formation was found. Interestingly, in these cells, cAMP showed an antagonizing effect on TGFβ-stimulated RhoA activation (Xing & Bonanno 2009). Furthermore, in rat hepatocytes, caffeine and 8-bromo-cAMP suppressed TGFβ-induced CTGF expression which was accompanied by a decrease in Smad2 and phosho-Smad1/3 levels. A dissociation of the Smad2/3-CBP/p300 complex was proposed as the underlying mechanism (Gressner *et al.* 2008). In neurons, lithium has been shown to inhibit Smad3/4 transactivation by a depletion of p300/CBP which forms a complex with phospho-CREB rather than with Smad3/4 in this context (Liang *et al.* 2008).

In fibroblasts derived from palmar fascia of the hand, a suppressing effect of cAMP on the TGFβ response on the RNA and, in part, also on the protein level of α-SMA, collagen (COL) 1A2, COL3A1 as well as on fibronectin 1-extra domain A (FN1-EDA) and connective tissue growth factor (CTGF) genes was described. Increasing the intracellular cAMP level alone did not alter gene expression (Satish *et al.* 2011). Using human dermal fibroblasts Schiller *et al.* could confirm an inhibitory effect of dibutyryl cyclic AMP and forskolin on the TGFβ-mediated expression of COL1A1, COL1A2, CTGF and also on PAI-1 and TIMP-1. Again, cAMP alone was not effective. As an explanation, the authors propose a cAMP-dependent depletion of the co-factor CBP, that, under these conditions, is not sufficient to mediate the Smad-dependent TGFβ effect (Schiller *et al.* 2010). A decreased level of PAI-1 protein in response to simultaneous treatment with TGFβ and forskolin as compared to TGFβ stimulation alone had been shown earlier in different epithelial and fibroblastic cells derived from different species (Thalacker & Nilsen-Hamilton 1992). The same group and others report reduced PAI-1 protein expression following cAMP elevation in most cell lines tested (Santell & Levin 1988, Thalacker & Nilsen-Hamilton 1992).

Schiller *et. al.* also report an opposite effect of cAMP on TGFb-mediated expression. In their study, a synergistical induction of hyaluronan synthase 2 by cAMP and TGFβ was found. The authors propose that this synergism is due to the close vicinity of a CREB and a Smad binding sites in the promoter of this gene (Schiller *et al.* 2010). In C2C12 muscle-derived cells, another group showed that cyclic AMP enhanced BMP signaling via complex formation of phospho-Smad1/5/8 with phospho-CREB and CBP (Ohta *et al.* 2008).

Another study provides additional evidence for an interaction between the two pathways by reporting the induction of CREB expression in the presence of TGFβ in MDA-MB-231 cells. This study showed that ectopic CREB expression enhanced the TGFβ-dependent expression of PTHrP, MMP2 and MMP9 and decreased expression of OPG in MDA-MB-231 cells (Son *et al.* 2010).

A different way of communication of the two pathways is described for cystic fibrosis transmembrane conductance regulator (CFTR) in alveolar epithelial cells. In this case, the cAMP-stimulated expression of this gene was inhibited by TGFβ via inactivation of the β2 adrenergic receptor, which induces a rise in the intracellular cAMP level upon interaction with G-proteins in these cells (Roux *et al.* 2009).

Some studies also showed an influence of cAMP on TGFβ-induced cell functions or phenotypes. Elevated intracellular cAMP levels suppressed the migration of fibroblasts and the ability of fibroblasts to contract collagen gels (Schiller *et al.* 2010). In addition, in fibroblasts and rabbit corneal keratocytes, cAMP reduced a TGFβ-induced myofibroblast differentiation (Satish *et al.* 2011, Xing & Bonanno 2009).

Taken together, most of the work focusing on the influence of cAMP elevation on the TGFβ responsiveness of genes found an antagonistic effect. However, there were also reports revealing a supportive role of cAMP on the TGFβ regulation of certain genes. Of note, besides on the gene the responses were also dependent on the cell type.

5. Objectives of this work

It has been shown that cAMP and TGFβ pathways are involved in breast cancer development and progression. The cross-talk of these pathways has been described for different cell types, but studies with breast cancer cells have not been conducted so far. Mechanisms underlying this interaction have been identified and show a high diversity among different cell types.

The aim of this study was to investigate the interplay of the cAMP and the TGFβ pathways in breast cancer cells, especially if and how cAMP modulates the expression of TGFβ-regulated genes that are important in breast cancer.

Apart from the conventional 2D cultures, where cells grow attached to a plastic surface, cells were also maintained and treated in 3D cultures. In 3D-cultures, attachment of the cells to the culture dish is prevented which allows the formation of cell-cell-interactions in three dimensions. Different 3D systems have been developed. For most of them, the addition of extracellular matrix components is required. It is known that breast cancer cells are capable of secreting their own matrix proteins and that the addition of different matrix proteins influences the expression of the cell`s surface receptors and cellular organization (Benton *et al.* 2009, Krause *et al.* 2010, Swamydas *et al.* 2010). In order to avoid these artificial responses, in this study, no matrix compounds were added to the 3D cultures. This 3D model might resemble either the *in vivo* situation of an early tumor stage, when tumor cells have not

yet evaded the ductal lumen (carcinoma in situ), or a very advanced tumor stage, when dissiminated tumor cells had formed a metastatic aggregate, e.g. in pleural effusions or cerebrospinal fluids. The 3D culture system also allows investigations of cell functions that cannot be observed in 2D cultures, such as homotypic aggregation (Dittmer *et al.* 2008).

The mesenchymal TGFβ-responsive triple negative breast cancer cell line MDA-MB-231 was chosen as a model system for aggressive breast cancer. These cells display a highly proliferative and invasive phenotype, while being sensitive to TGFβ-induced tumor promoting effects and being resistant to TGFβ-induced growth inhibition (Kalkhoven *et al.* 1995). Their invasive capacity allows them to penetrate the basement membrane. The molecular basis for the process is, in part, the expression of matrix-degrading enzymes. MDA-MB-231 cells express a variety of MMPs with MMP9 as the most prominent MMP responsible for invasion (Liu *et al.* 2003). Autocrine TGFβ signaling has been shown to be important for cell motility and invasion of this cell line (Farina *et al.* 1998).

Considering the low expression of E-cadherin and high expression of vimentin as well as N-cadherin and cadherin 11 (Karnoub *et al.* 2007, Tamura *et al.* 2008) MDA-MB-231 cells are considered as mesenchymal-type cells. Gene expression profiles clearly confirmed a mesenchymal–like signature (Charafe-Jauffret *et al.* 2006). However, MDA-MB-231 cells are regarded as cells that have not accomplished EMT entirely, but rather as having undergone a partial EMT. This is due to the retained epithelial cell shape and the maintained responsiveness to EMT-inducing stimuli (Karnoub *et al.* 2007).

The importance of TGFβ signaling for the formation of bone metastasis by MDA-MB-231 cells was shown by selectively blocking type II TGFβ receptor (Yin *et al.* 1999). Suppressing the activity of this receptor led to less bone degradation. Restoration of TGFβ signaling by transfecting the cells with a constitutively active type I TGFβ receptor restored PTHrP production and the capacity to induce bone destruction.

In MDA-MB-231 cells, also cAMP/PKA/CREB signaling participates in tumor progression. It has been shown that CREB regulates a number of genes found in advanced tumors, such as PTHrP, MMP2 and MMP9 and that a higher expression of CREB favors osteolysis (Son *et al.* 2010).

Therefore, to learn about the potential interactions between the TGFβ and cAMP pathways in breast cancer, MDA-MB-231 cells are an appropriate model to work with. Signals that interfere with TGFβ pathways would offer approaches to fight its tumor promoting actions.

MATERIALS AND METHODS

1. Materials

1.1. Chemicals

If not indicated differently, all chemicals were purchased from Applichem, Darmstadt, D.

1.2. Standard buffers and media

Table 2. Formulation of standard buffers and media used in this study.

Buffer/Medium	Formulation
Dulbecco`s PBS	Diluted from 10X solution (Promocell GmbH, Heidelberg, D)
5X TBE	54 g tris base, 27,5 g boric acid, 20 ml 0,5 M EDTA pH 8.0 ad 1000 ml *A. dest.*
LB medium	Made from LB medium powder according to Miller (25 g/l)
LB agar	Made from LB agar powder (40 g/l)
4X protein sample buffer	5.00 ml H_20 + 6.25 ml 1 M Tris, pH 7 + 20.00 ml 10 % SDS + 5.75 ml 0.4 % bromphenolblue + 19.50 ml glycerine + 2.50 ml ß-mercaptoethanol

Specialized buffers and media are mentioned in the corresponding method section.

1.3. Cell culture additives

Table 3. Additives used in cell culture experiments.

Additive	Solvent	Manufacturer
FSK	DMSO	Calbiochem/Merck Millipore, Billerca, MA, USA)
TGFβ1	1 mg/ml bovine serum albumin, 4 mM HCl	R&D Systems, Minneapolis, MN, USA
Actinomycin D	50 % DMSO	Calbiochem/Merck Millipore, Billerca, MA, USA)
Ly364947	DMSO	Tocris Bioscience, Bristol, UK
HDAC inhibitor 3	DMSO	Calbiochem/Merck Millipore, Billerca, MA, USA)

1.4. Antibodies

Table 4. Secondary antibodies used for Western blot (WB) analysis and immunocytochemistry (ICC).

Antibody	Source	Dilution WB	Dilution ICC	Manufacturer
α-ERK1/2	Rabbit polyclonal	1:1000		Cell Signaling, Danvers, MA, USA
α-PAI-1 AGD 25	mouse	1:200 – 1:1000	1:250	American Diagnostics, Stanford, CA, USA
α-YAP	Rabbit polyclonal	1:1000 – 1:1500		Cell Signaling, Danvers, MA, USA
α-P(S127)-YAP	Rabbit polyclonal	1:1000 – 1:1500		Cell Signaling, Danvers, MA, USA
α-Cox-2	Mouse monoclonal	1:1000		Dako, Glostrup, DK
α-TIMP-1	Rabbit polyclonal	1:1000		GeneTex, Irvine, CA, USA
α-CREB	Rabbit monoclonal	1:1000		Epitomics, Burlingame, CA, USA
α-P(S133)-CREB	Rabbit monoclonal	1:1000		Epitomics, Burlingame, CA, USA
α-Smad2/3	Mouse monoclonal	1:1000		Santa Cruz biotechnology, Santa Cruz¸ CA, USA
α-P(S423/S425)-Smad3	Rabbit polyclonal	1:500 – 1:1000		R&D Systems, Minneapolis, MN, USA
α-TβRI	Rabbit polyclonal	1:500		Cell Signaling, Danvers, MA, USA
α-ITGB1	Rabbit monoclonal		1:2000	Epitomics, Burlingame, CA, USA
α-CD44	Mouse monoclonal		1:4000	Lab vision, Fremont, CA, USA
α-Cyr61	Rabbit polyclonal		1:500	Santa Cruz biotechnology, Santa Cruz¸ CA, USA
α-Fibronectin	Rabbit polyclonal		1 :2000	Epitomics, Burlingame, CA, USA

Table 5. Secondary antibodies used for Western blot analysis.

Antibody	Dilution WB	Manufacturer
Anti-rabbit-HRP	1:2000	Cell Signaling, Danvers, MA, USA
Anti-mouse-HRP	1:2000	Cell Signaling, Danvers, MA, USA

1.5. Plasmids

Table 6. Plasmids used for ectopic protein expression.

Expression plasmid	Manufacturer/provided by
pEXL-Flag-Smad3	R.A. Weinberg, Cambridge, MA, USA (Liu *et al.* 1997)
pcDNA3.1-Six1	H.L. Ford, Aurora, CO, USA (Micalizzi *et al.* 2010)
pcDNA3.1zeo/TβRI(T204D)	T.A. Guise, Indianapolis, IN, USA (Yin *et al.* 1999)

Table 7. Reporter plasmids used for promoter activation studies.

Reporter plasmids	Manufacturer/provided by
pGL4.10[*luc2*]	Promega, Madison, USA
pGL4.74[*hRluc*/TK]	Promega, Madison, USA
pGL4.10[*luc2*]_TβRI	New construct see 2.6.1.
3TP-luc	Y. Sun, Rochester, GB (Wrana *et al.* 1992)

1.6. Primer

Table 8. Primer used for qRT-PCR.

Transcript	Forward primer (5`->3`)	Reverse primer (5`->3`)
GAPDH	GAAGGTGAAGGTCGGAGT	GAAGATGGTGATGGGATTTC
HPRT	GGACAGGACTGAACGTCTTGC	TGAGCACACAGAGGGCTACAA
MMP-1	CTGAAGGTGATGAAGCAGCC	AGTCCAAGAGAATGGCCGAG
TGFα	AGCCTTTTGTGGGCCTTC	GAATAACCCCAAGCAGACGG
ITGA6	GGCACCTACACTCACCTGCA	CAGCTAACGTGATGGACGTCTG
ITGB1	GGAAAACAGCGCATATCTGGA	CATCGAAACCACCTTCTGGAG
Ets-1	CGTACGTCCCCCACTCCTG	TTGATGATGGTGAGAGTCGGC
MMP-9	CCCGGACCAAGGATACAGTTT	GGAATGATCTAAGCCCAGCG
uPA	ATTCCTGCCAGGGAGACTCAG	CCAGTCAAAGTCATGCGGC
p21	CTGTGATGCGCTAATGGCG	CGGTGACAAAGTCGAAGTTCC
MMP-10	TGGAGCAAGGCTTCCCCTAGA	TGATGACTTTCCAGGAGTTGAGC
PTHrP	ACCTCGGAGGTGTCCCCTAAC	TCAGACCCAAATCGGACGG
TIMP-1	CTGTTGTTGCTGTGGCTGAT	TGGATAAACAGGGAAACACT
PAI-1	GGCCATGGAACAAGGATGAGA	GACCAGCTTCAGATCCCGCT
Cox-2	GCAAATTGCTGGCAGGGTT	TCTGTACTGCGGGTGGAACAT
Smad3	GTGGATGGCTTCACCGACC	TTGACATTGGAGAGCAGCCC
CREB	GCTGCCTCTGGAGACGTACAA	GCTAGTGGGTGCTGTGCGA
YAP	GGATGGTGGGACTCAAAATCC	CAATTCCTGAGACATCCCGG
Six1	TGCTTCAAGGAGGAGTCGAGG	GGATTGTGCGCGTACCACT
Eya2	GCTATGGCTCCAGCT	AGGTGTAGTGGCTCTGTCCAG
TβRI	CATTGCTGGACCAGTGTGCT	CAGTGCGGTTGTGGCAGAT
TβRII	AGAAAGCTGAGTTCAACCTGGGA	TGATGGCACAGTGCTCGC

Primers for other applications are mentioned in the corresponding method section.All primers were purchased from Eurofins MWG (Operon, Ebersberg, D).

1.7. siRNAs

Table 9. siRNAs used for protein knockdown.

siRNA	Sense strand (5`->3`)
siLuc	CUUACGCUGAGUACUUCGA
siCREB-624	UGACUAUCUUCUGAUGCA
siSmad3-869	CCAGUGACCACCAGAUGAA
siYAP-930	GACAUCUUCUGGUCAGAGA
siSix1-848	CCAACUCUCUCCUCUGGAA

All siRNAs were purchased from Eurofins MWG (Operon, Ebersberg, D).

1.8. Devices

Table 10. Devices used in the presented study.

Device	Manufacturer
Microscopes: Axioskop 40, Axiovert 135	Carl Zeiss, Jena, D
Laminar flow bench: Hera safe	Heraeus,/Thermo Fisher Scientific, Waltham, MA, USA
Electroporator: Gene Pulser Xcell	Bio-Rad, Munich, D
Centrifuges: Biofuge pico, Multifuge 3 S-R	Heraeus/Thermo Fisher Scientific, Waltham, MA, USA
PCR-Cycler: T-Gradient thermocycler	Biometra, Göttingen, D
qPCR-Cycler: iCycler	Bio-Rad, Munich, D
Incubators: innova 4230 (for bacteria)	New Brunswick Scientific, Edison, NJ, USA
IG150 (for eucaryotic cell culture)	Jouan/Thermo Fisher Scientific, Waltham, MA, USA
Spectro-photometer: Ultrospec 1000 (cuvettes)	Pharmacia Biotech/GE Healthcare Bio-Sciences, Uppsala, SE
Spectra Max 340PC (96 well plates)	Molecular Devices, Sunnyvale, CA, USA
Protein quantification: Qubit fluorometer	Invitrogen/Life Technologies, Carlsbad, CA, USA
Agarose gel electrophoresis: Agagel Standard G45/1	Biometra, Göttingen, D
SDS-PAGE: Mini-Protean 2 Cell	Bio-Rad, Munich, D
Western Blot: Mini Trans-Blot Cell	Bio-Rad, Munich, D
Microtome: Jung Histoslide 2000	Leica Microsystems, Wetzlar, D
Luminometer: Sirius Luminometer	Berthold Detection Systems, Pforzheim, D

1.9. Software

Table 11. Software used in the presented study.

Software	Manufacturer
GraphPad Prism	Version 4, GraphPad Software, San Diego, CA, USA
Microsoft Office	Microsoft, Redmont, WA, USA
iQ5 Optical System Software version 2.0	Bio-Rad, Munich, D
Primer Express v2.0	Applied Biosystems/Life Technologies, Carlsbad, CA, USA
AxioVision 4	Carl Zeiss Imaging Solutions GmbH, Jena, D
Canvas 8	ACD Systems (former Deneba Systems), Seattle, WA, USA

2. Methods

2.1. Mammalian cell culture

2.1.1. Cells

The human breast cancer cell lines MDA-MB-231, MCF-7 and BT20 were used. Bone marrow-derived human MSCs (hMSCs) from different donors were kindly provided by L.P. Mueller, University of Halle.

2.1.2. Cell culture

MDA-MB-231, MCF-7 and BT20 cells were grown in RPMI 1640 + GlutaMAX medium (Gibco/Life Technologies, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum (Pan Biotech GmbH, Aidenbach, D) in the absence of antibiotics and incubated at 37°C in a humidified incubator at 5 % CO_2. Monolayer (2D) cultures were grown on cell culture plates or flasks depending on application. For 3D cultures, cells were trypsinized (Trypsin/EDTA, Lonza, Basel, CH) and maintained in suspension on top of a thin layer of 2 % Seakem GTG agarose (dissolved in PBS) (Lonza, Basel, CH) in a 75 cm^2 cell culture flask. (Dittmer *et al.* 2008). The freely floating cells quickly formed aggregates of different shapes and sizes in the cell culture flask. For observation of single 3D aggregates, 5000 cells were seeded per well into a 96-well plate (TPP, Trasadingen, CH). When additives (Tab. 3; 1.3) were used, they were either added after cells had attached to the culture dish (2D) or administered simultaneously with cell seeding (3D). For mock treatment only the solvent in which the

additive was solved was added. The concentration used and the duration of treatment is given in the corresponding result section.

Human MSCs were maintained in DMEM (low glucose, PAA Laboratories, Pasching, AT), 15 % fetal calf serum (PAA Laboratories, Pasching, AT) and kept at low density to avoid differentiation. For co-culturing of breast cancer cells with hMSCs, hMSCs were first seeded in hMSC medium. After hMSCs have attached to the substratum, MDA-MB-231 cells were added and cells co-cultured in RPMI/10 % FCS. Incubation of MDA-MB-231 cells with hMSC-conditioned medium was performed applying 20 % conditioned medium in RPMI/10 % FCS.

2.2. Protein analysis

2.2.1. Cell lysis

Cells grown in 2D cultures were washed with PBS, scraped off the plate and harvested by centrifugation. Cells in 3D culture were collected by centrifugation and washed with PBS before cellular proteins were isolated.

Nuclear protein extraction was done according to a modified protocol described by (Lindemann *et al.* 2001). After centrifugation, cells were resuspended in 100 µl of buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA) and placed on ice for 15 min. After addition of 50 µl 5 % NP-40, the cell suspension was vortexed for 10 s and centrifuged in a microcentrifuge at full speed for 30 s. The pellet was resuspended in 60 µl of buffer C (20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT) and incubated on ice for 10 min. After centrifugation for 10 min, the supernatant was collected and stored at -80 °C.

Extraction of plasma membrane proteins was carried out as described (Cardone *et al.* 2007) with some modifications. Cells were resuspended in 100 µl buffer A as described above and homogenized by five passes through a 20-gauge needle. Cellular fractions were isolated by stepwise centrifugation at 3000 rpm (600 x *g*) and 6500 rpm (3500 x *g*) and then full speed in a microcentrifuge for 10 min each. The pellets after the first two centrifugations were discarded. The pellet of the last centrifugation contained the plasma membrane proteins and was dissolved in buffer D (5 mM HEPES pH 7.9, 0.5 mM K-EDTA pH 7.2, 1 mM DTT).

2.2.2. Protein quantification

Total protein contents of cellular lysates were measured by using a Qubit fluorometer following the instructions of the manufacturer (Invitrogen).

2.2.3. SDS-PAGE

SDS-PAGE and Western blot analyses were performed as described (Dittmer *et al.* 2006) with some modifications. While for nuclear or cytosolic protein lysates 10 µg of total protein was applied onto the gel, for plasma membrane extracts 5 µg was used. The corresponding volumes of lysates were diluted with 4X sample buffer. Conditioned cell culture medium was also diluted with 4X sample buffer and fourty microliters were loaded onto the gel. All samples were incubated at 99°C for 3 min, then cooled on ice. Proteins were separated by SDS-PAGE in electrophoresis buffer (192 mM glycine, 25 mM tris base). Gels contained a 10% separating gel overlaid by a 4 % stacking gel (Tab. 12). For the determination of molecular weights of proteins PageRuler Prestained Protein Ladder (Pierce/Thermo Fisher Scientific, Waltham, MA, USA) was used.

Table 12. Recipe for the separating and stacking gel for SDS-PAGE.

	Separating gel	**Stacking gel**
Acrylamide-Bis-acrylamide-solution (40 %, 29:1)	2.5 ml	0.5 ml
Gel buffer*	2.5 ml	2.5 ml
A. dest.	5.0 ml	2.0 ml
10 % APS**	50 µl	25 µl
TEMED	7.5 µl	5 µl

***separating gel: 1.5 M Tris pH 8.8 (HCl)**
Stacking gel: 0.5 M Tris pH 6.8 (HCl), 0.2 % SDS
****prepared freshly**

2.2.4. Western Blot analysis

After electrophoresis, SDS-gels were rinsed in electroblot buffer (192 mM glycine, 25 mM tris base, 20 % methanol) and electroblotted onto a Immobilon polyvinylidene difluoride membrane (Merck Millipore, Billerca, MA, USA) at 200V for 1 h. After blocking with 2 % dry milk in washing buffer (10 mM Tris/HCl pH 7.5, 100 mM NaCl, 1 mM EDTA) at room temperature for 10 min, the membrane was incubated with the primary antibody in washing buffer containing 0.2 % milk for 1 h at room temperature. The membrane was washed three times in washing buffer containing 0.05 % Tween 20 for 5 min each and incubated with the appropriate secondary antibody in washing buffer containing 0.2 % milk at room temperature

for 1 h followed by at least three washes in washing buffer containing Tween 20 for 20 min each. Peroxidase activity was visualized by chemiluminescence using ECLPlus (Lonza, Basel, CH) followed by exposure to Hyperfilm ECL (GE Healthcare Europe, Freiburg, D).

2.2.5. Coomassie gel staining

After blotting, gels were stained by using a modified SimplyBlue SafeStain procedure (Invitrogen/Life Technologies, Carlsbad, CA, USA). Gels were washed in water, heated in a microwave and placed on a shaker for 5 min. The procedure was repeated 3 times. After that, gels were stained with Coomassie blue solution (per liter; 80 mg Coomassie brilliant blue R-250 plus 3.5 ml 32 % HCl) and destained with water.

2.3. Quantitative reverse-transcription PCR

2.3.1. RNA isolation

Cells grown in 2D cultures were washed with PBS and lysed in lysis buffer. Cells in 3D culture were collected by centrifugation and washed with PBS before lysis. Total RNA was isolated by using NucleoSpin RNA II (Macherey-Nagel, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer`s instructions. DNA was digested on-column by using 30 Kunitz units of RNase-free DNase in 80 µl RDD buffer (Qiagen, Hilden, D). Total RNA content was determined by measuring the absorbance at 260 nm in a spectro-photometer.

2.3.2. Synthesis of cDNA

For cDNA synthesis, 1 µg of total RNA was mixed with 1 µl of 10 mM dNTPs (Eppendorf, Hamburg, D), 20 or 40 U RNasin Plus RNase Inhibitor (Promega, Madison, USA), and 1 µl of random hexamers (100 ng/µl; Amersham Biosciences/GE Healthcare Europe, Freiburg, D) in a total volume of 13 µl and incubated at 65 °C for 5 min and quickly cooled on ice. After addition of 4 µl of 5x strand buffer and 2 µl of 0.1 M DTT, the primers were allowed to anneal to the RNA at 25 °C for 2 min. cDNA synthesis was achieved by addition of 1 µl of Superscript II (200 units/µl; Invitrogen/Life Technologies, Carlsbad, CA, USA) and by consecutive incubations at 25 °C for 10 min and at 45 °C for 50 min. The reaction was stopped by keeping the mixture at 70 °C for 15 min.

2.3.3. Quantitative PCR

Quantitative PCRs were performed by using ABsolute QPCR SYBR Green Fluorescein mix (ABgene, Thermo Fisher Scientific, Waltham, MA, USA). To 10 µl of SYBR Green mix, 1.25 µl of each primer (2.5 pmol), 2 µl of cDNA (1:20 diluted), and 5.5 µl of water were added, and the mixture was run in a Bio-Rad iCycler. After activation of the polymerase at 95 °C for 15 min, 40 cycles were run. In each cycle, denaturing was performed at 95 °C for 15 s, annealing at 60 °C for 1 min, and synthesis at 72 °C for 1 min. Each sample was analyzed in duplicates. The relative RNA level of each gene of interest was calculated by the comparative *Ct* ($2^{-\Delta\Delta Ct}$) method. Housekeeping genes GAPDH and HPRT were used as reference genes. The combined expression of these two genes was used for normalization. Primers of all genes analyzed are listed in Tab. 8 (1.6).

2.4. Protein knockdown (RNA interference) and ectopic expression of proteins

2.4.1. Protein knockdown (RNA interference)

Small interfering RNAs (siRNAs) as used are listed in Tab. 9 (1.7). Cells were transfected with 5 µl of a siRNA stock solution in RNase-free water (100 pmol/µl) by electroporation (2.4.3.). In order to avoid dilution of siRNA via cell division cells were seeded at high density. As a control siRNA, siRNA targeting the firefly luciferase (siLuc) was used. This siRNA is supposed to have no specific target in human cells. After transfection cells were maintained in culture for 2-3 days to allow the siRNA to induce its knockdown effect, before cells were harvested for RNA and/or protein analysis.

2.4.2. Transfection with expression plasmid

For ectopic protein expression, cells were transfected with 5 µg expression plasmid by electroporation (2.4.3.). As a control, cells were electroporated without adding plasmids.

2.4.3. Electroporation

Cells were trypsinized, washed once with RPMI medium (serum-free), and resuspended in RPMI medium at a density of approximately 8 million cells per ml. For each transfection, 250 µl of the cell suspension were mixed with siRNA and/or expression plasmid and electroporated by using a Bio-Rad GenePulserX-Cell (250 V, 800 microfarads). After incubation on ice for 30 min, cells were mixed with growth medium and seeded into cell culture dishes (Nunc/Thermo Electron LED, Langenselbold, D).

2.5. Determination of the cellular cAMP level

Determination of intracellular cAMP levels was carried out by using the Cayman cyclic AMP Enzyme Immunoassay Kit by following the instructions of the manufacturer (Cayman Chemical, Ann Arbor, USA). In this assay, acetylcholinesterase-coupled cAMP competes with cAMP provided by the sample for binding to a cAMP-specific antibody. The more cAMP the sample contains, the less acetylcholinesterase-coupled cAMP can bind to the antibody and the less enzyme activity can be detected. Therefore, the level of enzyme activity is inversely related to the cAMP content in the sample. Cells were washed once with PBS and incubated with 0.1 M HCl at room temperature for 20 min and debris removed by centrifugation (10 min at 10400 rpm). Cells grown as monolayers were lysed in the culture dish. Cells kept in 3D culture were collected by centrifugation and washed and lysed in a reaction tube. The procedure for measuring low cAMP concentrations was used which required acetylation of the sample. Acetylation was carried out as described in the manufacturer's protocol. Acetylated supernatants were assayed after dilutions with EIA buffer. Fifty µl of standards or samples were incubated in the presence of 50 µl of tracer (acetylcholinesterase-coupled cAMP) and 50 µl of cAMP-specific rabbit antibody at 4°C for 17-18 h. Acetylcholinesterase activity was determined after addition of Ellman`s reagent by measuring absorbance at 412 nm in a Spectra Max 340PC spectro-photometer.

2.6. Molecular cloning

In order to examine the TβRI promoter inducibility by cAMP, the -392/+21 bp fragment of the human TβRI promoter was inserted into the pGL4-10[luc2] luciferase reporter plasmid.

2.6.1. Generation of a TβRI promoter fragment

A -392/+21 bp fragment of the human TβRI promoter was amplified from genomic DNA of MDA-MB-231 cells. For that purpose, a PCR reaction mix containing 120 pmol of each of the primers TβRI-392fw (TAGAGGAGGTTAGAAGAAAAGAGCGT) and TβRI+21rv (AGCAAACCTCGCCTCGC), 100 ng template DNA, 100 µmol of each dNTP (Fermentas/Thermo Fisher Scientific, Waltham, MA, USA), 1x *Pfu* polymerase buffer (+$MgSO_4$), 4 units *Pfu* polymerase (Fermentas/Thermo Fisher Scientific, Waltham, MA, USA) and 5 % DMSO in 4 aliquots with a total volume of 12 µl each was prepared. Amplification was performed by running an initial denaturation at 95°C for 20 min, followed by 40 repetitive cycles of denaturation at 95°C for 2 min, annealing at 57°C for 1 min, extension at 75°C for 3 min and one final extension at 75°C for 10 min in a thermocycler. The resulting PCR product was run on an agarose gel, the corresponding band was excised and DNA was extracted by using Zymoclean Gel DNA Recovery kit (Zymo Research, Irvine,

USA) by following the manufacturer`s protocol. The isolated DNA fragment was subjected to re-amplification PCR in order to increase purity by using the same PCR mix components and the same conditions of thermocycling. Agarose gel electrophoresis confirmed that by this step a cleaner PCR product was achieved.

2.6.2. PCR product purification and phosphorylation

The PCR product was purified by using QIAquick PCR Purification kit (Qiagen, Hilden, D). After cleaning the fragment was phosphorylated by mixing 20 µl of the product (1,5 µg) with 20 µl of 2X Quick Ligase buffer and 1 µl (10 units) of T4 PolyNTP polymerase (Fermentas/Thermo Fisher Scientific, Waltham, MA, USA) and incubating the mixture at 37°C for 30 min following enzyme inactivation at 65°C for 10 min.

2.6.3. Insertion of the PCR product into a reporter plasmid

The fragment was inserted into pGL4.10[*luc2*] (Promega, Madison, USA), a eukaryotic expression vector which encodes the luciferase gene of *Photinus pyralis* and, according to the company, does not contain DNA sequences that could act as promoter elements. For blunt end cloning into the multiple cloning site the single cutter *Eco*RV was selected. Two µg of vector DNA was digested by using 2 µl of NEB buffer 4 and 4 units of *Eco*RV (New England Biolabs, Ipswich, USA) in a total volume of 20 µl at 37°C for 1 h. After cooling on ice, the vector was dephosphorylated by incubating the digested plasmid in the presence of 2 µl of 10x Antarctic Phosphatase reaction buffer and 5 units of Antarctic Phosphatase (New England Biolabs, Ipswich, USA) at 37°C for 30 min. Enzyme inactivation was accomplished by keeping the reaction mix at 65°C for 10 min. Successful digestion was checked by agarose gel electrophoresis. The digested vector displayed a faster migrating band on the gel than the undigested one.

For ligation 25 ng of phosphorylated PCR product was mixed with 0.6 µl digested, de-phosphorylated plasmid DNA, 10 µl of 2X Quick Ligase buffer and 5 units of T4 DNA Ligase (Fermentas/Thermo Fisher Scientific, Waltham, MA, USA) in a total volume of 20 µl and incubated at room temperature for 1 h.

2.6.4. Transformation of *E.coli*

An aliquot of 50 µl of NEB Turbo competent *E.coli* (New England Biolabs, Ipswich, USA) was thawed on ice and transformed with 7.5 µl ligation mix. Then, the cell-plasmid-mix was kept on ice for 20 min, subjected to a heat shock at 42°C for 30 sec, cooled on ice for 5 min and incubated for 30 min at 37°C after addition of 350 µl SOC medium. Cells were plated

onto LB agar plates supplemented with 50 µl/ml ampicillin and left in an incubator at 37°C o/n.

2.6.5. Identification of positive clones

Positive clones were identified by colony PCR. Colonies were picked and boiled in 20 µl water at 95°C for 5 min. Four µl of the lysate were mixed with 1 µl of each primer specific for the inserted DNA fragment (100 µmol/l) and 6 µl 2X GoTaq Green Master Mix (Promega, Madison, USA) and the DNA amplified by using the following PCR program: 95°C 5 min; 40x (95°C 1 min, 57°C 1 min, 72°C 1 min); 72°C 5 min.

In addition to the colony PCR, PCRs with purified plasmid DNA was carried out. Plasmid DNA from 4 ml liquid LB medium bacterial culture supplemented with 50 µl/ml ampicillin was isolated by using the ZR Plasmid Miniprep-Classic kit according to the user`s manual of Zymo Research (Irvine, USA) and PCR reaction was conducted using the same procedure as described above for colony PCR. The primers were chosed such that it allowed the discrimination between the two possible orientations of the inserted fragment within the vector. A band of the size of 470 bp indicated the correct orientation of the insert when using the primers GL4_MCS_fw (GGCCTAACTGGCCGGTACC) which anneals within the vector on the sense strand and TβRI+21rv which matches the 3`-end of the insert. As a negative control, the primer pair comprising GL4_MCS_fw and TβRI-392fw was expected to result in no PCR product.

Correct insertions were confirmed with double digestion. For that purpose, plasmid DNA was mixed with 2 µl NEB buffer 4, 2 units *Kpn*I-HF (New England Biolabs, Ipswich, USA) and 10 units *Hind*III (New England Biolabs, Ipswich, USA) in a total volume of 20 µl and incubated at 37°C for 2 h. Subsequent agarose gel electrophoresis confirmed successful cleavage.

As a final validation for a successful and correct insertion the plasmids were sequenced by Eurofins MWG Operon (Ebersberg, D).

2.6.6. Plasmid amplification and purification

One clone containing the correct fragment in correct orientation (designated as pGL4.10_TβRI) was selected and amplified in 200 ml LB medium containing 50 µl/ml ampicillin by incubating at 37°C o/n. Plasmids were isolated by using the Plasmid Purification Maxi Kit (Qiagen, Hilden, D) by following Qiagen`s protocol. Plasmid

concentration was determined by measuring the absorbance at 260 nm in a spectro-photometer.

2.7. Luciferase assays for the determination of promoter activity

2.7.1. Transfection with reporter plasmids

For luciferase assays, cells were transiently transfected with 5 µg of plasmids by using electroporation (see 2.4.3.). Cells were seeded into 12-well plates (TPP, Trasadingen, CH). After incubation for 6-24 h, medium was replaced with fresh medium supplemented with additives. For the determination of TβRI promoter activity cells were co-transfected with 5 µg pGL4.10[*luc2*]_TβRI and 2.5 µg pGL4.74[*hRluc/*TK]. The latter plasmid containing the renilla luciferase gene downstream a constitutive active promoter was used for normalization. Cells were assayed for luciferase activity after 24 h of incubation.

2.7.2. Measurement of luciferase activity

After washing with PBS cells were lysed in 250 µl of PLB (Promega dual luciferase reporter assay, Promega, Madison, USA) under agitation at room temperature for 15 min. Ten µl of each supernatant were mixed with 50 µl of Luciferase Assay Reagent II and analyzed for luciferase activity (Sirius Luminometer, Berthold Detection Systems). Relative promoter activity was calculated by normalizing luciferase activity either against renilla luciferase activity (pGL4.74[*hRluc/*TK]) or total protein (3TP-luc). The latter was determined by using the Qubit fluorometer (Invitrogen/Life Technologies, Carlsbad, CA, USA).

Vector 3TP. To examine TGFβ/Smad-dependent transcription the 3TP-Luc reporter construct was used. (Wrana *et al.* 1992). The plasmid 3TP contains the firefly luciferase gene cloned downstream of an artificial promoter consisting of the TGFβ-responsive PAI-1 promoter fragment between positions -740 to -636 and three TPA (phorbol ester)-responsive elements (TPA`s; 1 in reverse and 2 in sense orientation) derived from a human collagenase gene (de Groot & Kruijer 1990). Smad3 and Smad4 have been described to cooperatively bind to the PAI-1 promoter between -684/-677 and -670/-664 (Stroschein *et al.* 1999).

2.8. Immunocytochemistry

2.8.1. Preparation of 2D culture slides

Cells were grown on Superfrost slides (Gerhard Menzel, Braunschweig, D) in the presence of additives as indicated, washed with PBS, fixed with 10 % formaldehyde solution for 15 min and air-dried. Immunochemical staining was performed as described in (2.8.3.).

2.8.2. Fixation, paraffin-embedding and preparation of slides with 3D-cultured cells

For 3D culturing in 96-well plates, 5000 cells resuspended in 200 µl medium were seeded into each well (TPP, Trasadingen, CH). After incubation in the presence or absence of additives for 3 days, 100 µl of medium was removed and replaced by 100 µl of a 10 % formaldehyde solution. Cell aggregates were fixed for 30 min at room temperature. Then, cells were collected in a 15 ml tube, sedimented by gravity and dehydrated by incubation in increasing concentrations of ethanol (1x 50 %, 2x 70 %, 2x 96 %, 2x 100 %) and finally by three washes with xylol. The 3D aggregates were then transferred into a 1.5 ml tube, submerged in liquid paraffin and incubated at 60°C o/n for infiltration of paraffin. The tube was cooled and the resulting hardened paraffin pellet was removed, transferred into a histology cassette and filled with liquid paraffin. After cooling on ice, 5 µm thick sections of paraffin-embedded cell aggregates were cut from the paraffin blocks with a microtome and placed onto Superfrost slides (Gerhard Menzel, Braunschweig, D).

2.8.3. Immuncytochemical staining

For deparaffinization, slides were incubated in 2 cuvettes filled with xylol for 10 min each. The cells were rehydrated with decreasing ethanol concentrations (2x 100 %, 2x 96 %, 2x 80 %, 2x 70 %) for 5 min each and rinsed in water. To inactivate cellular peroxidases, slides were incubated in 1 % hydrogen peroxide (Merck Millipore, Billerca, MA, USA) for 15 min. After rinsing with water slides were heated to 95°C in 1x ChemMate Target Retrieval (Dako, Glostrup, DK) or, for detection of the Cox-2 antigen, in Target Retrieval Solution, pH 9.0 (Dako, Glostrup, DK) for 45 min. After cooling down to room temperature in a water bath, slides were stained by using the Cover plate Technology (Thermo Fisher Scientific, Waltham, MA, USA) and the ZytoChem Plus HRP-Kit (Zytomed, Berlin, D). In detail, protein block by using blocking solution was performed for 10 min at room temperature followed by incubation with the primary antibody at 4°C o/n (Tab. 4; 1.4.). Antibodies were diluted in Real Antibody Diluent (Dako, Glostrup, DK). Slides were washed twice with PBS and incubated with a biotinylated secondary antibody for 10 min. Subsequently, antibodies were removed by washing twice with PBS and slides were then incubated with streptavidin-HRP-

conjugate for additional 10 min. After washing twice with PBS, slides were stained by incubating with a HRP substrate for 30 min. Slides were removed from the Coverplate Cassette, rinsed in water, stained with hematoxylin, again rinsed with water and coated with non-aqueous mounting medium on top of which a thin glass cover slip was placed (Mowiol, recipe taken from: http://www.laborjournal.de/rubric/tricks/tricks/trick65.lasso).

2.8.4. Hematoxylin and eosin staining

For hematoxylin and eosin staining, slides were prepared as described in 2.8.3 before antibody staining. After hydrogen peroxide incubation and rinsing with water, slides were stained with hem alaun solution according to Mayer (Roth, Karlsruhe, D). Then, slides were incubated in tap water to intensify the nuclear staining. Cytoplasmic counterstaining was performed using a 1 % eosin solution (w/v).

2.9. Functional assays

2.9.1. Cell viability assay

Cell viability was determined by using the ViaLight plus kit (Lonza, Basel, CH). This luminescence-based assay measures the cellular ATP content as a marker for viability. In this assay, ATP drives a luciferase-catalyzed reaction that leads to the emission of light in the presence of oxygen and luciferin. Cells are lysed by incubation in lysis buffer for 20 min. An aliquot of the lysate is mixed with AMR plus reagent which contains the enzyme luciferase as well as its substrate luciferin. As the ATP present in the cell lysate is the limiting factor for the luciferase reaction, the emitted light as determined by using a luminometer is linearly correlated with the cellular ATP content.

2.9.2. Cell-cell adhesion assay

Four million cells were seeded onto a confluent monolayer and incubated for 3 h. After incubation, the remaining non-attached cells in the supernatant were counted by using a Countess Automated Cell Counter (Invitrogen/Life Technologies, Carlsbad, CA, USA).

2.9.3. Cell migration assays

Four hundred thousand cells were seeded into the upper compartment of a ThinCert cell culture insert (Greiner Bio-one, Frickenhausen, D), that were placed into 6-well plates. The inserts contained a porous membrane (8 µm-pores) at the bottom that allowed cells to penetrate the membrane only by an active migratory process. In order to stimulate migration

(chemotaxis) towards the lower compartment, cells were suspended in serum-free medium, whereas the medium pipetted into the lower compartment contained 10 % fetal calf serum. If additives were used, they were added to both compartments. After 18 h of incubation, the inserts were removed. Cells that had remained onto the upper side of the membrane were wiped off and cells that migrated to the lower side of the membrane were fixed in 10 % formaldehyde solution and stained in 1 % eosin solution. After rinsing the inserts with tap water to remove excess staining solution, the membrane was destained in 1 ml water. OD450-values were determined by using a spectro-photometer.

2.9.4. Proliferation assay

Cell proliferation was determined by using the Cell Proliferation ELISA (Roche Diagnostics, Mannheim D). The test is based on the measurement of the pyrimidine analogue 5-bromo-2`-deoxyuridine (BrdU) that, when added to cell culture medium, is incorporated in place of thymidine into newly synthesized DNA. After incubation, cells were fixed and the DNA was denatured. BrdU is detected by using a specific antibody that is peroxidase-coupled. Enzyme activity as measured by reading absorbance of its colorimetric product in a spectro-photometer. Absorbance values correlated with the amount of incorporated BrdU and therefore with cell proliferation.

RESULTS

1. Activation of cAMP and TGFβ signaling pathways in MDA-MB-231 cells

MDA-MB-231 breast cancer cells were either grown in adherent monolayer cultures (2D culture) or in 3D cultures as freely floating cell aggregates on top of an agarose-coated plastic surface (Fig. 6).

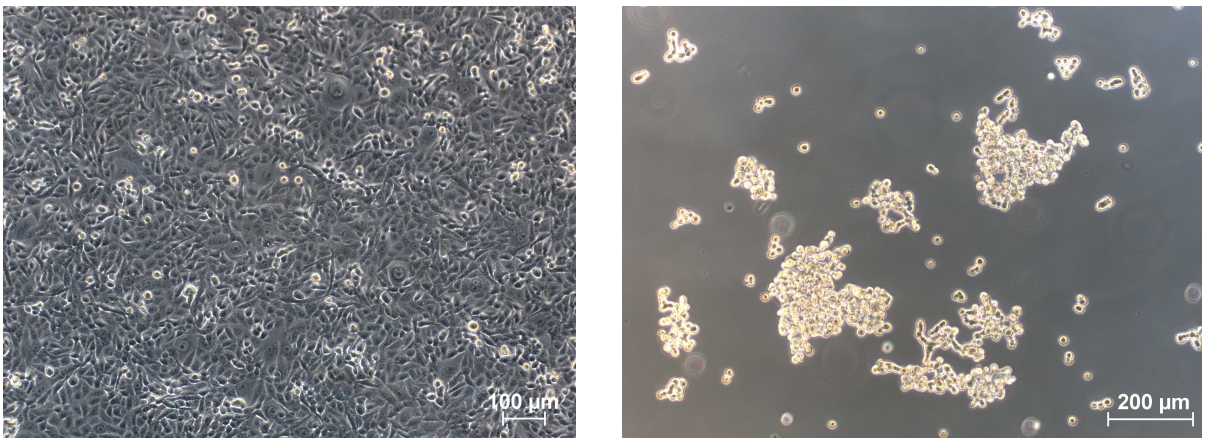

Figure 6. 2D- (left) and 3D-cultured MDA-MB-231 cells (right).

In order to activate the cAMP pathway cells were treated with 10 µM forskolin (FSK) which served as a stimulator of adenylyl cyclase activity. After incubation with forskolin, cells were lysed and cAMP concentrations were determined by a competitive enzyme immunoassay (EIA). As expected, the cells responded to FSK by increasing their intracellular cAMP levels. In 2D cultures, cAMP concentrations were 2.0-fold higher after 6 h and 2.8-fold higher after 24 h of incubation with FSK compared to mock treatment. In 3D cultures, FSK induced cAMP elevation to a similar extent of 3.4-fold after 24 h (Fig 7A). Concomitantly, phosphorylation of the downstream cAMP-dependent transcription factor CREB was also observed in nuclear cell extracts of 2D- and 3D-cultured cells as determined by Western blot analysis of phospho-CREB (Fig. 7B).

For the activation of the TGFβ pathway, human recombinant TGFβ1 was applied at a final concentration of 10 ng/ml. Successful activation was confirmed by Western blot analysis of the phosphorylated transcription factor Smad3 in nuclear extracts of 2D- and 3D-cultured cells (Fig. 7C).

These analyses also confirmed the different modes of activation of the transcription factors CREB and Smad3. While non-activated CREB is already present in the nucleus where it is phosphorylated in response to elevated cAMP levels, Smad3 translocates into the nucleus after being activated in the cytoplasm. Interestingly, the phosphorylation-dependent shuttling

of Smad3 into the nucleus can only be clearly observed in 2D-cultured cells. Cells grown in 3D culture contained nuclear Smad3 protein even before the TGFβ pathway was activated. Nevertheless, the level of phosphorylated Smad3 was increased upon TGFβ exposure. To ensure equal loading of proteins onto the gels, membranes were reprobed with an anti-ERK1/2 antibody. While 2D- and 3D-cultured cells showed different total Smad3 nuclear protein levels, they both displayed only weak phospho-Smad3 levels in the nucleus when unstimulated.

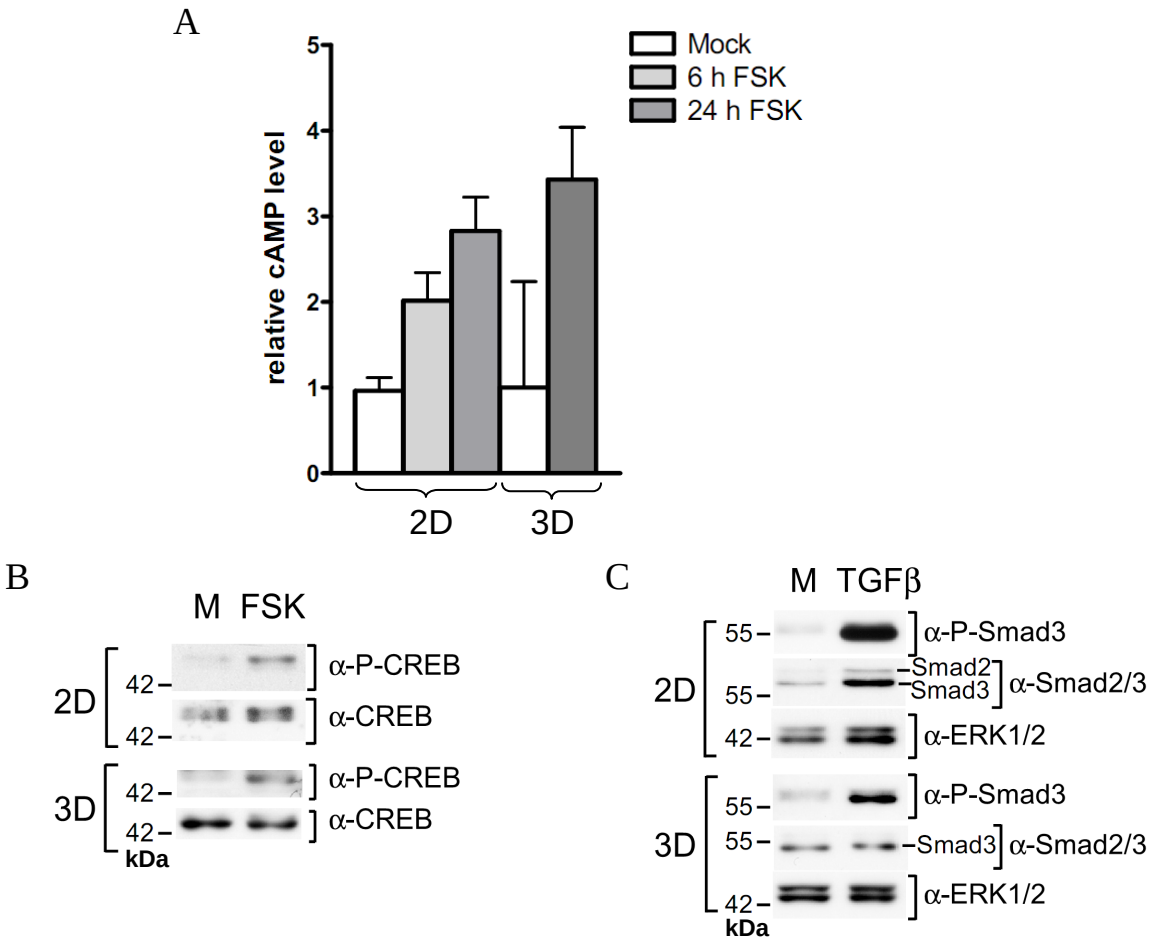

Figure 7. Forskolin and TGFβ1 activate the cAMP and TGFβ pathways in MDA-MB-231 cells.

A) cAMP levels as detected by EIA in cells treated with 10 µM forskolin for 6 or 24 h in 2D or 24 h in 3D cultures; normalized to mock-conditions separately for both 2D and 3D cultures and B) Western blot analysis detecting phosphorylation of the transcription factor CREB in response to forskolin in nuclear cell extracts after 24 h incubation; C) Western blot analysis detecting TGFβ-induced Smad3 phosphorylation in nuclear cell extracts after 24 h incubation with 10 ng/ml TGFβ or following mock treatment. M=Mock, P=phospho, α=anti (antibody)

To show that TβRI is the corresponding type I TGFβ receptor responsible for mediating TGFβ effects in MDA-MB-231 cells, the specific TβRI inhibitor Ly364947 was applied to TGFβ- or mock-treated cells at a concentration of 10 µM. The inhibitor effectively blocked TGFβ-induced Smad3 phosphorylation and gene expression of cyclooxygenase-2 (Cox-2), plasminogen activator inhibitor-1 (PAI-1) and tissue inhibitor of metallopreinase-1 (TIMP-1)

(Fig. 8). Therefore, TβRI is functional and responsible for mediating the TGFβ effects on Smad3 phosphorylation and gene expression in these cells.

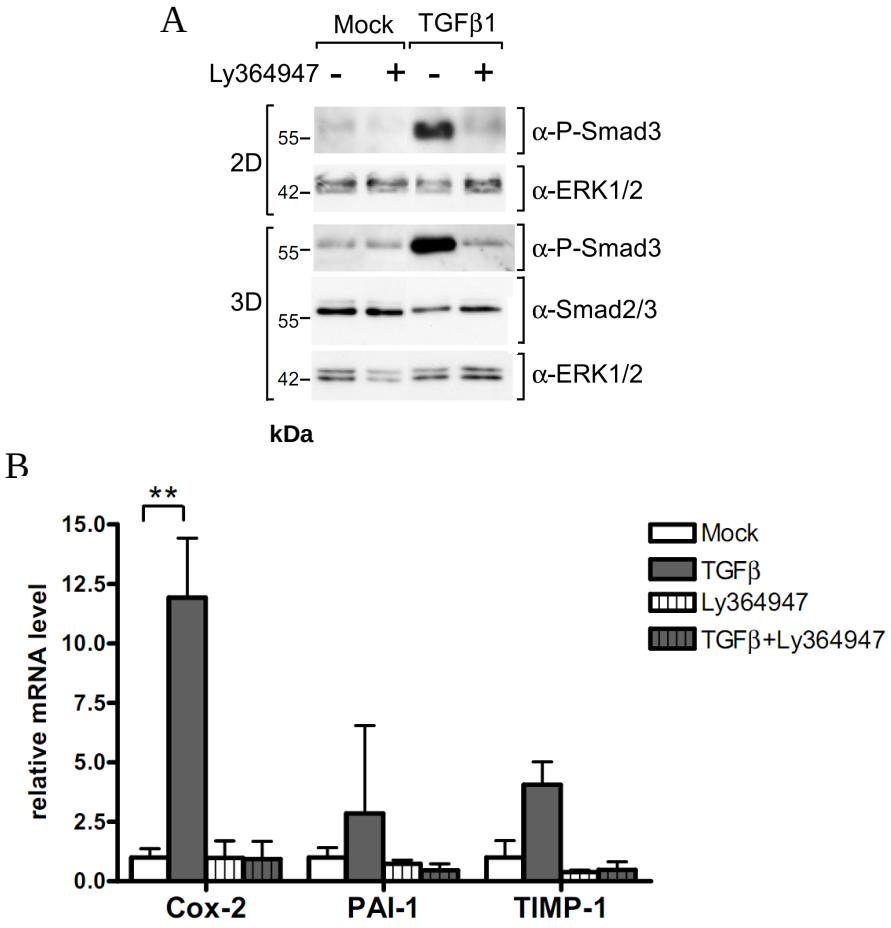

Figure 8. Activation of the TGFβ/Smad pathway is dependent on TβRI in MDA-MB-231 cells.
A) TGFβ-induced Smad3 phosphorylation is inhibited by 10 µM Ly364947.
B) TGFβ-dependent target gene expression is inhibited by 10 µM Ly364947.
Error bars indicate standard deviations (n=3). **p<0.01

2. Changes in expression of cancer-related genes in response to cAMP and TGFβ

Thirteen tumor-relevant genes known to be regulated by cAMP and/or TGFβ were selected for examination. Cells were treated with either forskolin (10 µM) or TGFβ (10 ng/ml) or both substances for 24 h and gene expression was measured by qRT-PCR and compared to mock treatment.

2.1. Cyclic AMP- and TGFβ-induced gene expression in 2D- and 3D-cultures

The 13 tested genes showed different strength of TGFβ-response from no response (1.1-fold induction) to a 160-fold induction in 2D- and 3D-cultured cells (Fig. 9). Parathyroid hormone-related peptide (PTHrP), PAI-1, transforming growth factor alpha (TGFα), urokinase-type plasminogen activator (uPA), and p21 showed a higher TGFβ-response in 3D as compared to 2D cultures. Exposure to the cAMP-elevating agent FSK affected genes differently. Among

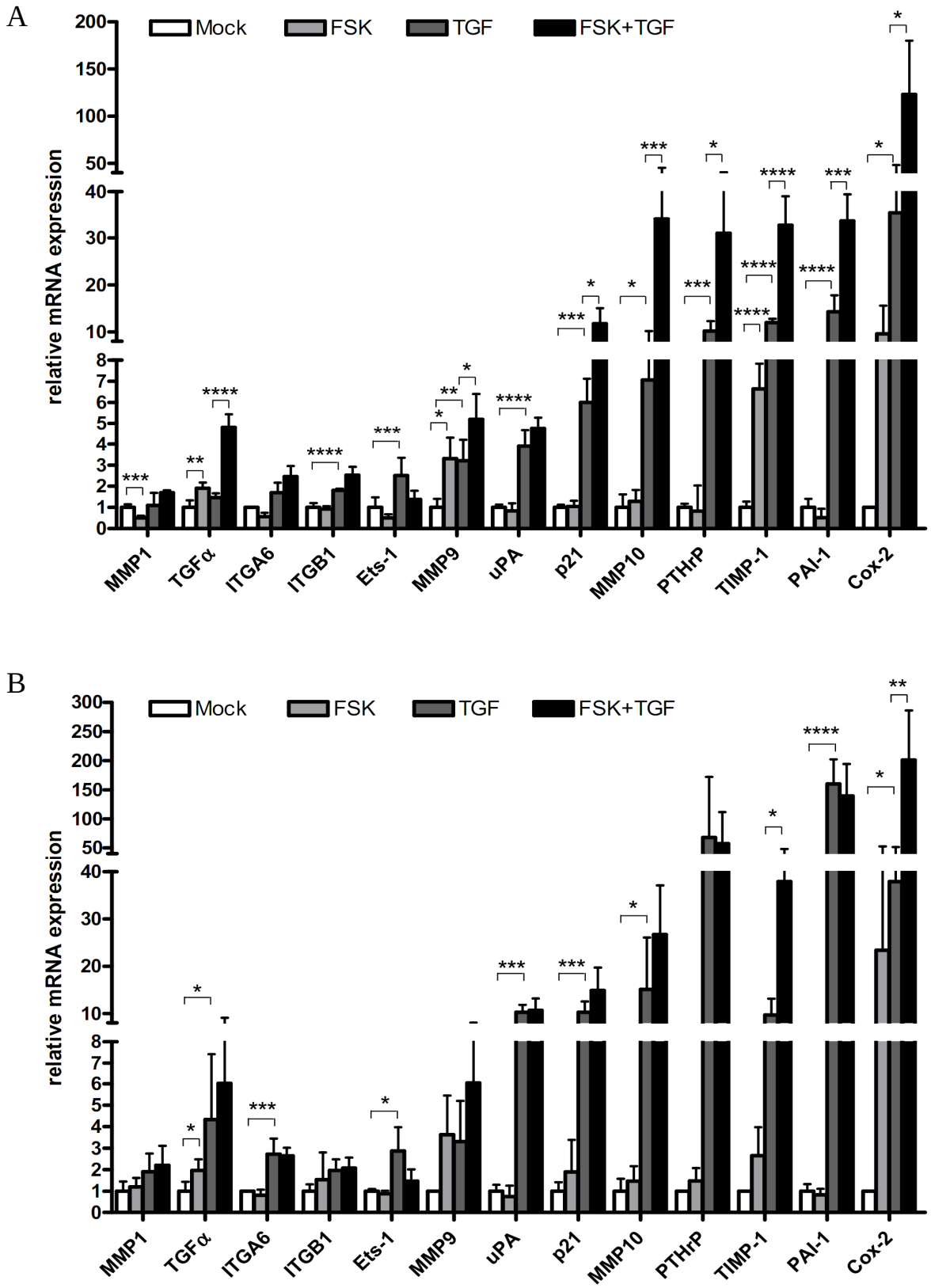

Figure 9. Regulation of cancer-associated genes in response to 10 µM forskolin, 10 ng/µl TGFβ or forskolin+TGFβ, in MDA-MB-231 cells.

Cells were incubated with 10 µM forskolin, 10 ng/ml TGFβ1 or both factors or were mock-treated in 2D culture (A) or 3D culture (B) for 24h and analyzed for mRNA expression by qRT-PCR. Bars represent fold inductions of RNA expression of cancer-associated genes which appear in the order of increasing TGFβ response in 2D culture. Error bars indicate standard deviations (n=4-5). *p-value<0.05, **p<0.01, ***p-value<0.005, ****p<0.001 (student`s t-test)

the genes that showed forskolin response, matrix metalloproteinase (MMP) 1 and PAI-1 were downregulated in 2D cultures. In 3D culture, none of these genes showed downregulation. TGFα, MMP9, TIMP-1 and Cox-2 were upregulated in both, 2D and 3D cultures.

2.2. The impact of cAMP elevation on TGFβ-dependent gene expression

Treatment with forskolin that upregulated the expression of Cox-2, TIMP-1 and TGFα, also increased the TGFβ-induced expression of these genes in 2D-cultured as well as in 3D-cultured cells. These genes also showed similar responsiveness to TGFβ and TGFβ/FSK in 2D and 3D cultures. The expression of some other genes was either not affected (PTHrP, MMP10, p21) or down-regulated (PAI-1) by forskolin alone but also showed an increased TGFβ responsiveness in the presence of forskolin. This effect could only be detected in 2D-cultured, but not 3D-cultured cells. Of note, in 3D-cultured cells, TGFβ alone was sufficient to increase gene expression to levels comparable to those obtained in the presence of both TGFβ and FSK in 2D cultures (Tab. 13). In 3D-cultured cells, this group of genes did not show any further increase in TGFβ-induced expression in the presence of forskolin. In summary, my data suggest that even without having a regulatory effect on gene expression alone, forskolin induced a higher gene expression of some genes in the presence of TGFβ under 2D-culture conditions. However, this effect was not found, when cells were maintained in 3D culture.

Table 13. Forskolin increases TGFβ responsiveness of cancer-associated genes.

	2D		3D	
	TGF	FSK+ TGF	TGF	FSK+ TGF
Cox-2				
TIMP-1				
TGFα				
PAI-1				
PTHrP				
MMP10				
p21				

low high

Relative expression level (heat map)

To test the possibility that the changes in the mRNA levels are translated into changes in protein expression, cells were treated as described for RNA analysis and harvested for isolation of proteins. As PAI-1 and TIMP-1 are known to be secretory proteins, conditioned cell culture medium (CM) was collected for their detection after 24 hours of incubation of the cells with forskolin, TGFβ or both agents. For Cox-2, a weak protein expression was expected in whole cell extracts due to its low mRNA levels as measured by qRT-PCR. In order to be able to detect such a low-abundant protein, a protein isolation method was used that allows the separation of subcellular compartments. With this method, plasma membrane extracts (PM) were prepared, in which the Cox-2 protein could be detected by Western blot analysis, when cells had been stimulated with TGFβ or forskolin/TGFβ under both culture conditions and also after forskolin treatment in 3D-cultured cells. The TIMP-1 protein could be detected in CM in all samples. PAI-1 was found in CM but also in PM, which indicated that this protein is partly attached to the plasma membrane. Also on the protein level, a higher expression of PAI-1 was found in 2D-cultured cells, when treated with forskolin in addition to TGFβ, whereas in 3D- cultured cells similar expression levels were found in the presence of TGFβ alone and TGFβ plus forskolin. This analysis showed (Fig. 10) that, under the different conditions used, the secreted PAI-1 and TIMP-1 as well as membrane-bound PAI-1 and Cox-2 proteins show expression patterns that are similar to those observed on the mRNA level. This indicates that, for these genes, changes in RNA expression as induced by forskolin and TGFβ are translated into protein expression changes.

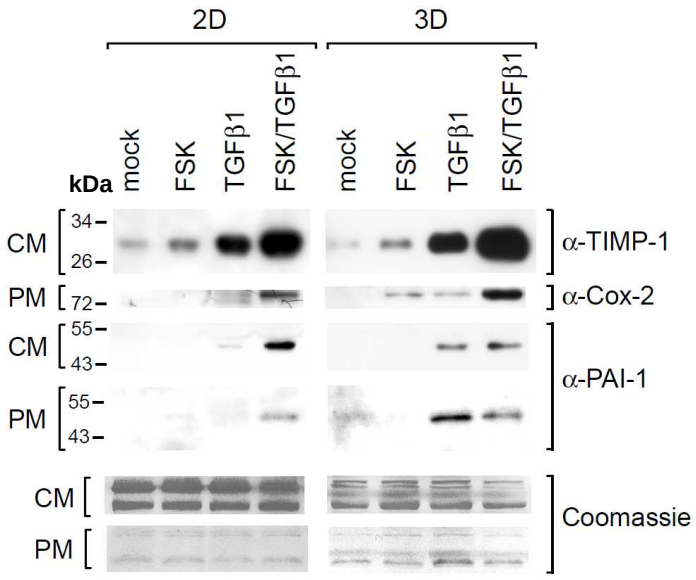

Figure 10. Forskolin increases Cox-2, TIMP-1 and PAI-1 protein levels in TGFβ-treated MDA-MB-231 cells.

Cox-2 protein levels were measured in membrane extracts (PM), those of TIMP-1 in conditioned medium (CM) and PAI-1 protein levels in both PM and CM by Western blot analysis. Samples were obtained from cells exposed to forskolin and/or TGFβ or from mock-treated cells (24 h). The Coomassie stain of the blotted gels shows equal protein loading.

3. Cross-talk of cAMP and TGFβ signaling pathways

The ability of forskolin to affect TGFβ-induced gene expression on the one hand and its failure to activate expression in some cases in the absence of TGFβ on the other hand raised the question of whether forskolin may act in a TGFβ-dependent manner. Therefore, the expression and activation of components of the TGFβ pathway were examined in the presence and absence of forskolin.

3.1. The impact of cAMP elevation on TGFβ-dependent Smad phosphorylation and promoter activity

First, the possibility was tested that forskolin enhances the TGFβ-dependent Smad3 expression and phosphorylation. As shown in Figure 11, elevated phosphorylation of Smad3 in response to forskolin was detected in the presence of TGFβ. This phenomenon was observed after 3, 6, 16 and 24 h of incubation in 2D-cultured cells and was most prominent after 16 h in the presence of forskolin. Nuclear Smad3 protein levels were also increased in forskolin-treated samples. This may have either been caused by nuclear translocation of Smad3 upon phosphorylation or by upregulation of Smad3 expression. Because Smad3 could not be detected in the cytosolic fraction in 2D-cultured cells by Western blot analysis, this question could not be answered. However, by using qRT-PCR, no upregulation of Smad3 mRNA expression by forskolin could be measured (Fig. 12A), suggesting that forskolin did not affect Smad3 expression. Therefore, a nuclear translocation of Smad3 induced by phosphorylation as induced by forskolin is rather likely. In 3D cultures, no changes in Smad3 phosphorylation and total Smad3 protein levels were found after forskolin treatment. Thus, forskolin affected TGFβ signaling by interfering with the activity of Smad3 which, however, could only be observed in 2D cultures. In addition, a striking difference in basal Smad3 protein levels was visible between 2D- and 3D-cultured cells. Cells in 3D culture exhibited much higher nuclear as well as cytoplasmic levels of Smad3 compared to 2D-cultured cells, whereas under neither culture condition could phospho-Smad3 be detected in mock-treated cells (Fig. 7C & 11B). The higher Smad3 protein level in 3D-cultured cells did not result from a higher Smad3 mRNA expression as shown by mRNA measurements (Fig. 12B). Interestingly, this higher total Smad3 level was associated with increased Smad3 phosphorylation in response to TGFβ (Fig. 11B).

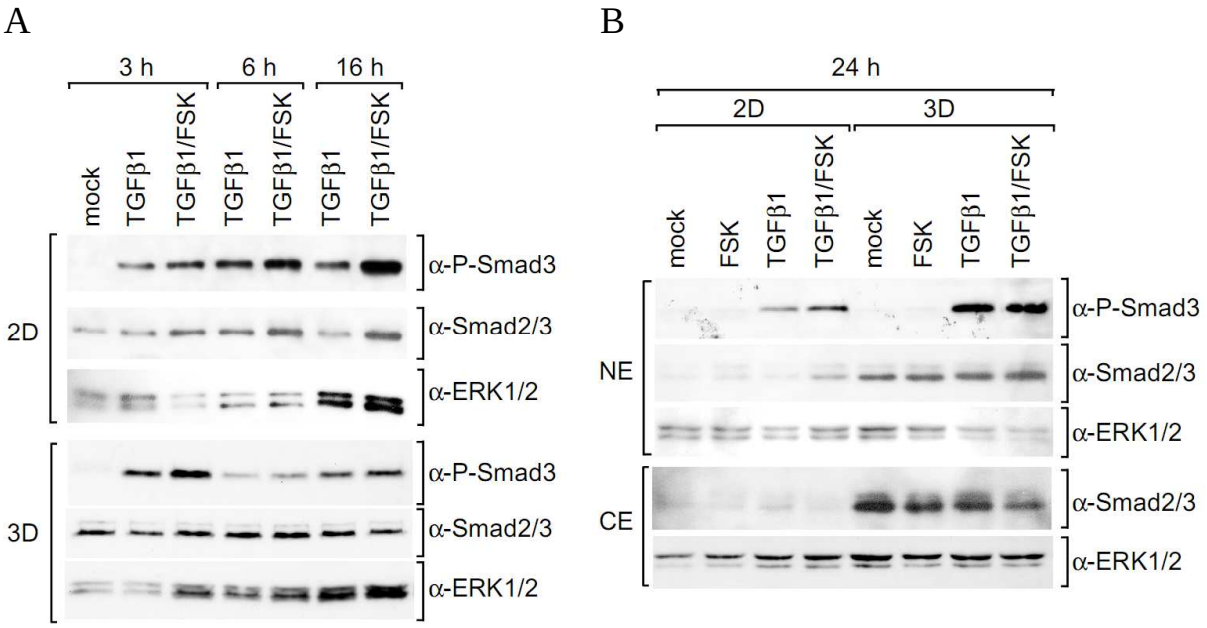

Figure 11. Forskolin enhanced TGFβ-dependent Smad3 phosphorylation in 2D-, but not in 3D-cultured cells.

A) Western Blot analysis of phospho (P)-Smad3, Smad3 and ERK1/2 (loading control) in nuclear extracts of cells treated as indicated for 3, 6 or 16 h; B) Western Blot analysis of P-Smad3, Smad3 and ERK1/2 (loading control) in nuclear (NE) and corresponding cytoplasmic extracts (CE) of cells treated as indicated for 24h.

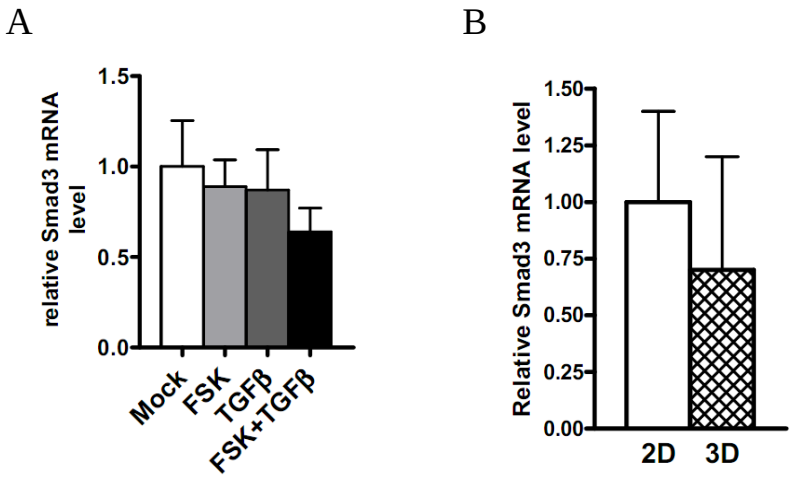

Figure 12. Smad3 mRNA levels are not affected by forskolin and are not different between 2D- and 3D-cultured cells.

A) Smad3 mRNA levels after 24 h of incubation with forskolin-, TGFβ-, forskolin/TGFβ- and mock-treated cells (2D culture).
B) Basal Smad3 mRNA levels in 2D- and 3D-cultured cells.
Error bars indicate standard deviations (n=3).

In order to further support the hypothesis that cAMP increases the activity of the TGFβ signaling cascade, cells were transfected with a reporter construct allowing to determine the TGFβ pathway activity on the gene promoter level. For this purpose, the 3TP-Luc plasmid containing the TGFβ-responsive promoter elements of the PAI-1 promoter cloned upstream of a luciferase gene was chosen (Wrana *et al.* 1992). Following transfection of cells with this

reporter construct, cells were mock-treated or incubated in the presence of forskolin, TGFβ or both factors. Twenty-four hours later cells were analyzed for luciferase activity. Cells treated with forskolin in addition to TGFβ showed a 1.5-fold higher promoter activity compared to cells grown in the presence of TGFβ alone (Fig. 13B left). This forskolin effect provides another evidence for a supporting role of cAMP in TGFβ signaling.

3.2. The impact of the Smad3 protein level on TGFβ-dependent Smad phosphorylation and promoter activity

To examine the possibility that the observed higher Smad3 levels in 3D cultures prevented the forskolin effect on TGFβ-treated cells, Smad3 was ectopically expressed in 2D-cultured cells. For this purpose, cells were transfected with the Smad3 expression plasmid pEXL-Flag-Smad3 (Liu *et al.* 1997) by electroporation. For comparison, control cells were electroporated without plasmids. Western blot analysis confirmed that Smad3 was successfully overexpressed (Fig. 13A).

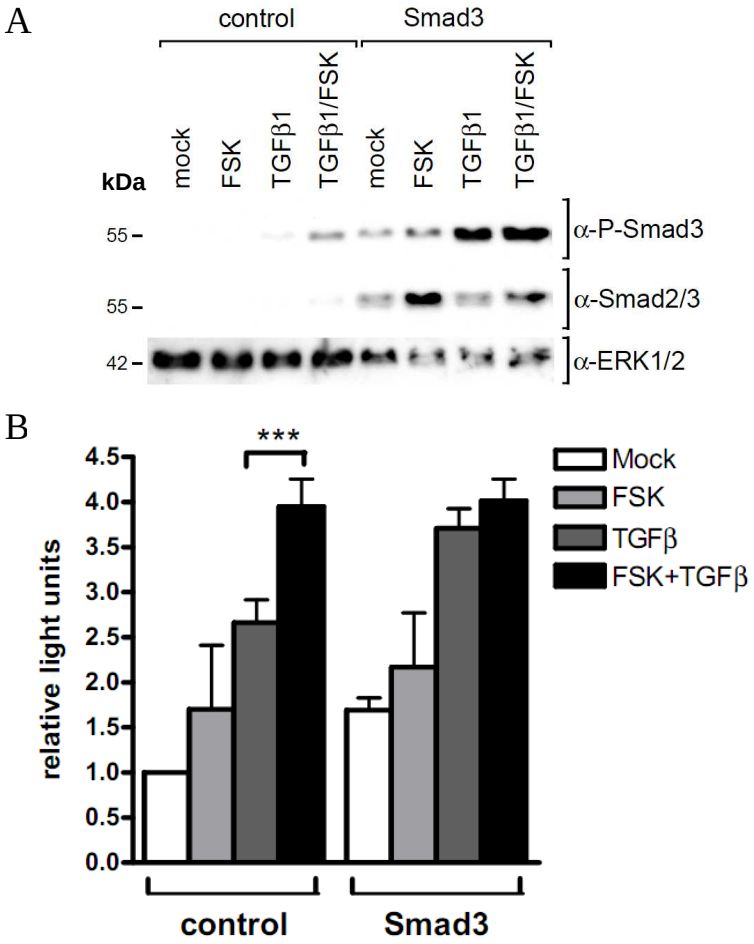

Figure 13. Ectopic Smad3 expression suppresses the forskolin-induced increase of TGFβ-dependent Smad3 phosphorylation and 3TP-Luc promoter activity.

Smad3 phosphorylation (A) and 3TP-Luc promoter activity (B) in Smad3-transfected or control cells treated with forskolin, TGFβ1, both substances or mock-treated. Promoter activities were normalized to mock-condition of control cells. Error bars indicate standard deviations (n=9). *** p-value<0.001 (student`s t-test)

Interestingly, the elevated Smad3 level was accompanied by increased basal Smad3 phoshorylation (Fig. 13A) and a 1.7-fold higher basal promoter activity in untreated cells (Fig. 13B). Treatment of Smad3-transfected cells with TGFβ led to a further increase in Smad3 phosphorylation (Fig. 13A) and in promoter activity (2.2-fold; Fig. 13B). The promoter activation by TGFβ was similar to control cells (2.3-fold). More importantly, when Smad3 was overexpressed, forskolin failed to induce a further enhancement of either Smad3 phosphorylation and promoter activity in TGFβ-treated cells (Fig. 13). Therefore, an elevated Smad3 protein level as observed in 3D-cultured cells is sufficient to suppress the stimulatory effect of forskolin on TGFβ signaling.

Since higher expression of Smad3 was accompanied with higher phosphorylation of Smad3, the question arose, whether the expression of Smad3 may have an impact on the expression of TβRI, the kinase that phosphorylates Smad3. To analyze the effect of Smad3 expression on TβRI expression, cells were either transfected with the Smad3 expression plasmid to overexpress Smad3 or with a Smad3-specific siRNA to downregulate Smad3 (Fig. 14). Neither did ectopic Smad3 expression nor did the siRNA-mediated Smad3 knockdown induce a change in TβRI expression (Fig. 14). These findings showed that the effects of higher Smad3 levels on Smad3 phosphorylation and TGFβ-dependent promoter activity cannot be explained by an upregulation of TβRI expression.

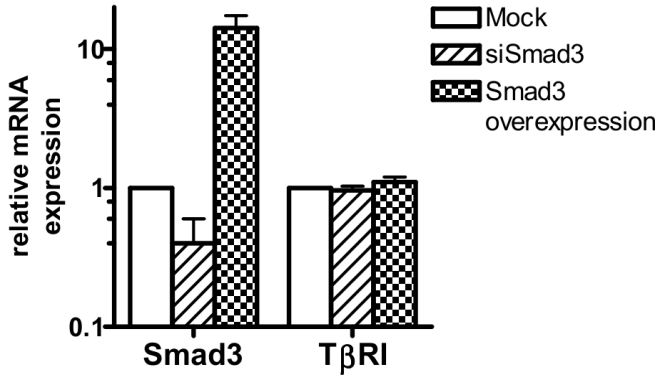

Figure 14. The TβRI level is regulated independently of the Smad3 level.

Smad3 and corresponding TβRI mRNA expression in cells treated with siRNA against Smad3, Smad3 expression plasmid or control cells. Y axis appears in log scale. Error bars indicate standard deviations (n=3).

3.3. Impact of Smad3 and Yes-associated protein (YAP) for the cAMP elevation of TGFβ-dependent gene expression

The importance of Smad3 for the forskolin effect on TGFβ-dependent gene expression was further investigated. Cells were transfected with siRNA against Smad3 (siSmad3) or siRNA against the firefly luciferase (siLuc) (which is not expressed in human cells) as a control and

incubated with TGFβ in the presence or absence of forskolin in 2D culture (Fig. 15). The mRNA expression of PAI-1, TIMP-1 and Cox-2 was analyzed (Fig. 16).

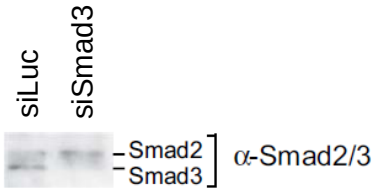

Figure 15. Smad3 protein level upon incubation with siRNA (Western blot analysis)

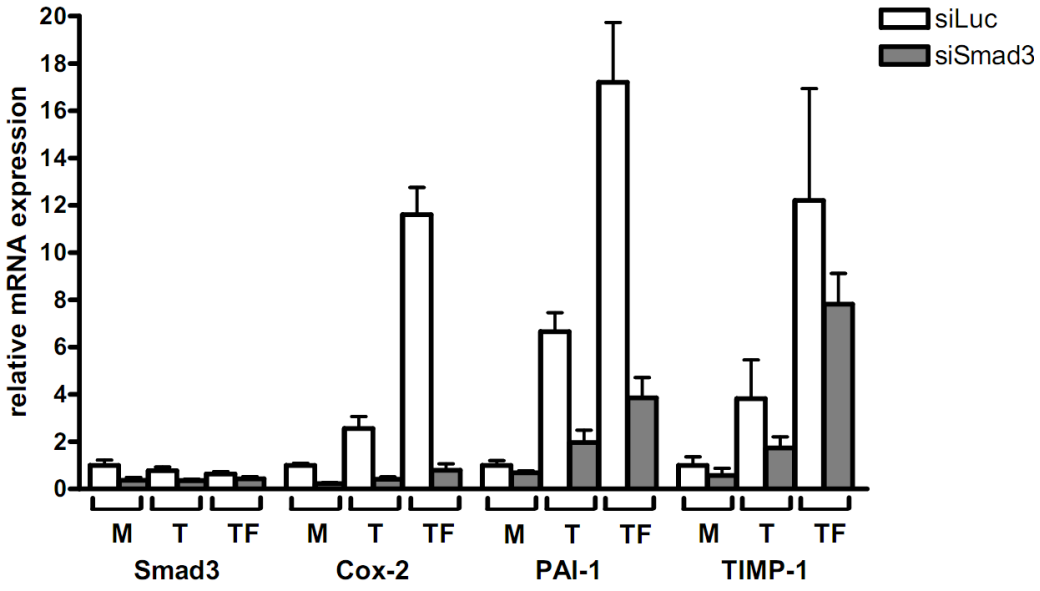

Figure 16. The forskolin effect on TGFβ-dependent gene expression is dependent on Smad3.

Cells were transfected with the siRNA against Smad3 (siSmad3, grey) or the control siRNA (siLuc, white), incubated for 2 days and treated with TGFβ or TGFβ/FSK or mock-treated for 24 h. Bars represent mRNA expression relative to the corresponding expression in mock-treated siLuc-transfected cells. Error bars indicate standard deviations (n=3).

Smad3 knockdown resulted in a significant reduction in basal gene expression as well as in the TGFβ and TGFβ/FSK responses. Basal expression of Cox-2, PAI-1 and TIMP-1 was reduced to 20 %, 68 % and 57 % of the control values, respectively. In the presence of TGFβ siSmad3-treated cells only reached 16 % (Cox-2), 35 % (PAI-1) and 45 % (TIMP-1) of the expression values obtained with control cells. In the presence of both TGFβ and forskolin gene expression under Smad3 knockdown conditions was down to 7 % (Cox-2), 22 % (PAI-1) and 64 % (TIMP-1) of the control values. These results show that first, as expected, Smad3 was necessary for the TGFβ-dependent induction of these genes. The fact that basal expression was also inhibited by siSmad3 suggests that MDA-MB-231 secrete TGFβ that activates TGFβ signaling in an autocrine TGFβ activation loop as has previously been shown (Dumont *et al.* 2003). More importantly, the data revealed that Smad3 knockdown affected the forskolin-enhanced TGFβ response of Cox-2 and PAI-1 (Tab. 14). The forskolin effect on

TIMP-1 seems to be less dependent on Smad3. It has to be taken into account that, for unknown reasons, the Smad3 knockdown effect is less prominent in the presence of TGFβ and forskolin (reduction of Smad3 RNA levels to 68 %, Fig. 16) than under mock condition (36 %, Fig. 16). This circumstance might have hidden a more pronounced and statistically significant reduction in the TGFβ/FSK response by siSmad3 and may point to a possible feedback loop to restore the cellular Smad3 level.

Table 14. Smad3 knockdown reduces forskolin-induced gene upregulation in the presence of TGFβ.

	Fold induction by forskolin in the presence of TGFβ		**Fold siSmad3 / fold siLuc**	**Student`s t-test**
gene	siLuc	siSmad3		p-value
Cox-2	4.53	1.95	0.43	0.1059
PAI-1	2.58	1.96	0.76	0.0449
TIMP-1	3.19	4.53	1.42	0.2111

Cells were transfected with siRNA against Smad3 or the control siRNA siLuc, incubated for 2 days and treated with TGFβ in the presence or absence of forskolin for 24 h. Values represent fold induction of mRNA levels by forskolin in the presence of TGFβ (n=3).

Next, the possibility was tested that YAP which acts as a modulator of Smad3 activity also plays a role in regulating the TGFβ/forskolin response. Phosphorylated YAP in the cytoplasm inhibits Smad complexes to translocate into the nucleus and therefore lower its transcriptional activity (Varelas *et al.* 2011). The phosphorylation status of YAP was examined by Western blot analysis (Fig. 17). This experiment revealed an induction of phospho-YAP levels in the cytoplasm of 2D-cultured cells by forskolin independently of the presence of TGFβ. In contrast to data previously reported (Varelas *et al.* 2011), this elevation of phospho-YAP levels was not accompanied by a higher abundance of Smad3 in the cytoplasm, at least not in 2D-cultured MDA-MB-231 cells (Fig. 17) suggesting that phospho-YAP and Smad3 translocation are likely to be independently regulated in MDA-MB-231 cells.

Cells in 3D cultures exhibited higher basal levels of phospho-YAP compared to 2D-cultured cells. This finding might be ascribed to the fact that 3D-cultured cells exist at a higher cell density, a condition known to induce higher phospho-YAP levels (Varelas *et al.* 2011). In 3D cultures, no forskolin-induced increase in YAP phosphorylation was observed (Fig. 17).

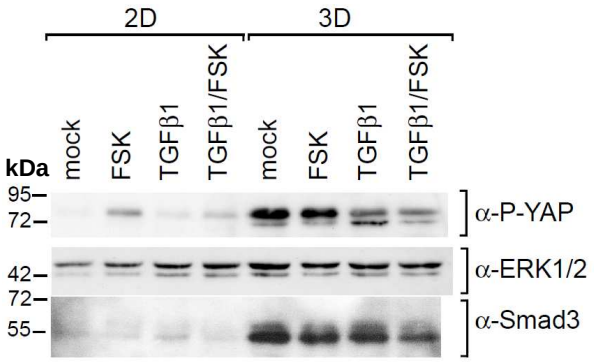

Figure 17. The cellular localization of the Smad3 protein is independent of phospho-YAP in MDA-MB-231 cells.

Western Blot analysis of phospho-YAP (P-YAP), Smad3 and ERK1/2 (loading control) in cytoplasmic extracts (CE) in mock-treated cells or cells incubated with forskolin and/or TGFβ for 24 h or of control cells (Mock).

In the following experiment, it should be clarified whether YAP is of importance for the forskolin-induced TGFβ response. Therefore, YAP expression was suppressed by siRNA (Fig. 18). Cells were then incubated in the presence of TGFβ with or without forskolin. Cellular lysates were analyzed for the mRNA levels of Cox-2, PAI-1, TIMP-1, PTHrP and p21. Most of these genes, although not statistically significant, surprisingly showed an induced expression in the presence of forskolin (Tab. 15). These results may suggest that YAP is a suppressor of the forskolin-induced TGFβ response. On the other hand, it seems that this activity of YAP is independent of its ability to regulate Smad3 nuclear export which was discussed above.

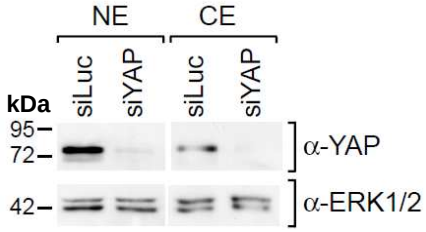

Figure 18. YAP level following siRNA-mediated knockdown.

Western Blot analysis of YAP and ERK1/2 (loading control) in nuclear (NE) and cytoplasmic extracts (CE) of cells transfected with siLuc or siYAP.

Table 15. YAP knockdown increases forskolin-induced gene upregulation in the presence of TGFβ.

	Fold induction by forskolin in the presence of TGFβ		Fold siYAP/ fold siLuc	Student's t-test
gene	siLuc	siYAP		p-value
Cox-2	1.78	3.46	1.94	0.0330
PAI-1	2.20	2.41	1.10	0.6302
TIMP-1	2.63	4.69	1.78	0.2952
PTHrP	1.76	3.43	1.95	0.1006
p21	1.02	1.59	1.56	0.1612

Cells were transfected with siRNA against YAP or the control siRNA siLuc, incubated for 3 days and treated with TGFβ in the presence or absence of forskolin for 24 h. Values represent fold induction of mRNA levels by forskolin in the presence of TGFβ (n=3).

3.4. Cyclic AMP-induced expression of the TGFβ receptor I

The forskolin-promoting activity on TGFβ-dependent Smad3 phosphorylation may imply that forskolin may activate a certain kinase either on the transcriptional or the post-transcriptional level. Since the type I TGFβ receptor is responsible for canonical Smad phosphorylation, changes in the expression and/or activity of this receptor may be involved. To explore this possibility, the effects of forskolin on the expression of type I and II TGFβ receptors (TβRI & II) were tested. In 2D-cultured cells, treatment with forskolin or with forskolin and TGFβ for 24 h raised the mRNA expression of TβRI by ~3-fold. In contrast, 3D-cultured cells showed no such response (Fig. 19). A comparison of the basal TβRI expression levels between 2D- und 3D-cultured cells revealed a 5.6-fold higher TβRI expression in 3D-cultured cells (Fig. 20A). Unlike TβRI, TβRII did not show higher, but rather lower expression in 2D-cultured cells in response to forskolin and TGFβ (Fig. 19). In 3D-cultured cells, TβRII expression was somewhat increased in the presence of forskolin alone. These data suggest that the forskolin-induced TβRI expression may account for the observed stimulatory effect of forskolin on TGFβ target gene expression in the presence of TGFβ.

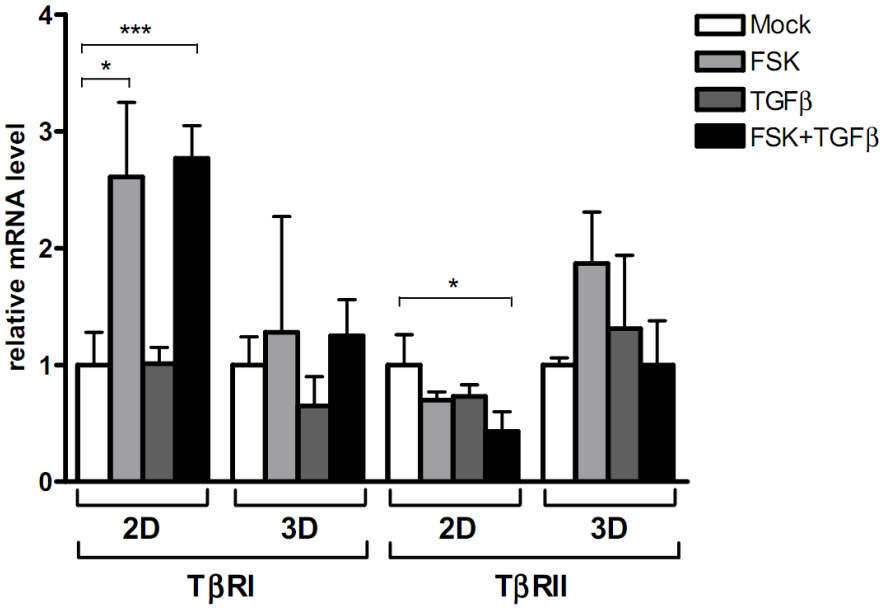

Figure 19. Different effects of forskolin on TGFβ receptor I & II expression in 2D and 3D cultures. RNA expression levels were normalized to mock-conditions separately for 2D- and 3D-cultured cells. Error bars indicate standard deviations (n=3). *p-value<0.05, ***p-value<0.005 (student`s t-test)

Moreover, the higher basal TβRI level in 3D-cultured cells might have partially be responsible for the abrogation of the forskolin effect in these cells. Interestingly, the higher TβRI expression coincided with a higher cAMP level in 3D-cultured cells (Fig. 20B) which together with the fact that cAMP can activate TβRI expression might suggest that the high level of cAMP in 3D-cultured cells has contributed to the high level of TβRI expression in these cells.

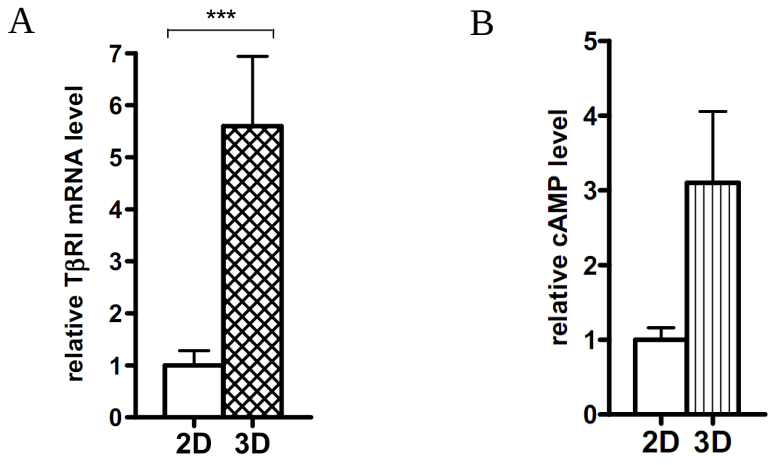

Figure 20. Comparison of TβRI mRNA expression (A) and cAMP level (B) in 2D- and 3D-cultured cells. Error bars indicate standard deviations (n=3). ***p-value<0.005 (student`s t-test)

When the high Smad3 level in 3D-cultured cells was mimicked in 2D-cultured cells by ectopic Smad3 expression, forskolin was still able to stimulate the upregulation of TβRI (Fig. 21). This suggests that the high Smad3 level in 3D-cultured cells is not responsible for the failure of forskolin to induce TβRI expression. But, even though TβRI could be upregulated by forskolin in 2D-cultured, Smad3-overexpressing cells, the higher TβRI level did not result in a rise of phospho-Smad3 (Fig. 13A). This suggests that, at high Smad3 levels, TβRI expression is not the limiting factor for Smad3 phosphorylation.

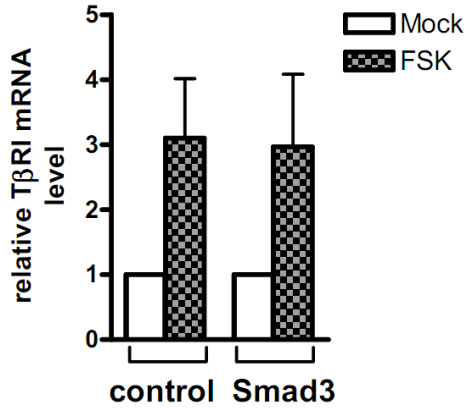

Figure 21. Forskolin upregulates TβRI expression independently of the Smad3 level.
FSK-mediated mRNA upregulation of TβRI was measured in Smad3-overexpressing and control cells. RNA expression levels were normalized to mock-conditions separately for each control and Smad3-treated cells. Error bars indicate standard deviation (n=3).

Next, it was tested, whether the cAMP effect on TβRI can also be detected in other breast cancer cell lines. A ~1.5-fold upregulation of TβRI expression in response to forskolin was found in MCF-7 and in BT20 breast cancer cells (Fig. 22). Therefore, forskolin is likely to modulate the TGFβ pathway also in other breast cancer cells.

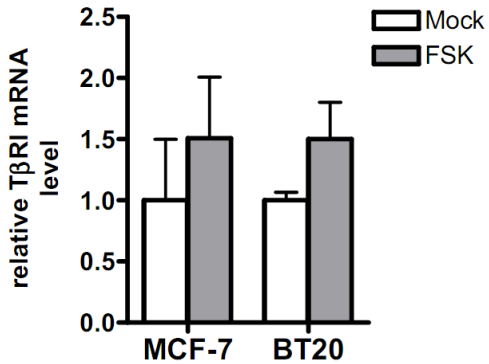

Figure 22. Stimulation of TGFβ receptor I expression by forskolin in MCF-7 and BT20 breast cancer cells.
MCF-7 cells were treated with forskolin or mock-treated. BT20 cells were treated with forskolin or mock-treated in the presence of TGFβ. TβRI RNA expression was measured. Error bars indicate standard deviations (n=3).

Since the TβRI upregulation seems to play an important role in the forskolin-induced elevation of TGFβ-dependent gene expression, experiments were performed to investigate the underlying mechanism. In an attempt to distinguish between a transcriptional and a post-transcriptional regulation of TβRI by forskolin, an inhibitor of transcription, actinomycin D, was applied to the cells alone or together with forskolin at a concentration of 5 µM. TβRI mRNA levels were monitored over 9 h. In the presence of actinomycin D, forskolin completely failed to induce TβRI mRNA expression, whereas, in control samples without actinomycin D, an increasing level of TβRI mRNA accumulated over time (Fig. 23). This finding provides evidence for the forskolin-induced TβRI upregulation to take place on the transcriptional level. Interestingly, treatment with actinomycin D alone or together with forskolin suppressed also basal TβRI mRNA expression suggesting that maintaining basal TβRI expression requires constant transcription.

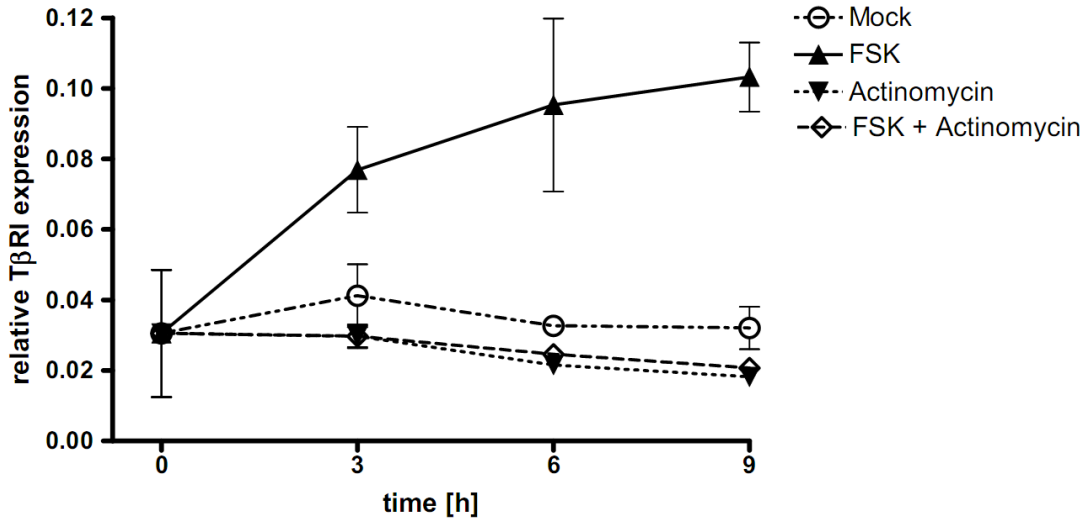

Figure 23. Time course of induction of TβRI mRNA expression in the presence of forskolin (10 µM), actinomycin (5 µM), both factors or in the absence of either factor.
Error bars indicate standard deviations (n=3).

4. Cyclic AMP regulation of TβRI expression

4.1. Cyclic AMP-induced TβRI promoter activity

In order to investigate whether forskolin directly affects TβRI transcription, TβRI promoter activation studies were performed. First, the TβRI promoter was screened for sequences that might be cAMP-regulated. For this purpose, an *in silico* search for putative transcription factor binding sites were conducted. Analyzing the TβRI promoter did not reveal any putative cAMP-responsive elements (CREs). Interestingly, PKA has been reported to be involved in tamoxifen-mediated transcriptional activation of the human $p27^{Kip1}$ gene. The PKA-activated $p27^{Kip1}$ promoter elements did not contain CREs either (Lee *et al.* 2003). The authors

demonstrated that two Sp1 consensus sequences were essential for this activation and that the binding of this transcription factor to these sequences was increased in the presence of tamoxifen. In order to check, whether the TβRI promoter contains similar elements, the tamoxifen-inducible sequences of the $p27^{Kip1}$ promoter were aligned with the human TβRI promoter sequence. The region containing the two PKA-responsive Sp1 sites and an adjacent GC-rich region within the $p27^{Kip1}$ promoter showed striking homology with a region lying -100/-51 upstream of the transcriptional start site in the TβRI promoter (Fig. 24). The transcription factor Sp1 binds to GC-rich sequences (GGGCGG/CCCGCC or similar) (Dittmer *et al.* 1994). It is conceivable that the two identified PKA-responsive Sp1 sites or further potential Sp1 sites within the GC-rich region contribute to the induction of TβRI expression by forskolin.

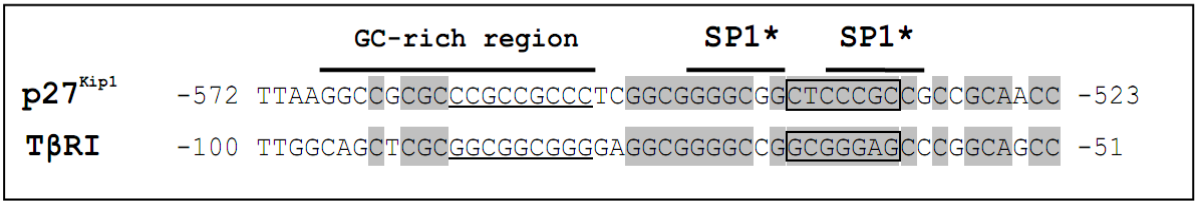

Figure 24. Alignment of $p27^{Kip1}$ with TβRI promoter sequences.

Homology was found with the two PKA-responsive Sp1 sites and an adjacent GC-rich region. Sp1*: PKA-inducible Sp1 sites in the $p27^{Kip1}$ promoter; underlined: complementory sequence; grey box framed: inverted sequence; grey box: identical sequence

A fragment comprising bases from -392 to +21 of the TβRI promoter, containing the Sp1 sites, was cloned into the pGL4.10[*luc2*]-luciferase reporter plasmid to create pGL4.10[*luc2*]_TβRI. Cells were co-transfected with this construct and, for normalization, with the plasmid pGL4.74[*hRluc/*TK] coding for a renilla luciferase gene under the control of a constitutively active promoter. Transfected cells were incubated in the absence or presence of forskolin for 6, 18 and 24 h. Promoter activity was moderately, but statistically significantly increased by ~1.2-fold in the presence of forskolin (Fig. 25). The weakness of the effect may be caused by the lack of sufficient levels of co-factors, like Sp1, that are required for transcriptional activation or by certain repressors that may bind to the fragment and may counteract the response. The importance of the transcription factor Six1, which has recently been shown to regulate TβRI expression, for the forskolin response was also studied (Micalizzi *et al.* 2010). To test if Six1 is required for TβRI upregulation by forskolin, Six1 expression was elevated by transfecting the cells with the plasmid pcDNA3_Six1. Although ectopic expression of Six1 increased TβRI promoter activity 2.5-fold, Six1 could not further increase the forskolin effect. This result suggests that the cellular Six1 level was not a limiting factor for the response of the TβRI promoter to forskolin.

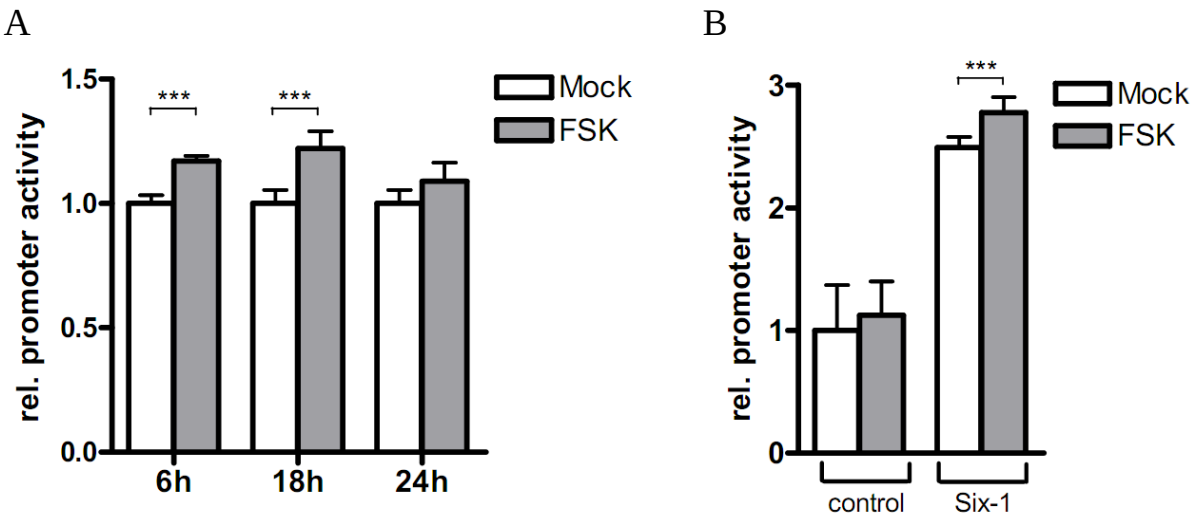

Figure 25. Forskolin induces TβRI promoter activity.

A) Stimulation of the relative (rel.) activity of the TβRI promoter by forskolin after 6, 18 and 24 h
B) Stimulation of the relative activity of the TβRI promoter by forskolin after 24 h following ectopic Six1 expression. Error bars indicate standard deviations (n=5). ***p-value<0.005 (student`s t-test)

4.2. Transcription factors and co-activators involved in TβRI upregulation

Next, I wondered, if Six1 or its co-factor Eya2 is upregulated by forskolin. I also tested the effect of forskolin on CREB expression. No upregulation was observed for these factors in the presence of forskolin (Fig. 26).

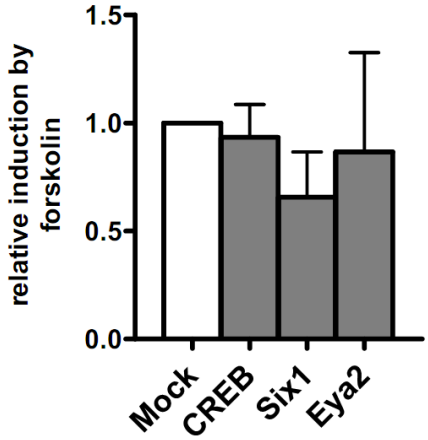

Figure 26. Forskolin did not induce RNA expression of CREB, Six1 or Eya2.

Bars represent fold induction of CREB, Six1 and Eya2 RNA expression by forskolin. Error bars indicate standard deviations (n=3).

CREB is directly phosphorylated by PKA when cAMP levels are elevated. To answer the question whether CREB activation is needed for forskolin to induce TβRI transcription, CREB expression was suppressed by a CREB-specific siRNA. CREB mRNA levels were effectively reduced after 3 and 24 h (Fig. 27A). However, in the presence of siCREB, forskolin treatment increased TβRI mRNA expression to a similar extent as found in the presence of control siRNA (Fig. 27B). This result shows that CREB is not involved in TβRI regulation as expected based on the finding that the TβRI promoter does not contain CREs.

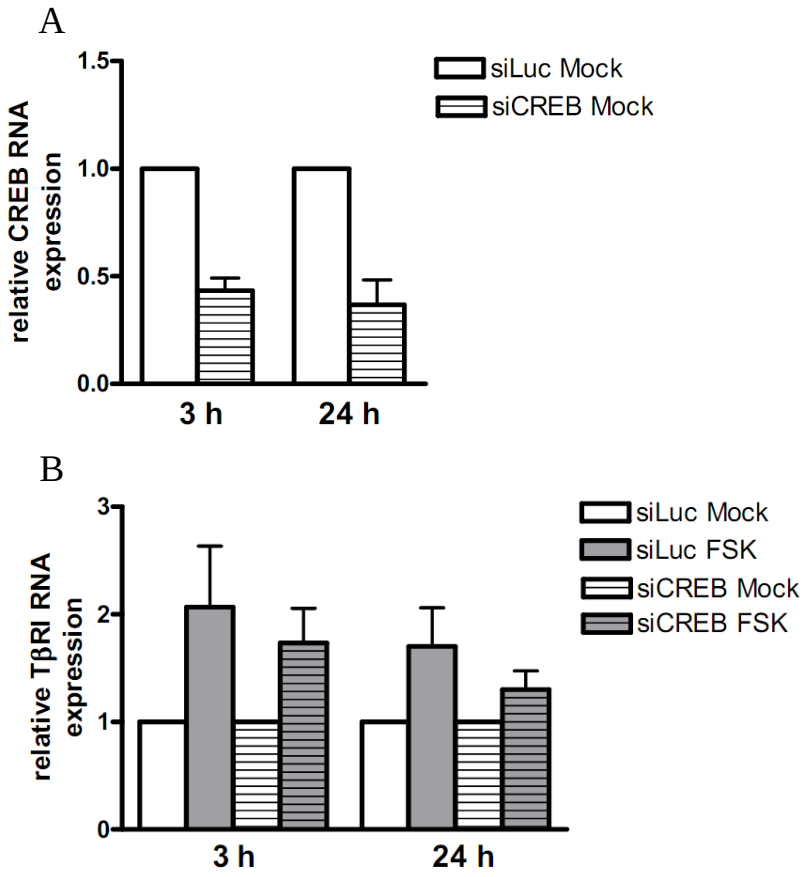

Figure 27. Forskolin-induced TβRI upregulation is independent of CREB.

A) CREB mRNA levels in response to CREB-specific siRNAs
B) Forskolin response of TβRI RNA in the presence of siCREB or control siRNA (siLuc) normalized to mock-conditions separately for siCREB and siLuc. Error bars indicate standard deviations (n=3).

Next, the influence of Six1 on the forskolin response of TβRI was tested. SiRNA-mediated Six1 knockdown was unable to inhibit TβRI upregulation by forskolin suggesting that Six1 does not contribute to this effect (Fig. 28).

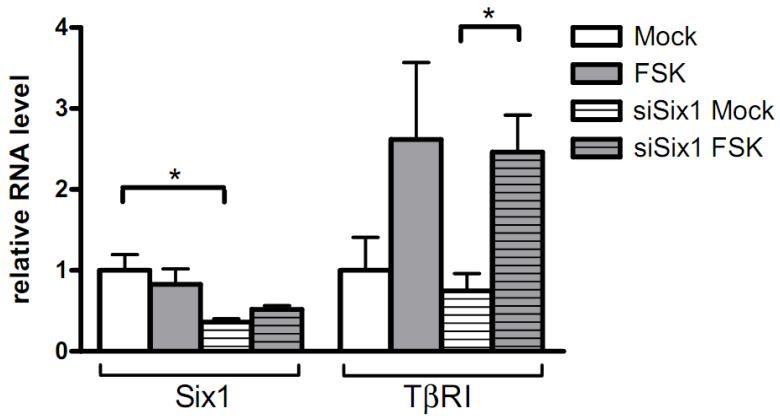

Figure 28. Forskolin-induced TβRI upregulation is independent of Six1.

Six1 and TβRI mRNA levels in the presence of siSix1 or control siRNA (siLuc) after mock-treatment or incubation with forskolin for 24 h. Error bars indicate standard deviations (n=3). *p-value<0.05 (student`s t-test)

In addition to transcription factors, histone modifications play an important role in transcriptional regulation (Leggatt & Gabrielli 2012). It has been shown that TβRI expression can be stimulated by inhibitors of histone deacetylases (HDACs) in breast cancer cells suggesting that histone acetylation plays a role in TβRI transcription (Ammanamanchi & Brattain 2001). To check the importance of histone acetylation for the forskolin effect on TβRI expression, cells were incubated with an HDAC inhibitor (2 µM) in the presence and absence of forskolin. HDAC inhibition led to an elevated TβRI expression which could not be further increased by the addition of forskolin (Fig. 29). This suggests that, at least in part, forskolin may also interfere with histone acetylation to enhance the transcription of TβRI. This observation may explain the low rise in promoter activity in response to forskolin. Promoter assays use plasmid DNA, which, unlike genomic DNA, is usually not correctly wrapped around histones (Mladenova *et al.* 2009). Therefore, this assay only partially reflects the response of the genomic promoter to a histone modifying factor.

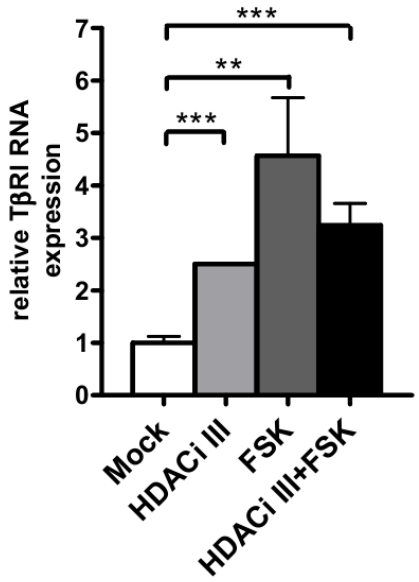

Figure 29. HDAC inhibition induced TβRI upregulation.

Bars indicate relative expression of TβRI RNA of mock-treated cells and after treatment with 2 µM HDAC inhibitor III, 10 µM forskolin or both factors. Error bars indicate standard deviations (n=3). *p-value<0.05, **p-value<0.01, ***p-value<0.005 (student`s t-test)

4.3. Impact of ectopic TβRI expression on TGFβ-dependent gene expression

The ability of forskolin to elevate TβRI expression may be the reason for the increased TGFβ responsiveness of some genes in the presence of forskolin. To prove that the TβRI upregulation is the molecular mechanism mediating the forskolin effect on TGFβ-dependent transcription, TβRI was ectopically expressed in 2D-cultured MDA-MB-231 cells. Cells were transfected with a plasmid coding for TβRI(T204D). TβRI(T204D) is a constitutively active form of TβRI in which the amino acid threonine at position 204 is replaced by aspartate, mimicking phosphorylation of threonine as required for TβRI to phosphorylate Smads. Different amounts of the plasmid (1, 2.5 and 5 µg) were used which induced TβRI mRNA expression by 1.7-, 5.0- and 12.7-fold, respectively (Fig. 30A). Increased TβRI mRNA expression and plasma membrane abundance could be confirmed by qRT-PCR and Western blot analysis, respectively (Fig. 30B). Detection of the PAI-1, Cox-2 and TIMP-1 proteins showed higher expression in TβRI overexpressing cells compared to untransfected cells. The levels of these proteins depended on the amount of the TβRI(T204D) expression plasmid transfected into the cells. After transfection of 1 µg plasmid, the resulting TβRI levels resembled those as induced by forskolin. Strikingly, at this TβRI(T204D) level, the extent by which PAI-1, Cox-2 and TIMP-1 protein expression was upregulated was comparable to that in response to forskolin. Therefore, it is likely that forskolin promoted TGFβ signaling by increasing TβRI expression.

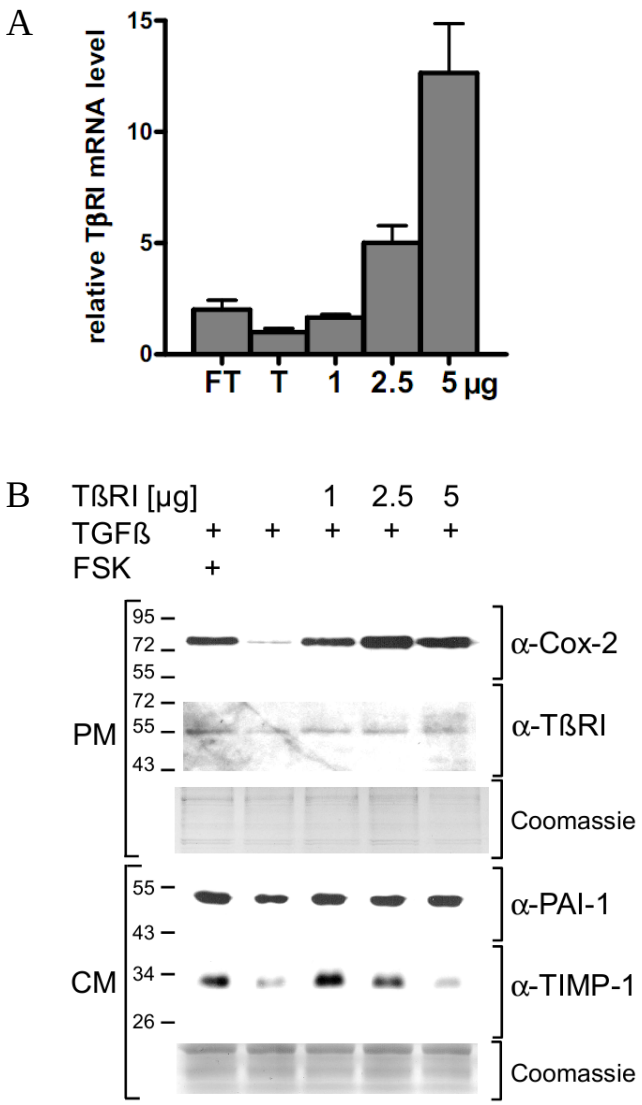

Figure 30. Ectopic TβRI expression mimics forskolin response.

Cells were transfected with 0, 1, 2.5 or 5 µg of TβRI expression plasmid and incubated for 24 h following treatment with TGFβ (T) or TGFβ/FSK (FT) or following mock-treatment for further 24 h. Subsequently, cells were analyzed for A) TβRI mRNA levels (error bars indicate standard deviations, n=3) and B) TβRI and Cox-2 protein level in plasma membrane extracts and PAI-1 and TIMP-1 in conditioned medium by Western blot analysis.

5. Relevance of the cross-talk between cAMP and TGFβ pathways for the response of MDA-MB-231 cells to stromal cells

In a tumor, the activities of tumor cells are modulated by neighboring stromal cells, such as mesenchymal stem cells (MSCs). MSCs are resident in the bone marrow and are attracted by inflammatory signals as secreted by tumor cells. Once invaded into the tumor tissue, their interactions with tumor cells may promote tumor progression (Dittmer *et al.* 2011). To study the possibility that MSCs may induce the same pathways in breast cancer cells as induced by forskolin or TGFβ, MDA-MB-231 cells were co-cultured with human bone marrow-derived

MSCs for two to four days in 2D culture. MSCs were mixed with MDA-MB-231 cells at a ratio of 1:300. After incubation, samples were analyzed for the activation of the cAMP and TGFβ signaling pathways. Breast cancer cells incubated with MSCs showed elevated phospho-CREB after two and four days and phospho-Smad3 levels after two days of incubation (Fig. 31).

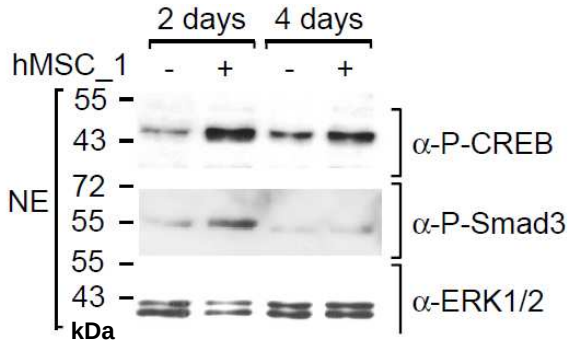

Figure 31. Human MSCs (hMSCs) induce the activation of cAMP and TGFβ pathways in breast cancer cells.
Western Blot analysis of phospho-Smad3, phospho-CREB and ERK1/2 (loading control) in nuclear extracts of breast cancer cells co-cultured with MSCs for 2 and 4 days.

These data indicate that the interaction of MSCs with breast cancer cells induces the activation of the cAMP and TGFβ pathways. Whether this activation allows a stimulation of forskolin and TGFβ target gene expression remains to be determined. Furthermore, it stays unanswered whether MSCs induce gene expression patterns in a similar way like forskolin drives an enhanced TGFβ response.

6. Impact of cAMP and TGFβ on the morphology and function of MDA-MB-231 cells

It is possible that gene expression changes result in a different cell morphology or behavior. A number of *in vitro* tests were performed to examine morphological and functional changes induced by forskolin and TGFβ in 2D and 3D cultures.

6.1. Morphological changes induced by forskolin and TGFβ

In 2D cultures, forskolin-treated cells exhibited a more spindle-like morphology with some cells exposing thin protrusions (Fig. 32B). In contrast, in the presence of TGFβ, cells appeared spread-out with large cytoplasms (Fig. 34C). Simultaneous treatment with both agents resulted in a morphology that resembled the morphology obtained by treatment with forskolin alone (Fig. 32D).

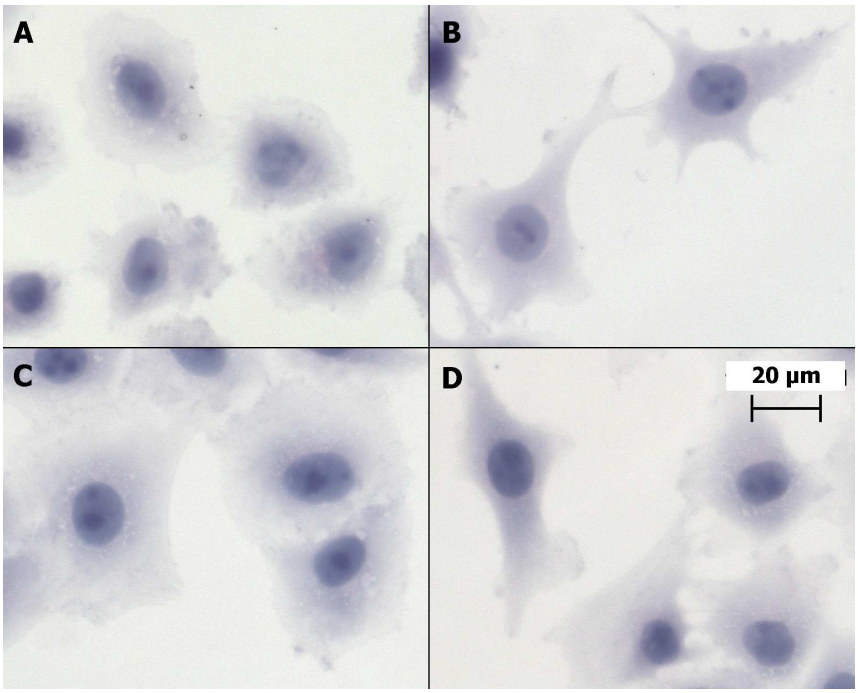

Figure 32. Morphological changes induced by forskolin and/or TGFβ in 2D-cultured MDA-MB-231 cells. Microscopic images of H&E stained mock- (A), forskolin- (B), TGFβ- (C) and forskolin/TGFβ-treated cells (D).

In 3D suspension cultures in 75 cm^2 flasks, MDA-MB-231 cells form multiple irregularly-shaped, loose aggregates of different sizes (Dittmer *et al.* 2009). In order to obtain single 3D cellular aggregates, a defined number of cells were seeded into 96-well plates. In the presence of forskolin, TGFβ or both factors smaller aggregates were formed (Fig. 33).

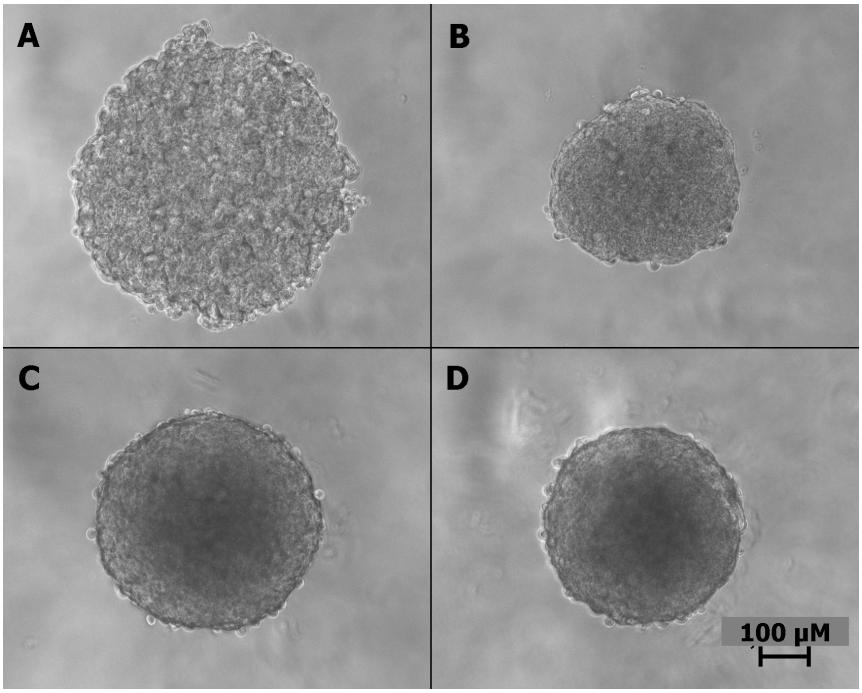

Figure 33. Forskolin and TGFβ treatment reduce cell aggregate size in 3D culture.

Microscopic images of mock-treated 3D-cultured MDA-MB-231 cells (A), 3D-cultured cells treated with forskolin (B), TGFβ (C) or forskolin plus TGFβ (D) in a 96-well plate after 3 days of incubation.

In the microscopic examination, allowing a view from the top onto the cell clusters, all aggregates appeared circular with either showing a smooth (TGFβ or TGFβ+FSK-treated) or a more rippled boundary (mock, forskolin-treated). The microscopic examination did not allow to distinguish between spheroids and discs. To address this issue, the aggregates were formaldehyde-fixed and embedded in paraffin. Then, histological slides were prepared and H&E stained (Fig. 34). Mock and forskolin-treated cells formed flat, disc-like aggregates with forskolin-treated cells appearing smaller and exposing dense nuclei surrounded by only a small stretch of cytoplasm. In contrast, TGFβ-treated cells formed aggregrates of spherical shape. This shape was also found in the presence of both forskolin and TGFβ but, as observed with forskolin alone, aggregates appeared more compact. TGFβ also induced a relaxation of the cell structure and exposed large, round nuclei and extended cytoplasm.

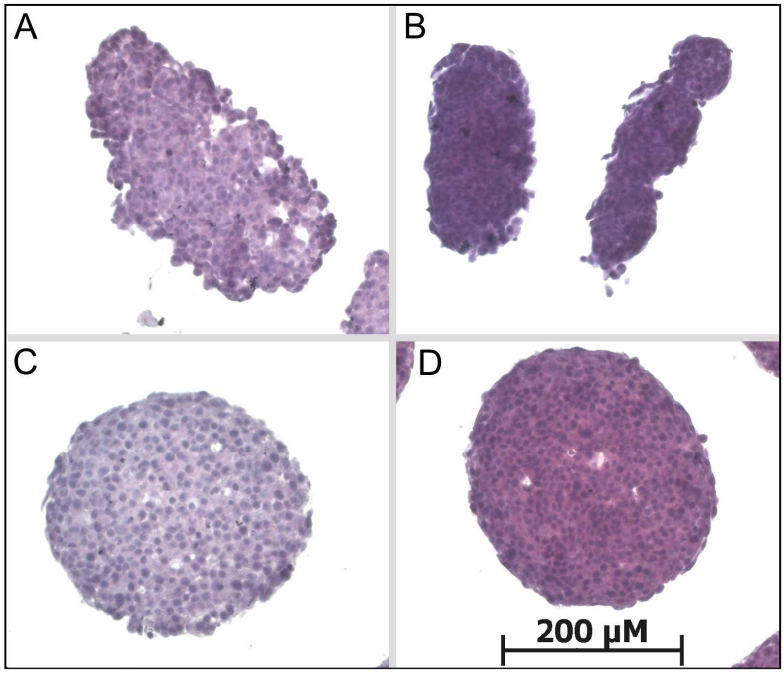

Figure 34. Morphologies of mock-treated 3D-cultured MDA-MB-231 cells (A) and 3D-cultured cells treated with forskolin (B), TGFβ (C) or forskolin plus TGFβ (D).

Cells were incubated for 3 days in the presence or absence of the stimulators, formaldehyde-fixed and embedded in paraffin. Sections were prepared and H&E stained.

Spheroids are frequently observed with other cell lines. MCF-7 epitheloid breast cancer cells form spheroids that become hollow in cultures resembling the development of normal mammary alveolar units (Dittmer *et al.* 2009). When mesenchymal MDA-MB-231 cells are used, spheroids are usually not formed, even in the presence of TGFβ (Dittmer *et al.* 2008). Therefore, it was surprising to find that spheroids are formed in 96-wells in the presence of TGFβ. It is suggested that the conical shape of these wells generated a frame allowing these cells to form a spheroid under these conditions. Immunocytochemical staining revealed that MDA-MB-231 spheroids show a distinct pattern for the expression of certain proteins (Fig. 35). Integrin beta1 (ITGB1), fibronectin (FN), PAI-1, cysteine-rich angiogenic inducer 61 (Cyr61) and CD44 are predominatly expressed by the outer cell layers of the spheroids. ITGB1 is known to be expressed at cell-cell- and cell-matrix junctions (Lahlou & Muller 2011). Besides its importance in cell adhesion, integrins are signaling receptors transducing extracellular signals into the cell. Importantly, signaling is also mediated “inside-out”, which means that intracellular conditions may regulate the extracellular adhesive properties of integrins. ITGB1 has been described as a binding partner for the ECM protein FN that is expressed in MDA-MB-231 spheroids at a position similar to ITGB1. It is therefore likely, that these two proteins contribute to shaping the spheroids. Cyr61 is an ECM-associated protein (Lin *et al.* 2012) and has been shown to be a secreted protein that acts as a ligand of several integrins (Jandova *et al.* 2012). CD44 is a receptor binding to matrix hyaloronan (HA) and signaling through HA/CD44 increases the activities of matrix-degrading enzymes (Montgomery *et al.* 2012).

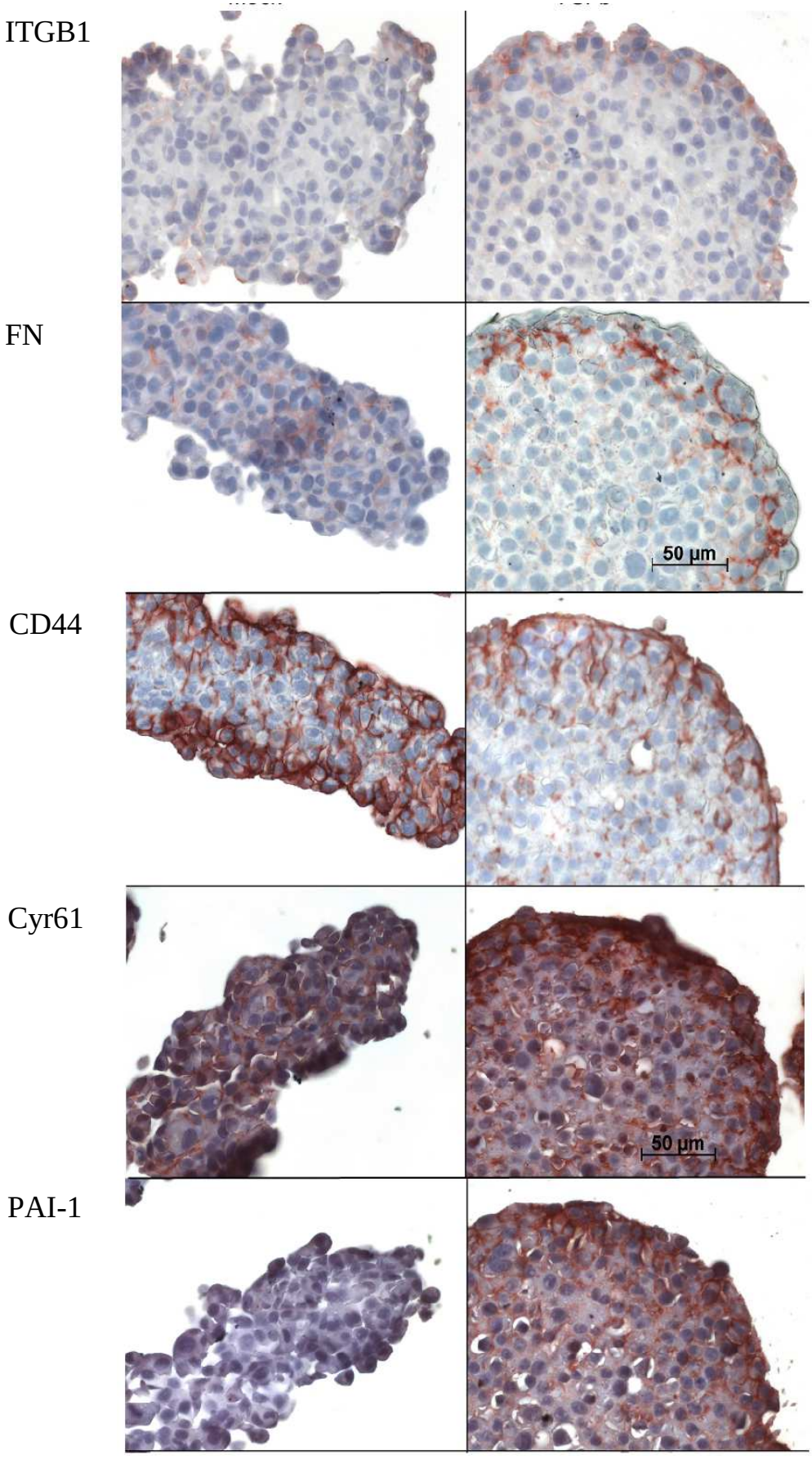

Figure 35. Imunocytochemical characterization of TGFβ-induced MDA-MB-231 spheroids.

Sections of mock- (left panels) and TGFβ-treated aggregates (right panels) were immuno-stained for ITGB1, FN, CD44, Cyr61 and PAI-1 (red). Nuclei are stained blue.

PAI-1 contributes to matrix remodeling by regulating plasmin-dependent matrix proteolyse. Interestingly, PAI-1 also stimulates FN matrix assembly and activates ITGB1 (Vial & McKeown-Longo 2008).

Together, these data suggest that MDA-MB-231 spheroids are organized in a specific way expressing markers of cell-matrix interaction preferentially at the outer cell layers that potentially cross-talk to maintain this structure.

Aggregates appeared to be more compact in the presence of forskolin. To examine to what extent forskolin changes the size of the aggregates, I measured the area of the aggregates in a 2D projection. However, the different 3-dimensional morphologies (disc vs. sphere) of forskolin- and TGFβ-treated aggregates did not allow a comparison of all four conditions. Hence, discs and spheres were compared separately. In the absence of TGFβ, the forskolin effect on the disc size was examined and, in the presence of TGFβ, the forskolin effect on the sphere size was measured. Forskolin significantly decreased the sizes of the aggregates in the presence and absence of TGFβ by 18 % and 57 %, respectively (Fig. 36).

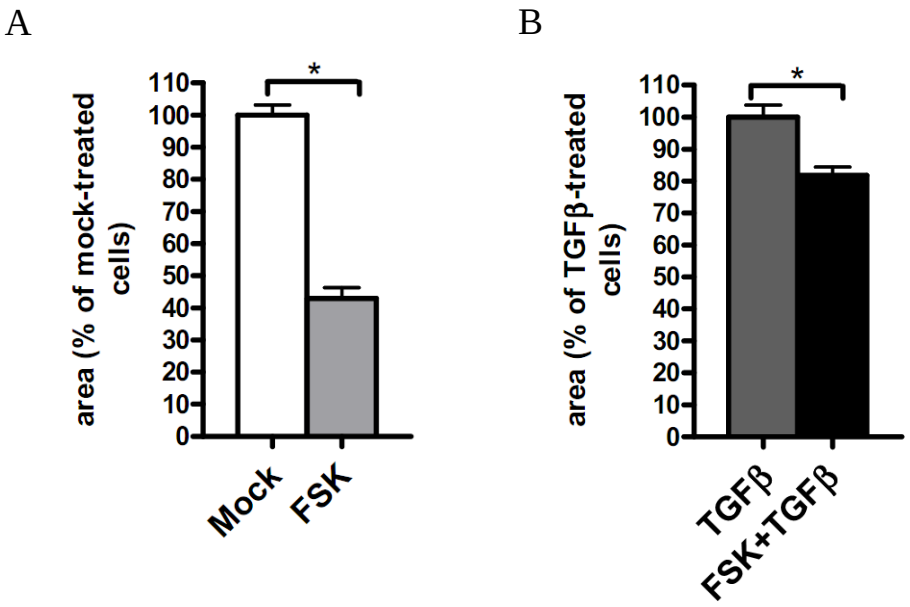

Figure 36. Forskolin reduced the sizes of MDA-MB-231 3D aggregates.

Cells were seeded into a 96-well plate and treated with forskolin, TGFβ or both factors or mock-treated for 3 days. Microscopic images were taken and the areas of the aggregates in a 2D projection were measured. The effects of forskolin on disc-shaped aggregates (A) and spheroids (B) were separately examined. Error bars indicate standard deviations (n=16). * p-value<0.05 (student`s t-test)

6.2. Functional changes induced by forskolin and TGFβ

Changes in cell morphology might reflect alterations in cell viability or adhesive or migratory properties of the cells. In order to check for changes in cellular behavior, intracellular ATP levels as an indicator for cell viability were determined after exposure to forskolin, TGFβ or both factors. In 2D culture, cell viability was slightly, but significantly reduced when cells were treated with TGFβ alone, whereas the other treatments did not affect cell viability (Fig. 37A). Interestingly, 3D-cultured cells responded more sensitive. Here, TGFβ substantially increased the viability of the cells which implies that the spheroid state might be advantageous to MDA-MB-231 cells in suspension cultures. In contrast, forskolin treatment reduced viability in the presence and absence of TGFβ (Fig. 37B).

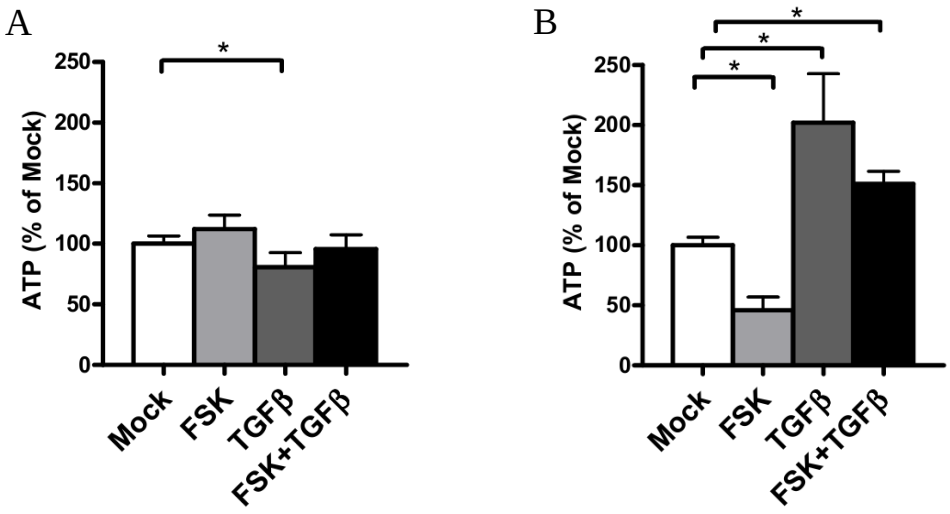

Figure 37. Changes in MDA-MB-231 cell viability in response to forskolin and/or TGFβ in 2D culture (A) and 3D culture (B).

Cells were seeded into uncoated 96-well plates for 2D culturing (A) or into plates coated with agarose for 3D culturing (B) and incubated for 3 days. Cells were lysed and the ATP content was determined by using a luciferase assay. Values are shown relative to mock treatment (100 %). Error bars indicate standard deviations (n=4). * p-value<0.05 (student`s t-test)

In an attempt to measure the effect of forskolin and TGFβ on cell-cell adhesion, the number of cells that attach to a preformed cell monolayer was measured. For this purpose, cells were plated on a culture dish and grown to confluency. A cell suspension containing 4 million cells was poured onto the cell monolayer and incubated for 3 h. The remaining non-attached cells were removed and counted. In the presence of forskolin, the number of cells that remained in the supernatant was significantly reduced suggesting an increased cell-cell-adhesion (Fig. 38). Treatment with TGFβ did not affect cell adhesion. Co-treatment with forskolin and TGFβ resulted in a slight, but not significant reduction in the number of non-attached cells. This result shows that forskolin increases cell adhesive properties in 2D cultures.

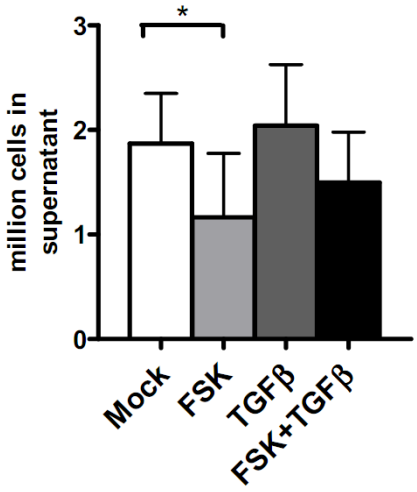

Figure 38. Forskolin increased cell-cell adhesion in 2D-cultured MDA-MB-231 cells.

Cells were seeded onto a confluent cell layer in the presence of forskolin and/or TGFβ or in the absence of these stimulators. Cells that remained in the supernatant after 3 h of incubation were counted. Error bars indicate standard deviations (n=6). *p-value<0.05 (student`s t-test)

Next, the possibility that forskolin or TGFβ could influence cellular migration was explored. Therefore, Boyden chamber assays were performed. First, monolayer cultures were pre-incubated with forskolin and/or TGFβ or none of these substances overnight. Then, cells were detached from the dish and seeded onto a porous membrane with 8 µm pores and incubated for another day under the same condition as before. Non-migrated cells that remained on the top side of the filter were removed and cells that migrated through the pores and remained attached to the bottom site of the filter were stained. This assay revealed that, under the influence of forskolin, cells migrated faster, whereas migration was significantly reduced in the presence of TGFβ (Fig. 39). Simultaneous treatment with forskolin and TGFβ resulted in stimulation of migration but to a smaller degree than forskolin alone.

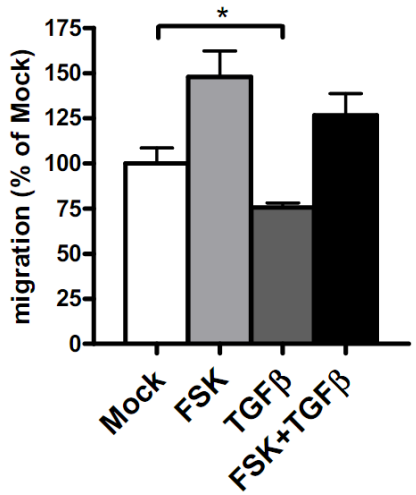

Figure 39. Forskolin increased and TGFβ decreased the migration of MDA-MB-231 cells.

After preincubation with forskolin and/or TGFβ or mock-treatment cells were analyzed for migratory activity in a Boyden-chamber assay for 17 h under the same conditions as used in the pre-treatment. Cells that have migrated through the filter pores were stained. OD values of eluated dye were used to measure migrated cells. Error bars indicate standard deviations. *p-value<0.05 (student`s t-test)

Since TGFβ is known to be a regulator of cell proliferation the ability of forskolin to modulate the TGFβ effect on DNA synthesis was tested by measuring the incorporation of the pyrimidine analogue 5-bromo-2`-deoxyuridine (BrdU). Cells were seeded into 96-well plates and incubated in medium supplemented with BrdU plus forskolin and/or TGFβ or solvents (Mock) for 24 h. Subsequently, the cells were fixed and BrdU that had been incorporated into newly synthethized DNA was detected with an enzyme-coupled antibody and a colorimetric substrate reaction. Using this assay, a significant reduction of cell proliferation by 19 % in the presence of TGFβ was observed. Furthermore, when forskolin was supplied simultaneously with TGFβ, proliferation was reduced by 25 %. The further reduction of TGFβ-induced inhibition of proliferation may reflect changes in gene expression, especially of the cell cycle inhibitor p21 which was shown to underlie the forskolin/TGFβ effect. Additional experiments will be required to prove this notion.

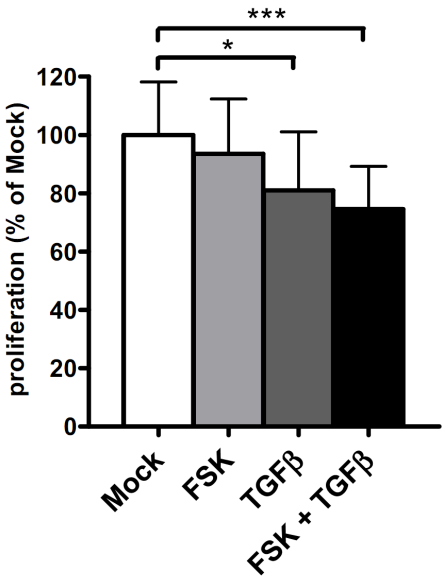

Figure 40. Forskolin increased the inhibitory effect of TGFβ on the proliferation of MDA-MB-231 cells.
Cells were grown in the presence of forskolin and/or TGFβ or were mock-treated. The culture medium was supplemented with the pyrimidine analogue BrdU (10 µM). After 24 h BrdU incorporation was measured. Cells were fixed, incubated with a peroxidase-coupled anti-BrdU antibody and enzyme activity was determined by reading absorbance that indicated substrate turnover. Error bars indicate standard deviations (n=10). *p-value<0.05, ***p-value<0.005 (student`s t-test)

In conclusion, forskolin and TGFβ could be shown to regulate cell shape and behaviour. In the presence of forskolin, single cells appeared thinner and the size of 3D cell aggregates was decreased suggesting that the smaller size of the cells may have caused the smaller size of the aggregates. Smaller aggregates might have resulted from the increase in cell-cell-adhesion as observed in the adhesion assay and/or also from cytoskeletal rearrangements that have not been investigated here. An unexpected observation was that TGFβ induced the formation of regularly shaped spheroids of MDA-MB-231 cells in 96-well plates. Concomitantly, cells exhibit improved viability and a distinct pattern of the expression of certain proteins. Despite the dramatic changes in cell morphology and function as caused by forskolin and TGFβ, most of these changes did not correlate with the changes in gene expression as found in response to these stimulators. With respect to cell proliferation, there might be a correlation between the reduction in proliferation and the induction of the cell cycle inhibitor p21, which has to be examined in more detail in the future. Taken together, this means that TGFβ and forskolin induce a complex pattern of response of which the effects on the analyzed genes are only a part of it.

DISCUSSION

1. Cross-talk between the cAMP and TGFβ pathways

In this study, the cross-talk between the cAMP and TGFβ pathways was examined in aggressive breast cancer cells. Hitherto, the interplay of both pathways has been studied in a number of cell types but not in breast cancer cells. Different mechanisms of interactions were reported which seem to be dependent on cell type and context. In most cases, an antagonism between both pathways was observed. By using dermal fibroblasts, one study showed an inhibition of the transcriptional co-activator CBP/p300 by cAMP signaling leading to insufficient Smad-CBP/p300 complex formation and thus to a downregulation of TGFβ-dependent gene expression by cAMP (Schiller *et al.* 2010). Alterations in Smad-CBP/p300 complex formation has also been observed in other studies as did a suppression of Smads or phospho-Smads (Liang *et al.* 2008, Liu *et al.* 2006). In contrast, the data I presented here do not suggest such mechanisms to exist in MDA-MB-231 cells. Instead, a novel mechanism was found by which cAMP stimulates TGFβ signaling. In 2D-cultured cells, activation of the cAMP pathway led to enhanced TGFβ-dependent Smad3 phosphorylation and activation of TGFβ-responsive genes. The combined activation of the cAMP and TGFβ pathway here led to an additive or even to a synergistic effect on TGFβ-responsive genes. An additive effect was preferentially seen with genes, such as TIMP-1 or Cox-2, that show cAMP responsiveness also in the absence of TGFβ. A synergistic effect was found with genes, such as PAI-1, that do either not respond to or whose expression is downregulated by forskolin.

2. Roles for Smad3 and YAP in cAMP-enhanced TGFβ signaling

Smad3 knockdown affected autocrine as well as paracrine TGFβ signaling and was shown to interfere with the forskolin/TGFβ effect on gene expression. Cox-2 expression was most dramatically reduced in response to Smad3 downregulation. PAI-1 also showed a significant loss of TGFβ and forskolin inducibility but to a lesser extent. In contrast, TIMP-1, though showing a reduced response to TGFβ in the presence of siSmad3, retained full forskolin response. These data may indicate that different genes require different levels of Smad3 for a maximum response to TGFβ. Previous studies showed that a reduced Smad3 expression induced non-canonical TGFβ signaling (Parvani *et al.* 2011). These pathways may also affect the expression of certain genes in a different way.

Other factors have been identified that modulate TGFβ/Smad signaling such as YAP. Phosphorylated YAP impedes TGFβ signaling by preventing the nuclear translocation of Smads (Varelas *et al.* 2011). Furthermore, cell polarity and density are connected to YAP. In high-density cells, TGFβ signaling is reduced because Smad3 bound to YAP is preferentially

translocated from the nucleus to the cytoplasm. The data of the analyses performed here with MDA-MB-231 cells do not support this view. Though forskolin elevated cytosolic phospho-YAP, this was not accompanied by Smad3 translocation or reduced TGFβ pathway activation. Moreover, despite the fact that 3D-cultured cells exhibited higher phospho-YAP levels, Smad3-dependent TGFβ signaling was not diminished and some Smad3 target genes showed even increased responsiveness to TGFβ. As a novel finding, YAP seems to inhibit the forskolin-dependent enhancement of TGFβ signaling, as knockdown of YAP resulted in a higher forskolin response of TGFβ target genes. Taken together, in MDA-MB-231 cells, YAP does not seem to regulate Smad3 cellular location and hence activity. Yet, YAP was found to suppress the forskolin effect on TGFβ responsiveness. The mechanism underlying this effect is not yet known.

3. Cyclic AMP-mediated upregulation of TβRI expression

Forskolin treatment led to upregulation of TGFβ receptor I potentially allowing a higher activation of TGFβ downstream factors (Fig. 41). Introduction of a constitutively active type I TGFβ receptor mutant was found to raise phospho-Smad3 levels and TGFβ-dependent gene expression in a similar way as forskolin did. Therefore, it is likely that TGFβ pathway activation by forskolin is caused by an increase in receptor expression. This is in line with the notion that expression of type I TGFβ receptor is a limiting factor in the TGFβ response. In a previous study, an increase of the TGFβ response was also found after TβRI overexpression in a dose-dependent manner (Micalizzi *et al.* 2010). The importance of TGFβ receptor expression for TGFβ response and breast cancer cell activity has been addressed by a number of studies which are summarized in Tab. 16.

In essence, it is suggested that, in early breast cancer, TβRII inhibition leads to a higher risk of tumorigenesis and progression and that TβRI stimulation prevents tumor formation and metastasis (Micalizzi *et al.* 2009, Siegel *et al.* 2003). In contrast, once breast cancer progressed, blocking TβRII activity inhibits and TβRI activation enhances bone metastasis (Tang *et al.* 2003, Yin *et al.* 1999). In high-grade breast tumor cells, inactivation of TβRII also significantly inhibited lung metastasis (Tang *et al.* 2003). These seemingly contradictory results have led to the hypothesis of TGFβ having two activities, one that is tumor-suppressive in early stages of breast cancer and another that is tumor-promoting in later stages (Imamura *et al.* 2012). Since MDA-MB-231 cells are aggressive cancer cells derived from an advanced breast cancer, TGFβ has likely a tumor-promoting effect on these cells. Hence, an upregulation of TβRI by cAMP may foster metastasis by these cells *in vivo*. This assumption is particularly based on the fact that the cAMP effect was also observed on genes, such as Cox-2, that are known to be involved in metastasis (Hiraga *et al.* 2006).

Table 16. Effect of TGFβ receptor modulation on breast cancer development and progression.

	TβRI/TβRI* upregulation	**TβRII inactivation**
mouse model (xenograft/ transgene)	▪ via Six1 overexpression in MCF-7 promotes EMT but inhibits metastasis (Micalizzi *et al.* 2009, (Micalizzi *et al.* 2010) ▪ inhibits tumorigenesis in *Her2* transgenic mice with conditional TβRI expression in breast epithelium (Siegel *et al.* 2003) ▪ induces bone metastasis formation of MDA-MB-231 via expression of PTHrP (Yin *et al.* 1999)	▪ conditional dnTβRII expression induces mammary tumorigenesis (Gorska *et al.* 2003) ▪ dnTβRII expression induces metastasis formation of MCF-7 (Micalizzi *et al.* 2010) ▪ increased risk of development of IBC from hyperplasia (Tang *et al.* 2003) ▪ dnTβRII in high-grade cell line reduced lung metastasis (Tang *et al.* 2003) ▪ dnTβRII expression inhibits bone metastasis formation of MDA-MB-231 by suppressing PTHrP expression (Yin *et al.* 1999)
patients	▪ no correlation found so far	▪ reduced TβRII expression correlates with higher tumor grade in DCIS, IBC (Tang *et al.* 2003)

TβRI*=constitutive active form of TβRI
dnTβRII=dominant negative form of TβRII (binds ligand, but is unable to transduce signal into the cell)

Only a few factors that regulate TβRI expression have been identified so far. In MCF-7 breast cancer cells, the transcription factor Six1, when overexpressed, induces TGFβ-dependent EMT and lymph node metastasis, at least partly by upregulating TβRI expression. This upregulation results in increased Smad3 phosphorylation and TGFβ-dependent 3TP promoter activity (Micalizzi *et al.* 2010). Recently, Eya2 was identified as a co-factor of Six1 that is required for the tumor-supporting functions of Six1 (Farabaugh *et al.* 2012). My data suggest an alternative mechanism that leads to upregulation of TβRI without changes in the Six1 or Eya2 expression. Here, I showed that the TβRI expression is induced by cAMP. This effect required active transcription, but did not rely on the cAMP-responsive transcription factor CREB. Whether other transcription factors that are controlled by cAMP, such as ATF-1 or ATF-2, are involved in this upregulation remains to be explored.

Previous investigations showed an increased TβRI expression after incubation of cells with a histone deacetylase (HDAC) inhibitor or in response to ectopic expression of the histone acetylase (HAT) p300 (Ammanamanchi & Brattain 2004). It has also been reported that

cAMP enhanced HAT activity (Yoo *et al.* 2012). Therefore, it is possible that cAMP enhanced TβRI transcription in MDA-MB-231 by interfering with chromatin remodelling. I could show that an HDAC inhibitor (HDACi) induced TβRI expression to a similar extent as forskolin and that forskolin and HDACi together did not have a stronger effect than each agent applied separately which suggests that the two activities are connected with each other. There is evidence for an activation of Sp1 by cAMP (Cheng *et al.* 2000) and a collaboration of Sp1 with p300 to stimulate TβRI transcription (Ammanamanchi & Brattain 2001, Ammanamanchi & Brattain 2004). Hence, an involvement of the p300/Sp1 complex is possible. Further studies are required to identify the cAMP-regulated enzyme that modulates histone acetylation such that it increases TβRI transcription. The cAMP pathway could either activate a histone acetyltransferase or inhibit a histone deacetylase. Besides p300, ATF-2 may be a candidate through which cAMP regulates TβRI transcription, since ATF-2 is a target of the cAMP pathway, has an intrinsic acetyltransferase activity and interacts with p300. (Karanam *et al.* 2007, Kawasaki *et al.* 2000, Sands & Palmer 2008). Furthermore, it remains to be tested whether Sp1 binds to the predicted Sp1 binding sequence within the TβRI promoter and whether this binding is increased after HDAC inhibition.

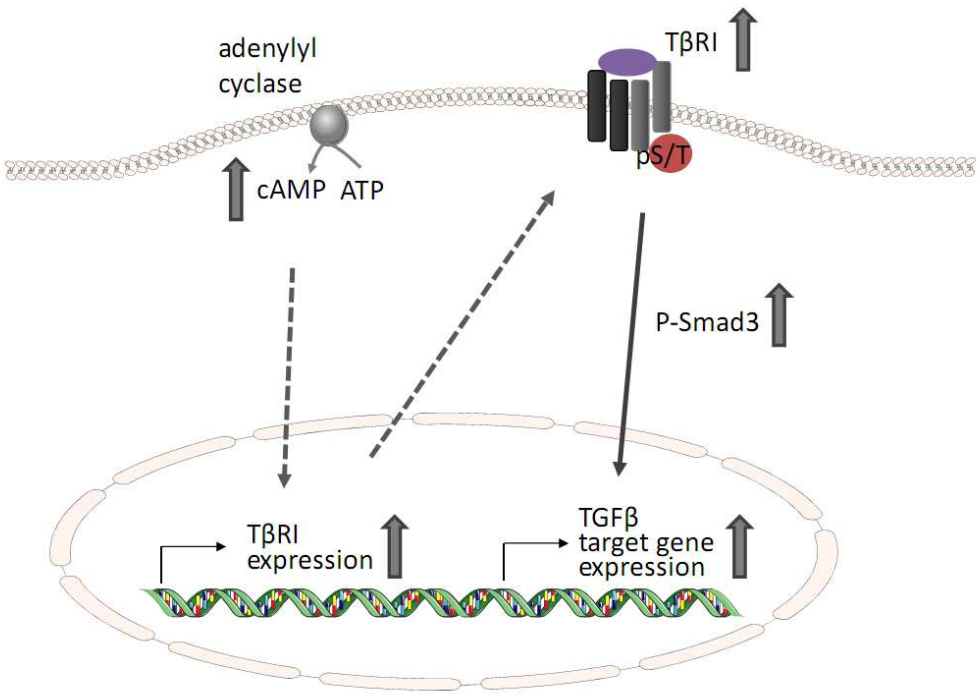

Figure 41. Proposed mechanism of cAMP-induced TGFβ signaling.
1) Elevation of the cAMP level results in an upregulation of TβRI and
2) higher TβRI expression in the plasma membrane.
3) This elevated receptor expression leads to enhanced TGFβ-dependent Smad3 phosphorylation and activation of TGFβ-responsive genes.

4. Role of Smad3 expression in TGFβ signaling in MDA-MB-231 cells

TGFβ induces the phosphorylation of regulatory Smad proteins, such as Smad3, and their subsequent translocation to the nucleus, where they regulate transcription. Here, I present evidence that the extent of Smad3 phosphorylation and hence TGFβ-dependent gene expression in MDA-MB-231 cells is also dependent on the level of total Smad3 protein. Forskolin-induced TβRI upregulation did not enhance TGFβ signaling, when Smad3 was overexpressed in 2D-cultured cells. Hence, the Smad3 protein expression level seems to be a limiting factor for the TGFβ response in 2D-cultured MDA-MB-231 cells. However, in 3D-cultured cells, high Smad3 protein expression levels coincided with high levels of cAMP and TβRI and unresponsiveness of TβRI to cAMP. Hence, it is unclear if the high Smad3 levels in 3D-cultured cells played a role in the loss of the effect of forskolin on TGFβ signaling. Interestingly, even in MDA-MB-231 cells transfected with an impaired TβRII, TGFβ-responsive transcription can be enhanced when Smad3 and Smad4 are overexpressed (Dumont *et al.* 2003). The reason for this activation in the absence of a functional TGFβ receptor complex that is required to phosphorylate Smad3 was not investigated. The reason why Smad3 protein levels in 3D-cultured cells are high, remains also unclear. Since a change in Smad3 mRNA level was not observed, mechanisms that regulate Smad3 on the protein level are likely responsible. One possibility is the involvement of microRNAs that regulate protein translation. Interestingly, miR-140 regulates Smad3 on the protein, but not on the mRNA level and might therefore be involved in the upregulation of the Smad3 level in 3D-cultured cells (Pais *et al.* 2010). It has also been reported, that Smad3 protein turnover is regulated by proteasomal degradation (Inoue *et al.* 2004). Thus, inhibition of Smad3 degradation resulting in an accumulation of Smad3 may also explain the high Smad3 levels in 3D-cultured cells.

5. Potential relevance of the cAMP/TGFβ cross-talk for tumor progression

Some of the TGFβ target genes that responded to cAMP are tumor promoter genes and/or serve as markers of tumor progression. Overexpression of the TGFβ target TIMP-1 alone has been shown to induce tumor growth of MDA-MB-231 cells in mice (Bigelow *et al.* 2009). Cox-2 and PTHrP are known enhancers of bone and lung metastasis formation, respectively (Gupta *et al.* 2007, Hiraga *et al.* 2006). Additionally, Cox-2 knockdown in MDA-MB-231 cells resulted in less tumor formation and lowered the lung metastatic potential of these cells (Stasinopoulos *et al.* 2007). PAI-1 expression correlated with stimulation of migration of MDA-MB-231 cells (Dittmer *et al.* 2006) and has long been known as a predictive and prognostic marker in breast cancer (Schmitt *et al.* 2011). Furthermore, the proteinase MMP9

investigated here plays a role in the invasion of tumor cells and the osteolysis following bone metastasis.

It is likely that the TGFβ/cAMP combined effect on tumor-supporting genes contributes to tumor progression. Forskolin was used to raise the cAMP artificially. Naturally, a rise of cAMP in tumor cells may be achieved by different mechanisms. One mechanism involves $G\alpha_s$-protein-coupled receptors. Interestingly, PTHrP and Cox-2 can induce an elevation of cAMP levels in cells. PTHrP activates the cAMP signaling pathway by activating the PTH receptor I (Juppner *et al.* 1991). Cox-2 is responsible for the production of prostaglandin E2 (PGE2) which acts on breast cancer cells via $G\alpha_s$-protein-coupled EP2/EP4, DP or IP receptors in an autocrine fashion (Timoshenko *et al.* 2003). EP2 and EP4 receptors have been shown to be expressed in MDA-MB-231 cells. Hence, high PTHrP and Cox-2 levels might contribute to an upregulation of TβRI and increased TGFβ responsiveness, e.g. in the bone microenvironment where the extracellular matrix is rich in latent TGFβ. Both factors by elevating the cellular cAMP level may further stimulate TGFβ responsiveness in bone-metastasized breast cancer cells (Fig. 42). The absence of the cAMP/TGFβ effect in 3D-cultured cells may be ascribed to the high basal cAMP level found in these cells. These may be due to hypoxia that arises within the cell aggregates (Hardelauf *et al.* 2011). Recently, a higher cAMP level and PKA phosphorylation have been detected together with an increased expression of carbonic anhydrase IX which is a marker for hypoxic conditions (Ditte *et al.* 2011). I could also observe a strong increase in carbonic anhydrase IX expression in 3D- compared to 2D-cultured MDA-MB-231 cells (data not shown). It is therefore possible that, at sites exposing non-hypoxic conditions, as found in microtumors or micrometastases, cAMP levels are low and cAMP-elevating factors in conjunction with TGFβ co-operate by the herein described mechanism. On the other hand, in hypoxic areas or niches, elevated cAMP levels may help maintaining a higher TβRI expression. Hypoxia has been shown to increase the cancer stem cell population in breast cancer cells (Conley *et al.* 2012) that exhibits a high TGFβ pathway activity (Hardt *et al.* 2012, Shipitsin *et al.* 2007). It is therefore conceivable that, under hypoxic conditions, at which an elevation of cAMP has been described, TβRI is upregulated and contributes to the elevation of the cancer stem cell pool with a highly active TGFβ pathway.

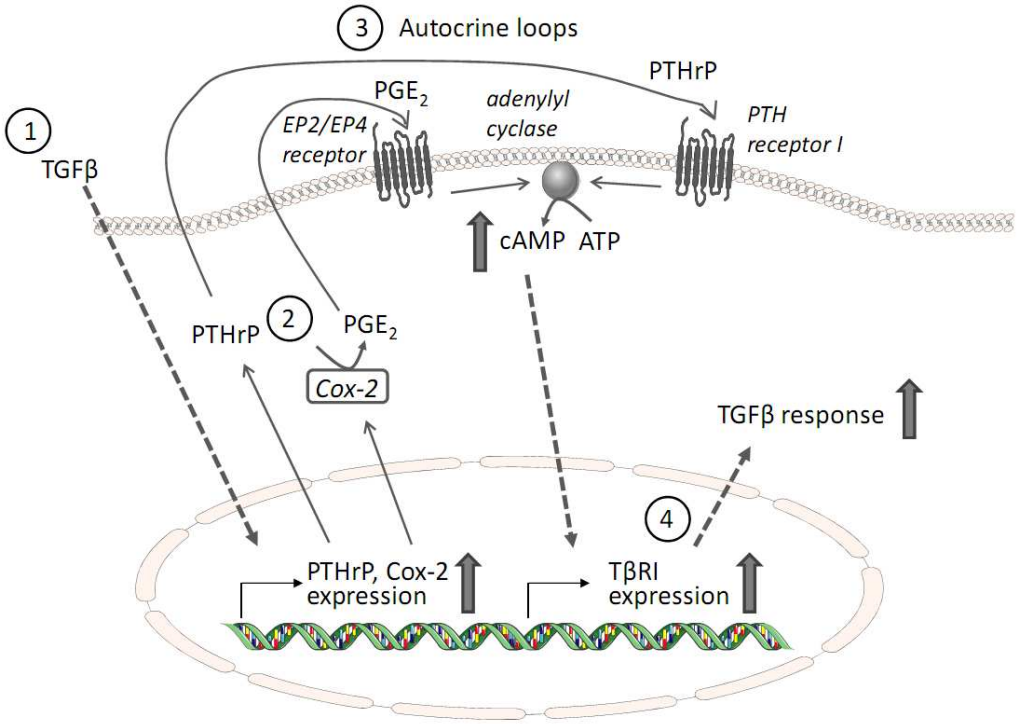

Figure 42. Proposed mechanism by which TGFβ-induced Cox-2 and PTHrP expression further stimulates TGFβ response by rising the cAMP and TβRI levels in the cell.

1) **TGFβ pathway activation leads to increased expression of Cox-2 and PTHrP.**
2) **Cox-2 generates PGE2, PGE2 and PTHrP are being secreted.**
3) **PGE2 and PTHrP activate the corresponding receptors in an autocrine fashion and thereby induce cAMP production.**
4) **cAMP stimulates TβRI expression which increases responsiveness of cells to TGFβ.**

6. Potential relevance of the cAMP/TGFβ cross-talk for the interaction between mesenchymal stem cells and breast cancer cells

Mesenchymal stem cells (MSCs) are stromal cells known to invade wounds where they are able to differentiate into cell types needed for tissue repair and to alter the inflammatory microenvironment and therefore to contribute to wound healing. Tumors resemble wounds in different aspects and are also a target for MSCs. Once MSCs have entered the tumor they interact with tumor cells in a mutual fashion and influence their behavior (Dittmer 2010, Dittmer *et al.* 2011). A number of studies performed with different cancer cells, including MDA-MB-231 cells, show that MSCs promote tumor cell migration and formation of breast cancer metastasis (Dittmer *et al.* 2011, Goldstein *et al.* 2010, Karnoub *et al.* 2007). The data of these studies suggest complex paracrine loops to be involved in these interactions. A number of mechanisms have been proposed. Here, it is shown that MSCs induce the activation of cAMP and TGFβ pathways in breast cancer cells as indicated by an increased phosphorylation of CREB and Smad3. Therefore, an interaction of these two pathways by means of a cAMP-induced TGFβ response as described here may take place.

7. Correlation of altered gene expression with phenotypic changes

Despite the fact that cAMP affected TGFβ target genes that have been linked to tumor progression, no correlation of this effect with cellular functions, including cell viability, cell adhesion and migration, could be observed. It is possible that the genes responsible for these changes are different ones than those being addressed in this study. It might also be that the observed gene expression changes play a role in tumorigenesis or the formation of metastases *in vivo* which cannot be detected with the methods as used here. E.g., TIMP-1 overexpression in MDA-MB-231 cells enhanced tumor growth in a xenograft model, but did not change tumor cell biology *in vitro*, although approximately 200 genes were found to be differentially expressed. In an additional analysis, cells of the xenograft were tested again and an even higher number of genes (600) was found to be deregulated suggesting additional alterations occuring *in vivo* (Bigelow *et al.* 2009). The colonization of organs by metastasizing cancer cells is a process that is not well understood. Metastasis is a multi-step process where intravasation, extravasation and the microenvironment in the recipient organ are of great importance. These factors were not examined in the *in vitro* tests as performed in this study. It might well be that the importance of the cAMP/TGFβ effect on tumor biology can only be observed by *in vivo* experiments. Therefore, further studies have to be conducted in order to analyze the functional consequences of the described cAMP/TGFβ effect.

It is also possible that cAMP and TGFβ regulate pathways leading to cytoskeletal rearrangements and migration in a way that is independent of the activities of the TGFβ/cAMP target genes studied here. Consistent with this notion, a study conducted by Dumont *et al.* showed that, in a MDA-MB-231 subline that harbors an impaired TGFβ receptor II, ectopic Smad3 and Smad4 expression restored TGFβ-dependent transcriptional activation but not TGFβ-dependent motility. Along these lines, TGFβ was shown to regulate migration of MCF-7 and MDA-MB-468 cells independently on TβRI (Imamichi *et al.* 2005).

This findings may indicate the presence of independent Smad and non-Smad pathways and may suggest that the migratory potential results from non-Smad TGFβ pathway activities.

One assay showed an decrease in cell proliferation by TGFβ which was amplified by forskolin. This result points to the possibility that the proliferation is directly dependent on the expression of the cell cycle inhibitor p21 that is upregulated by TGFβ and even further by forskolin and TGFβ. Further investigations are neccessary to confirm this idea.

The comparison of cells in 2D and 3D cultures with respect to forskolin and TGFβ responses revealed that TβRI upregulation and enhanced TGFβ signaling in the presence of forskolin as found in 2D-cultured cells does not occur in 3D-cultured cells. Nevertheless, TGFβ has an effect on the 3D-cultured cells as indicated by the spheroid formation of MDA-MB-231 cells

that only occurs under the influence of TGFβ. This was unexpected, since MDA-MB-231 cells rather form irregular aggregates or stellate structures in suspension or reconstituted basement membrane-based cultures, respectively (Dittmer *et al.* 2009, Wang *et al.* 2002). Working with 2D cultures alone would not have revealed this finding, but it might provide further insights into the activities of TGFβ. In former studies with murine breast cancer cells, a 3D cell culture model was used to distinguish between a non-malignant (S-1 cells) and a malignant phenotype (T4-2 cells) based on their "non-polarized" or "polarized" structure in reconstituted basement membranes. The authors state that the presence of E-cadherin and the lack of integrin ß1 on the cell surface characterize the non-malignant type and show evidence for a phenotypic reversion of the malignant to the non-malignant type by inhibiting integrin ß1 function (Weaver *et al.* 1997). The formation of regularly-shaped MDA-MB-231 spheroids in 96-well plates might be considered as a phenotypic reversion as induced by TGFβ under certain conditions. Wang *et al.* succeeded in reverting MDA-MB-231 cells by incubation with both, integrin ß1 and PI3K inhibitors. Alternatively, re-expression of E-cadherin in combination with one of these inhibitors generated the same phenotype (Wang *et al.* 2002). Whether the treatment of MDA-MB-231 cells with TGFβ interferes with the E-cadherin or integrin ß1 status or the state of PI3K activation needs to be determined. Similarities between TGFβ- and integrin-mediated responses during mammary tumor progression have been described before (Parvani *et al.* 2011). The studies assessing the molecular mechanism that leads to a phenotypic reversion did not include assays that would test changes in the functional characteristics of whole spheroids. In a recent study, TGFβ-treated mammary epithelial cells have been reported to form compact, dense spheroids in reconstituted basement membrane-based cultures (Wendt *et al.* 2010). These spheroids have been designated as invasospheres due to their ability to invade synthetic basement membranes as single units and to form metastases *in vivo*. TGFβ-induced EMT and elevated EGFR expression were linked to these functions. Since the cells described in this model have undergone EMT like MDA-MB-231 cells it is possible that also MDA-MB-231 cells are more invasive in the spheroid state. Thus, it still remains unclear whether the TGFβ-induced spheroid formation more resembles a reverted or an advanced state of the tumor cells.

8. Conclusions

The presented study revealed a novel mechanism by which cAMP modulates TGFβ signaling. By upregulation of type I TGFβ receptor cAMP enhances the TGFβ response of a group of important oncogenes via the Smad3-dependent pathway. Since upregulation of the type I TGFβ receptor as well as TGFβ target genes in advanced breast cancer cells has been associated with metastatic properties, this mechanism most likely contributes to tumor progression. Interestingly, in 3D-cultured cells, where the basal cAMP and type I TGFβ receptor levels were much higher than in 2D-cultured cells, TGFβ alone showed maximum activation of its target genes and cAMP had no effect. This suggests that, under *in vivo*-like conditions, basal cAMP levels are sufficient to support maximum TGFβ response. This study also reveals that, in a 3D culturing system, TGFβ induces morphological changes independently of the cAMP/TGFβ effect on gene expression. Under certain conditions, TGFβ could be shown to induce spheroid formation of MDA-MB-231 cells, which is a rare event. This phenotype resembled invasospheres and might hint to a more invasive behavior under the influence of TGFβ. Taken together, this study demonstrates that cAMP is able to enhance TGFβ signaling and that modulation of the TGFβ pathway activity is highly relevant in advanced breast cancer cells, such as MDA-MB-231 cells, that have already partially undergone EMT.

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SUMMARY

Aberrant activation of the cAMP/CREB and TGFβ/Smad signaling is involved in breast cancer progression. Little is known about the cross-talk between these two signaling pathways. I investigated the interplay of these pathways in breast cancer cells grown in 2-dimensional (2D) as well as in 3D cultures. In the presence of either cAMP or TGFβ, mRNA expression levels of many cancer-related genes were significantly altered. Simultaneous activation of both pathways affected genes differently. In 2D cultures, key genes of cancer progression (PAI-1, TIMP-1, Cox-2, PTHrP, TGFα, MMP10) displayed increased TGFβ responsiveness in the presence of forskolin, which was accompanied by an increase in TGFβ-dependent Smad3 phosphorylation. Furthermore, I found that forskolin induced a significant upregulation of TGFβ receptor I (TßRI) expression. This upregulation could be inhibited by actinomycin D, but not by a CREB-specific siRNA suggesting that forskolin increases TßRI transcription in a CREB-independent manner. Likewise, siRNA-mediated knockdown of the transcription factor Six1, which was shown to be involved in TßRI transcriptional regulation, was unable to inhibit the forskolin-induced TßRI upregulation. Promoter assays with a TßRI construct containing a TßRI promoter fragment from position -392 to +21 revealed a moderate, but signigficant increase in promoter activity in the presence of forskolin. Inhibition of histone deacetylase activity resulted also in an increase in TßRI mRNA, which could not be further stimulated by forskolin. This result suggests that histone modification is, at least partially, involved in forskolin-induced TßRI transcription. Ectopic expression of a constitutively active form of TßRI mimicked the response to forskolin. In contrast to 2D-cultured cells, 3D-cultured cells showed no forskolin response in the presence of TGFβ. Nor did forskolin stimulate TGFβ-dependent Smad3 phosphorylation or upregulated TßRI expression under these culture conditions. Interestingly, 3D-cultured cells exhibited *per se* higher levels of Smad3 and TßRI as well as of cAMP. The increased levels of these three factors under 3D growing conditions may have prevented the forskolin effect on TGFβ signaling as was observed in 2D cultures. Apart from alterations in gene expression, morphological and functional changes (cell viability, cell-cell-adhesion, migration) were induced by forskolin and TGFβ, which did not correlate with the observed gene expression changes. Surprisingly, under certain conditions, TGFβ was found to induce spheroid formation of 3D-cultured MDA-MB-231 cells that is rarely found with these cells. These spheroids resembled invasospheres and might hint to a more invasive behavior under the influence of TGFβ. Taken together, this study suggests that, under certain conditions, cAMP can interfere with TGFβ signaling in breast cancer cells by upregulating the expression of TßRI and that cAMP and TGFβ induce morphological and functional alterations independently of these mechanism.

ERKLÄRUNG

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst und keine anderen als die von mir angegebenen und zulässigen Quellen und Hilfsmittel benutzt habe.

Textstellen, die von mir wörtlich oder inhaltlich aus anderen Werken entnommen wurden, sind als solche erkenntlich. Die verwendeten Quellen sind im Abschnitt Referenzen zu finden.

Die Hersteller von Materialen (Plasmide etc.), die ich nicht selbst hergestellt habe, sind an den entsprechenden Stellen angegeben.

Ich versichere, dass ich mich zuvor noch nicht um einen Doktorgrad beworben habe. Diese Arbeit wurde weder in der vorliegenden noch einer anderen Fassung bereits an einer anderen Fakultät oder Hochschule zur Promotion eingereicht.

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