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DIPLOMARBEIT

Titel der Diplomarbeit

The Role of Activins and Their Antagonists in Human Melanoma Cell Lines

angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag. rer.nat.)

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Wien, im	März, 2011

MEDIZINISCHE UNIVERSITÄT WIEN

Herewith, I declare that this diploma thesis was performed by me at the Medical University of Vienna, Department of Medicine-I, Institute of Cancer Research and that I did not utilize any other sources than those specified in the references.

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Acknowledgement

I would like to thank all those who participated in making this piece of work a reality. Special thanks go to my supervisor Dr. Michael Grusch, who has supported, helped and encouraged me during my diploma thesis.

I would like to thank my family for supporting me throughout all my studies at University and I am equally thankful to my darling who actively motivated me at all levels.

Abstract

BACKGROUND: Activins are secreted dimeric polypeptides belonging to the TGF-β family of growth and differentiation factors. They have been shown to regulate cell growth, differentiation, migration and angiogenesis in multiple tissues and organs. Activins interact with various binding proteins which include antagonists in the extracellular space (follistatin and FLRG) and antagonistic co-receptors at the cell membrane (cripto). Deregulation of activin signals has been found in several tumor types and linked to the malignant growth of tumor cells.

OBJECTIVES: The purpose of this study was to (1.) analyze the expression of activins and activin antagonists in melanoma cell lines, (2.) functionally characterize melanoma cell lines overexpressing follistatin and (3) investigate potential signaling mechanisms of activin beta E.

METHODS: The expression levels of all four human activin subunits and antagonistic factors were investigated in a panel of human melanoma cell lines by quantitative RT-PCR. Three melanoma cell lines were transfected with follistatin and clones stably overexpressing follistatin (confirmed using enzyme-linked immunosorbent assay, ELISA) were analyzed with in vitro migration and proliferation assays. The effect of follistatin-mediated inhibition of activin activity on tumour growth was also investigated by xenotransplantation experiments in SCID mice. The impact of activin E, activin A and follistatin on smad signaling was assessed by immunoblotting.

RESULTS: Elevated expression levels of activin beta C (83%) were found in malignant melanoma cell lines compared to primary melanocytes. The expression of activin beta B (75%) and activin beta E (92%) in contrast, was downregulated, whereas activin beta A showed up- or down-regulation depending on the cell line. Expression levels of follistatin (75%) and cripto (100%) showed upregulation. The other follistatin related proteins, FLRG and follistatin-like-1 were up- or down-regulated, depending on the cell line. Only one of the two isoforms of follistatin (Fst-

315), encoding a 27 amino acid extension at the C-terminus, was found in nearly all melanoma cells, including primary melanocytes.

Supernatants of follistatin overexpressing VM21 and VM1 cells contained more than 10-fold elevated levels of follistatin and were also confirmed to inhibit smad phosphorylation induced by treatment with recombinant activin A. In vitro characterization of follistatin overexpressing cells revealed a tendency towards reduced proliferation but partly increased clonogenicity. Migration of melanoma cells was reduced ranging from 20% to 80% by overexpression of follistatin. Follistatin overexpressing VM21 cells were also tested by a xenotransplantation experiment in SCID mice, but did not show a difference in tumor growth when compared to mock controls.

Experiments with conditioned media showed that activin E, in contrast to activin A, does not lead to phosphorylation of smad2. Also no evidence was found that expression of activin beta E together with activin beta A could inhibit or enhance activin A-induced signals either by heterodimerization or by competition for activin receptors or follistatin.

Zusammenfassung

EINLEITUNG: Activine sind sezernierte dimere Polypeptide, die zur TGF-β Familie der Wachstums- und Differenzierungsfaktoren gehören. Activine regulieren Zellwachstum, Differenzierung, Migration und Angiogenese in verschiedenen Geweben und Organen. Die Funktion von Activinen wird seinerseits durch extrazelluläre Antagonisten (Follistatin und FLRG) und antagonistische Co-Rezeptoren an der Zellmembran (Cripto) limitiert. Deregulierung von Activin Signalen wurde in mehreren Tumorarten gefunden und in direkten Zusammenhang mit dem malignen Wachstum von Tumorzellen gebracht.

ZIELE: Das Ziel dieser Studie war es, (1) die Expression von Aktivinen und Aktivinantagonisten in Melanomzellen zu untersuchen, (2.) den Einfluss von Follistatinüberexpression auf Melanomzellen zu testen und (3.) mögliche Signaltransdutionsmechanismen der Aktivin Beta E Untereinheit zu analysieren.

METHODEN: Die Expression aller vier Aktivin Untereinheiten und antagonistischer Faktoren wurden in einer Reihe von humanen Melanomzellen durch quantitative RT-PCR untersucht. 3 Melanomzelllinien wurden stabil mit Follistatin transfiziert und die Überexpression wurde mittels ELISA bestätigt. Diese Zellen wurden dann in-vitro-in Migrations- und Proliferations-Assays getestet. Für Follistatin überexprimierende VM21 Zellen wurden auch Xenotransplantationsexperimente in SCID-Mäusen durchgeführt. Die Auswirkungen von Activin E, Activin A und Follistatin auf den Smad Signalweg wurde mittels Western-Blot Analyse untersucht.

ERGEBNISSE: Das Expressionsniveau von Activin C war in Melanomzelllinien erhöht (83%) im Vergleich zu primären Melanozyten. Die Expressionniveaus von Activin beta B (75%) und Activin beta E (92%) dagegen reduziert, während Activin beta A unterschiedliche Regulation in Abhängigkeit von der Zelllinie zeigte. Expression von Follistatin (75%) und Cripto (100%) war erhöht. Die Expression der anderen dem Follistatin

verwandten Proteinen, FLRG und Follistatin-like-1 war je nach Zelllinie erhöht oder reduziert. Nur eine der beiden Isoformen von Follistatin (Fst-315) wurde in nahezu allen Melanomzellen und in primäre Melanozyten gefunden.

Überstände von stabil transfizierten Zellen enthielten mehr als 10-fach soviel Follistatin wie die Kontrollzellen. Überstände Follistatin exprimierender Zellen waren auch in der Lage Smad-Phosphorylierung durch zugegebenes Aktivin A zu neutralisieren. In-vitro, zeigten die Follistatin überexprimierenden Zellen eine Tendenz zu verminderter Proliferations, aber teils erhöhter Klonogenizität. Die Migrationsfähigkeit der Melanomzellen wurde um 20%-80% reduziert, wenn Follistatin überexprimiert wurde. Die Rolle der Follistatin vermittelten Hemmung des Activins wurde auch durch Xenotransplantation Experimente von VM21 Zellen in SCID-Mäusen getestet. Allerdings war hier kein Unterschied im Tumorwachstum verglichen mit GFP-Kontrollen zu erkennen. Bezüglich möglicher Signalwege von Activin E wurde nachgewiesen, dass durch Aktivin E konditionierte Medien im Gegensatz zu Aktivin A konditionierte Medien keine Smad2 Phosphorylierung aktiviert wird. Auswirkungen der Konditionierung von Medien mit unterschiedlichen Kombination von Activin E, Aktivin A und Follistatin liessen auch weder eine Blockierung noch eine Verstärkung von Aktivin A induzierten Signalen durch Aktivin beta E - wie etwa durch Ausbildung von AE Heterodimeren oder Konkurrenz um Aktivinrezeptoren oder Follistatinbindung - erkennen.

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1. Introduction

1.1. Cellular and Molecular Mechanisms of Melanoma Development and Progression

1.1.1 Melanocyte Homeostasis and Melanomagenesis

Melanoma develops from malignant transformation of melanocytes, which are pigmented, differentiated cells localized at different places of the human body, found in the bottom layer of human epidermis (Uong and Zon), the inner ear (Markert and Silvers 1956), the middle layer of the eye (Barden and Levine 1983), meninges (Mintz 1971), heart (Theriault and Hurley 1970), and bones (Nichols and Reams 1960). Melanocytes are responsible for the production of melanin pigments and transporting to keratinocytes (Figure 1.1). Melanin is synthesized inside of melanosomes. Melanocytes develop from melanoblasts, which are derived from migratory neural crest cells. These cells are induced in gastrulation, in the area between the neural and non-neural ectoderm (Hirobe 1994; Erickson and Reedy 1998).

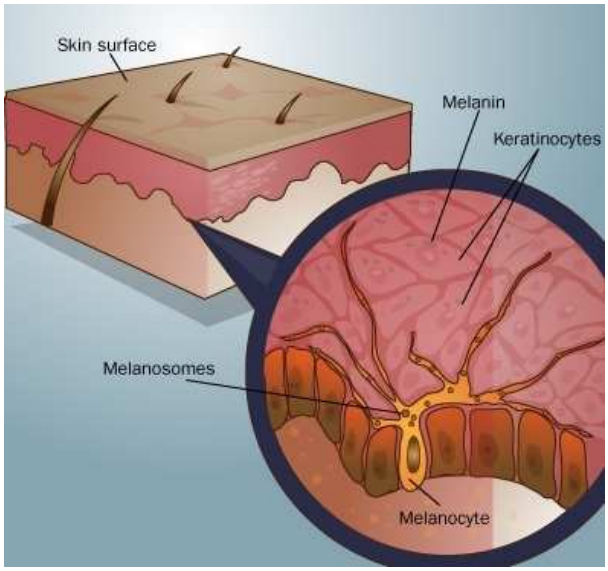

Figure 1. 1 Schematic model of skin cells. The melanocytes are located in the basal layer, produce and secrete melanin to surrounding keratinocytes. (http:// health. howstuffworks. com/ skincare/ problems/ medical/ albinism1.htm. accessed on 2010-12-07)

There is a relationship between melanocytes and keratinocytes, in which one melanocyte transports pigment containing melanosomes through its dendrites to about 36 associated keratinocytes (Fitzpatrick and Breathnach 1963; Jimbow, Quevedo et al. 1976). In this case, melanocytes are located in the basal layer of the epidermis, where they maintain a life-long stable ratio of 1:5 with basal keratinocytes (Haass and Herlyn 2005). Regulated induction of melanocyte division keeps this balance, and this ratio is only disturbed during transformation into a melanoma or a nevus. The expansion of the total skin surface during childhood requires melanocyte proliferation to maintain a stable ratio with the basal keratinocytes. In order to proliferate, melanocytes need to decouple from the keratinocytes and from the basement membrane, retract their dendrites, divide, and migrate along the basal layer of the membrane, before they finally recouple to the matrix and to keratinocytes to form another epidermal melanin component (Figure 1.2.) (Haass and Herlyn 2005). Cell division for melanocytes probably begins with the upregulation of growth factors which are produced by fibroblasts or by keratinocytes that leads to decoupling of melanocytes from keratinocytes and downregulation of cadherins. Mitogenesis is driven and regulated by other growth factors from epidermis or dermis.

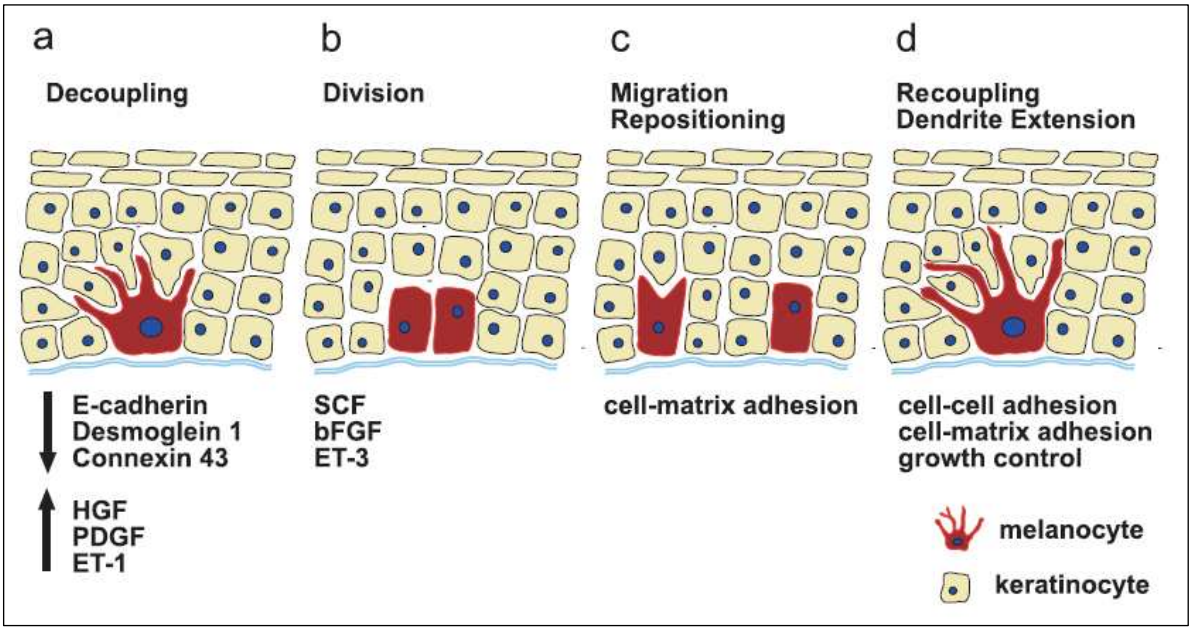

Figure 1. 2 Steps of melanocyte proliferation in normal human skin and associated molecular events. For proliferation, melanocytes need to decouple from the basal layer of membrane and from the keratinocytes, retract their dendrites (Figure 1.3a), divide (Figure 1.3b), and migrate along the basal membrane (Figure 1.3c) before they finally recouple to keratinocytes and to the matrix to form another epidermal melanin unit (Figure 1.3d) (Haass and Herlyn 2005).

Homeostasis physiologically determines whether a cell proliferates, differentiates, remains quiescent or undergoes apoptosis (Bissell and Radisky 2001). Imbalance of homeostasis may disturb the regulation of the epidermal melanin unit and stimulate continuous proliferation of the melanocytes, which may cause the development of melanoma. Hallmarks for melanoma cells include independence from exogenous growth factors through the production of autocrine growth factors or the constitutive activation of growth factor-related signaling cascades, unlimited life span, escape from control by keratinocytes, unresponsiveness to inhibitory growth factors such as transforming growth factor beta (TGF-β), invasion into the dermis, recruitment of fibroblasts for matrix and growth factor production, and recruitment of a neovasculature (Hanahan and Weinberg 2000). It is likely that melanoma cells escape from control by keratinocytes through the following mechanisms: (1) downregulation of

receptors important for communication with keratinocytes, (2) upregulation of receptors and signaling molecules important for melanoma cell–melanoma cell and melanoma cell–fibroblast interactions, (3) deregulation of morphogens that directed them during development to the epidermis and helped maintain their proper position, (4) loss of anchorage to the basement membrane because of an altered expression of cell–matrix adhesion molecules, and (5) increased production of metalloproteinases (Haass and Herlyn 2005).

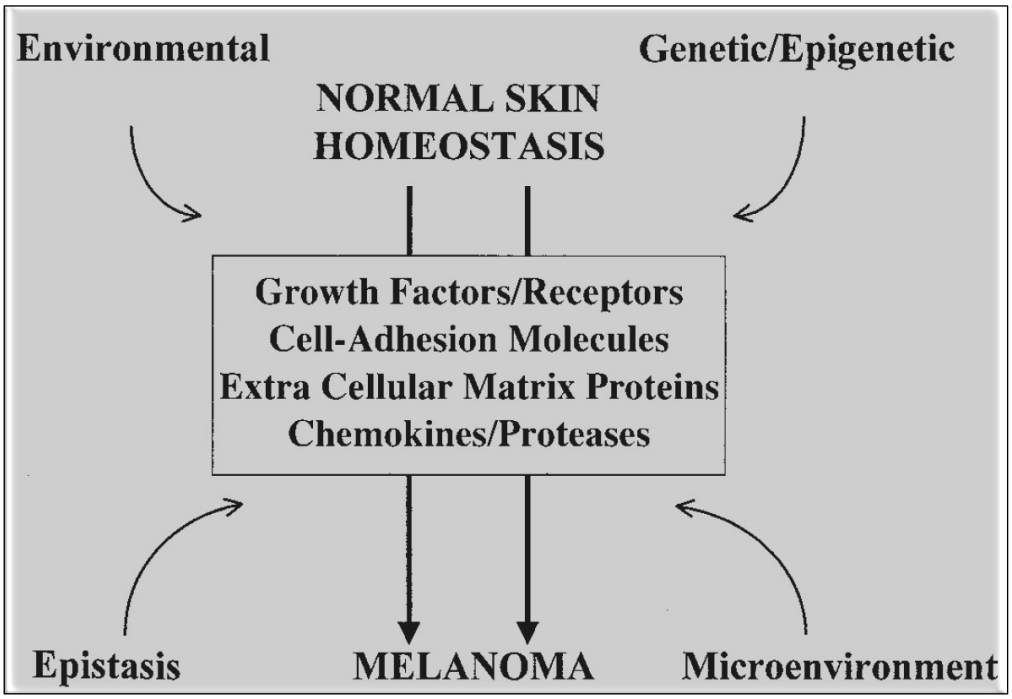

Figure 1. 3 The effective factors during melanoma progression. At the cellular level, environmental, genetic and epigenetic alterations play a key role in epistatic changes. The model suggests that alterations in the expression of some specific proteins give rise to homeostatic imbalance in the skin and support melanoma progression. (Satyamoorthy and Herlyn 2002)

Aberration between the normal melanocyte genome and genomes of melanoma cells has been clearly shown by genome analysis; this alteration can be categorized as environmental (UV radiation, sunburn), genetic (mutations, deletions, amplifications and translocations) and epigenetic (promoter hypermethylation), leading to epistatic changes (Figure 1.3). Experimental and epidemiological studies have indicated that strong exposures to UV radiation in early childhood may lead to melanoma in adults, but genetic and molecular studies have demonstrated additional autosomal abnormalities (Satyamoorthy and Herlyn 2002; Meyle and Guldberg 2009).

It has become clear that several components in the skin influence homeostatic balance and tumour development. These include dermal fibroblast, epidermal keratinocytes, inflammatory and endothelial cells. The balance between keratinoctyes and these components keeps melanocytes from consistent proliferation.

1.1.2. Cell–Cell Communication in Melanoma

Expression of a number of cell surface molecules is acquired by melanocytes during progression to melanoma. These molecules allow melanocytes to survive, grow, adhere and migrate and they block some genes like E-cadherin, which is known as invasion suppressor and is expressed in the normal skin (Herlyn, Berking et al. 2000). Cadherins which are a family of cell surface glycoproteins play a key role in promoting Calcium-dependent cell adhesion. Besides, they promote intracellular communication and are a supplementary part of cell-cell adherens junctions (Steinberg and McNutt 1999; Vleminckx and Kemler 1999). E-cadherin, expressed by keratinocytes and melanocytes, helps homophilic interaction and prevents melanocytes from aggressive cell division. On the other hand, expression of E-cadherin is progressively lost

during melanoma development, becomes heterogonous and gets distributed in the cytoplasm of the nevus cells, and is even predominantly absent in melanoma (Li and Herlyn 2000). It has been demonstrated that overexpression of E-cadherin in melanoma cells as well as co-culture with keratinocytes induces cell death and prevents growth, as well as invasion into the dermis (Hsu, Meier et al. 2000). In case of the loss of E-cadherin in melanoma cells, N-cadherin is expressed and this switching of the cadherin activity leads to tumor cell invasion, tumor-host cell interaction, migration as well as altered gene expression (Furukawa, Fujii et al. 1997) (Figure 1.3). N-cadherin is able to interact with fibroblast growth factor receptor (FGFR) to promote neurite formation and growth (Utton, Eickholt et al. 2001). The FGFR ligand FGF2 plays a significant role in promoting autocrine and paracrine growth effects in melanoma cells and inhibition of fibroblast growth factor receptor-1 (Becker, Lee et al. 1992; Wang and Becker 1997) leads to inhibition of tumor growth and cell death. In addition, there are also some other molecules, which can be required for the homeostatic growth network. Alterations in melanoma include inhibition of supressor proteins such as Kiss1, NM23, and melastatin (Betke, Korabiowska et al. 1998; Deeds, Cronin et al. 2000), activation of matrix metalloproteinases and expression of cell-adhesion melocules such as αvß3 integrin, which increases with melanoma development from the radial to the vertical growth stage in primary melanoma and further to metatstasis (Hsu, Shih et al. 1998).

As described above, melanoma cells express high levels of N-cadherin but have lost E-cadherin expression (Figure 1.4) (Haass and Herlyn 2005). The switching of cadherin profiles during melanoma progression enables melanoma cells to interact with other N-cadherin expressing cells such as fibroblasts, leading to tumor stroma adhesion, tumor cell migration and invasion as well as altered gene expression. It has been demonstrated that in melanma cells downregulation of E-cadherin is associated with gap junction incompatibility with keratinocytes (Hsu, Andl et al. 2000).

However changes in expression of gap junction molecules such as connexin 43 (Cx43) may result in a reduction or the loss of GJIC, which plays a cooperative role in melanoma progression (Haass, Smalley et al. 2004) and is able to regulate the growth, differentiation and migration of keratinocyte (Pitts, Finbow et al. 1988; Brissette, Kumar et al. 1994; Goliger and Paul 1995). It has been clearly demonstrated that all areas of melanomas are deficient in expression of gap junction molecules Cx26,Cx30 and Cx43. Suprisingly, Cx26 and Cx30 are induced in the epidermis of melanomas but they are not found in the epidermis of melanocytic nevi (Haass, Wladykowski et al. 2006).

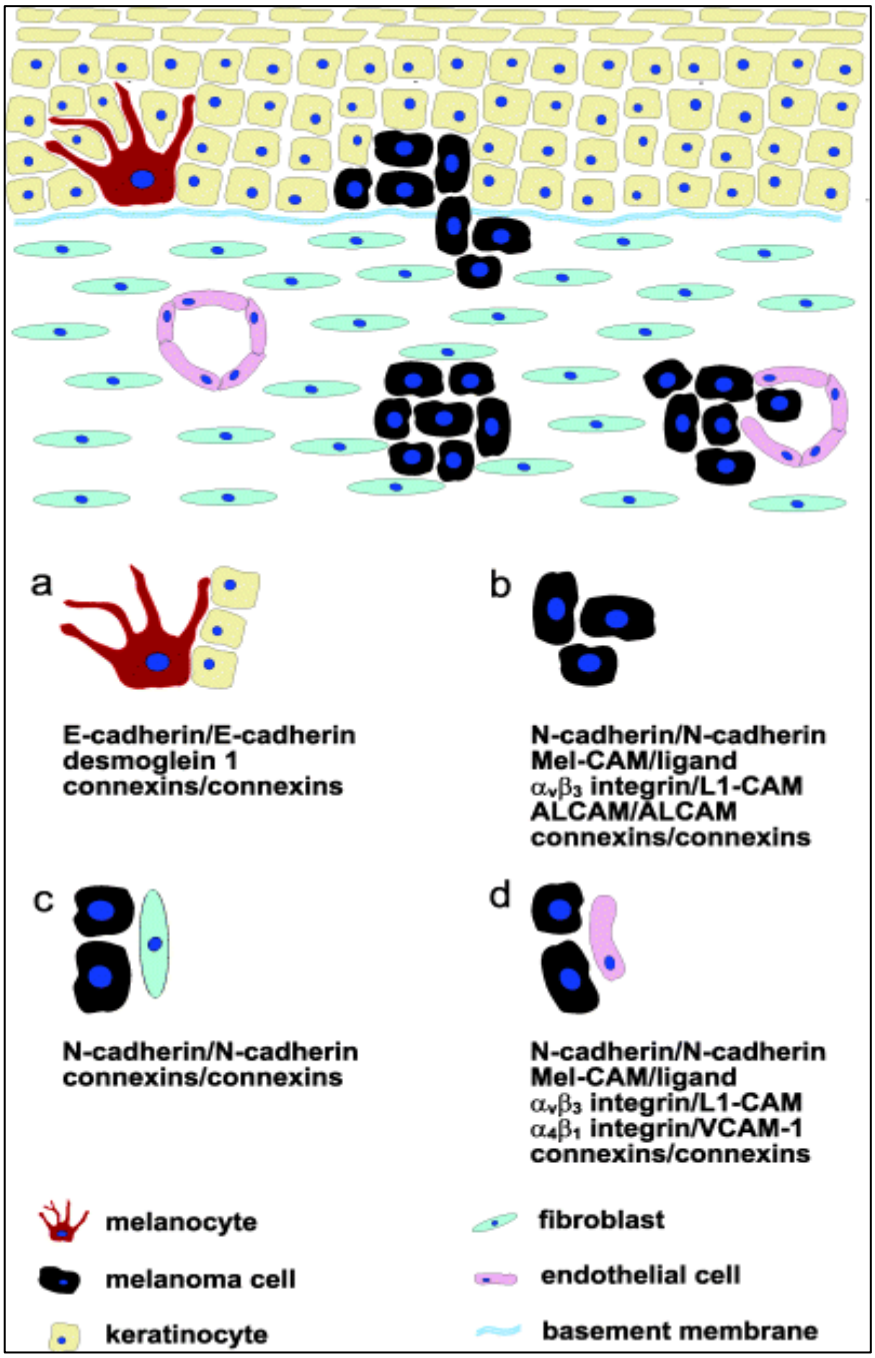

Figure 1. 4 Cell–cell communication in melanoma. E-cadherin, connexins and desmoglein 1 mediated interaction of normal epidermal melanocytes with keratinocytes (a). The cadherin profile can be changed from E- to N-cadherin during melanoma development, freeing the cells from epidermal keratinocytes as well as presenting new adhesive and communication characteristics. Besides melanoma cells have interaction with each other (b) with fibroblasts through N-cadherin, αvβ3 integrin/L1-CAM, Mel-CAM/Mel-CAM ligand, ALCAM and connexins, (c) with endothelial cells through connexins and N-cadherin, and (d) through N-cadherin, αvβ3 integrin/L1-CAM, Mel-CAM/Mel-CAM ligand, α4β1 integrin/VCAM-1 and connexins (Haass and Herlyn 2005).

1.1.3. Genetic Aberrations and Molecular Pathways in Melanoma Progression

Despite strong exposure of melanocytes to environmental risk factors such as UV radiation and chemicals in the skin, melanomas do not only signify UV mediated mutations or aberrations. Mutations in the tumor suppressor gene p53 have been observed in less than 5% of melanoma patients. On the other hand, mutations or deletions in the tumor suppressor $p16^{INK4A}$ have been found in more than 25%, whereas the mutations in the RAS oncogene have not exceeded more than 21% (Satyamoorthy and Herlyn 2002). Besides, about 20-40% germ line mutations or deletions in chromosome 9p21 have been observed. This chromosome region includes cell-cycle inhibitors and tumor suppressors such as $p15^{INK4B}$, $p16^{INK4A}$ and a differently coded locus of $p16^{INK4A}$ – p14/ARF (Sherr and Weber 2000).

Although the majority of the mutations have been found in the p16INK4A locus, nearly 40% affect both p16INK4A and p14/ARF (Rizos, Puig et al. 2001). The investigation for mutations or deletions in other genes such as PTEN, β-catenin, CDK4 did not yield significant occurrences of mutations in melanoma. Interestingly, despite its wild type status, p53 was shown to be functionally inactive in melanoma cells. (Satyamoorthy and Herlyn 2002), (Satyamoorthy, Chehab et al. 2000). This indicates that activity of p53 is blocked either by post-transcriptional mechanism or other factors. It has been demonstrated that, melanoma cells overexpress the MDM2 protein, which is implicated in inactivating p53 by targeting it for degradation (Rocco and Sidransky 2001). In addition, p53-dependent and independent apoptotic mechanisms can be inhibited by expression of the anti-apoptotic Bcl-2 protein (Hsu and Hsueh 2000).

In melanoma cells also Apaf-1 (apoptotic protease activating factor-1) which initiates the protease cascade and activates caspase-9 has been shown to be inactivated through several ways, for example DNA

methylation (Soengas, Capodieci et al. 2001). Activation of caspase-9 is initiated by Apaf-1/cytochrome c and inactivated by serine/threonine kinase Akt/PKB signaling (Soengas, Alarcon et al. 1999; Zhou, Li et al. 2000). Besides, bcl-2 is anti-apoptotic but is not absolutely cytoprotective against cell death by Apaf-1-caspase-9 signaling (Newmeyer, Bossy-Wetzel et al. 2000). Akt-mediated caspase-9 phosphorylation leads to its reduced protease activity (Fujita, Jinbo et al. 1999). It has been shown that several substrates of Akt/PKB play a role in cellular metabolism, cell survival and anti-apoptotic pathways.

The MAPK pathway can be activated in melanoma cells in two ways, (1) production of autocrine growth factors HGF and FGF (2) by BRAF gene mutation (Brose, Volpe et al. 2002). Inhibition of expression of autocrine HGF and FGF lead to downregulation of activation of ERK. This suggests that activation of members of the MAPK pathway is due to growth factors and genetic aberrations in distinct genes. Thus, there are multiple pathways involved in melanoma development. As presented in figures 1.5 and 1.6, activation of STAT3 or of the PI3-kinase/Akt pathway, as well as NF-kB signaling indicates the involvement of a broad spectrum of endogenous or exogenous factors.

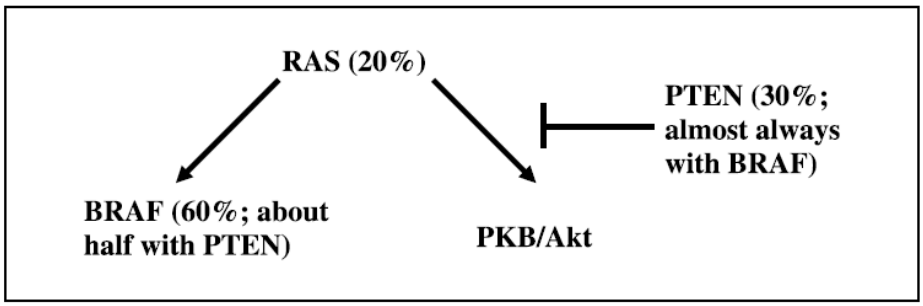

Figure 1. 5 Relationship between mutations in Ras, BRAF and PTEN. Percentages of mutation in melanoma are indicated in parentheses. Flat line is for inhibition, arrow for activation (Haluska, Tsao et al. 2006).

BRAF and RAS are important molecules belonging to the MAPK signaling pathway. There are three highly conserved RAF gens: ARAF, BRAF and CRAF (Raf-1) (Emuss, Garnett et al. 2005; Zebisch, Staber et al. 2006) ARAF and Raf-1 mutations are rare in human malignant cells, although they are also capable to activate the MAPK pathway (Beeram, Patnaik et al. 2005; Palmieri, Capone et al. 2009). Most mutations in the BRAF gene lead to a sigle substitution of valine with glutamic acid at amino acid position 600 (V600E) (Davies, Bignell et al. 2002). This mutation activates BRAF and leads to activation of the MEK-ERK signaling pathway in cells (Wan, Garnett et al. 2004). The activation of BRAF induces stimulation of downstream expression of the microphthalmia-accociated transcription factor (MITF) gen, which has been shown to play a role as the master regulator of melanocytes (Carreira, Goodall et al. 2005). It has been reported that there is a relationship between mutations in RAS, BRAF and PTEN. Concurrent BRAF-PTEN mutations function like NRAs mutation (Haluska, Tsao et al. 2006). NRAS mutations have been found in 10-20% of melanomas, loss of PTEN have been observed in 10-30% and c-KIT mutations seen in 23% of acral melanomas (Figure 1.5). The activating BRAF mutations have been observed in 60-80% of cutaneous melanoma tumors (Algazi, Soon et al. 2010).

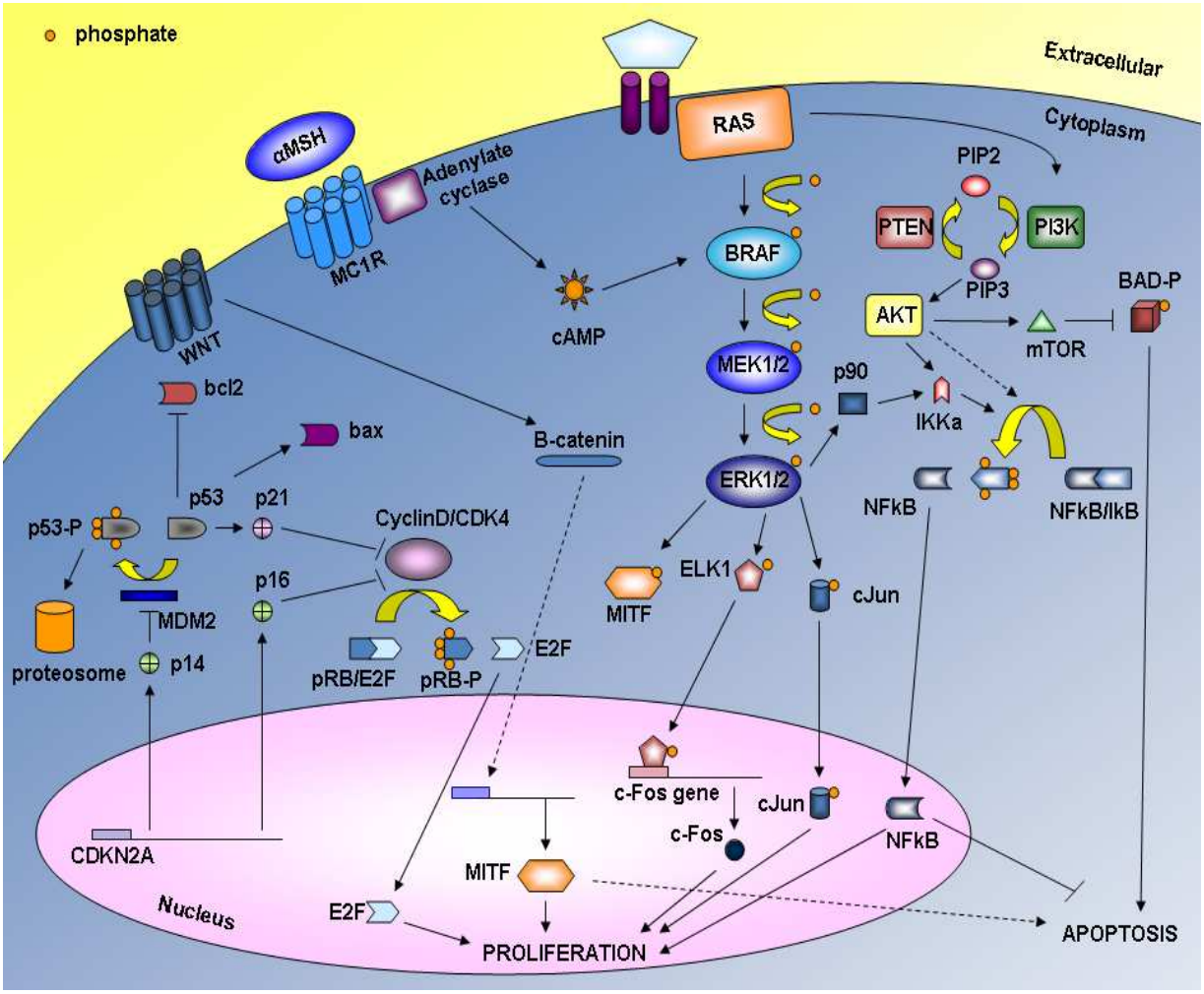

Figure 1. 6 Pathways involved in melanoma. Pathway associated with N-RAS, BRAF, and mitogen-activated protein kinase (MAPK) as well as with CDKN2A and MITF are schematically represented. Arrows, activating signals, interrupted lines, inhibiting signals. BAD, BCL-2 antagonist of cell death; cAMP, cyclic AMP; CDK4, Cyclin-dependent kinase 4; CDKN2A, Cyclindependent kinase inhibitor of kinase 2A; ERK1/2, Extracellular-related kinase 1 or 2; IkB, inhibitor of kB protein; IKK, inhibitorof- kB-protein kinase; MC1R, melanocortin-1-receptor; MITF, Microphthalmia-Associated Transcription Factor; MEK1/2, Mitogen-activated protein kinase-extracellular related kinase 1/2; PI3K, Phosphatidylinositol 3 kinase; PIP2, Phosphatidylinositol bisphosphate; PIP3, Phosphatidylinositol trisphosphate; PTEN, Phosphatase and tensin homologue (Palmieri, Capone et al. 2009).

1.2. Activins

1.2.1. Introduction to Activins

Another signaling system that is frequently deregulated in human malignancies is mediated via ligands of the the TGF-β family, their receptors and connected downstream effectors. The TGF-β family includes multiple factors that have not only tumor suppressor but also oncogenic effects (Piek and Roberts 2001). TGF-β, which is overexpressed in human melanoma cells, stimulates stroma cells to increase production and deposition of extracellular matrix proteins (Lopez-Bergami, Fitchman et al. 2008). The activation of stroma contributes to increased metastasis and a tumor cell survival advantage (Berking, Takemoto et al. 2001). Melanoma cells are resistant to the tumor-suppressive effects of TGF-β. In contrast to other tumor types, however, no detectable defects are found at the TGF-β receptor or smad level in melanoma. On the other hand, inhibitors of the TGF-β family pathway such as follistatin, SKI, filamin and endoglin can act as oncogens, because they interact with smad or membrane receptors by inhibition of tumor suppressive TGF-β signaling (Hussein 2005; Reed, Lin et al. 2005).

In normal physiology TGF-β has been shown to play a key role as a multifunctional polypeptide that regulates proliferation, differentiation, angiogenesis, embryonic development, wound healing and other functions in diverse cell types (Korpal and Kang 2010). The biological effects of TGF-β are dependent on cell type, growth conditions, and the presence of other polypeptide growth factors (Jakowlew 2006). TGF-β is the eponymous member of a large superfamily of secreted proteins including activins, growth and differentiation factors (GDFs), bone morphogenetic proteins (BMPs), inhibins, nodal and anti-Mullerian hormone (Figure 1.7) (Korpal and Kang 2010). Like other members of this superfamily, inhibins and activins are multifunctional factors. They were first identified as

gonadal proteins and their names are based on their ability to inhibit or activate release of follicle-stimulating hormone (FSH) from the pituitary (Mayo 1994). In subsequent years, the bioactivities of activin were defined and were found to be exerted in various cell types at almost all stages of development. Furthermore, intracellular and extracellular proteins may regulate signaling and actions of activin (DePaolo, Bicsak et al. 1991). The extracellular activin binding-protein follistatin (Fst) is a monomeric secreted glycoprotein which binds activin with high affinity and blocks the ability of activin to adhere to its cell surface receptors (de Winter, ten Dijke et al. 1996).

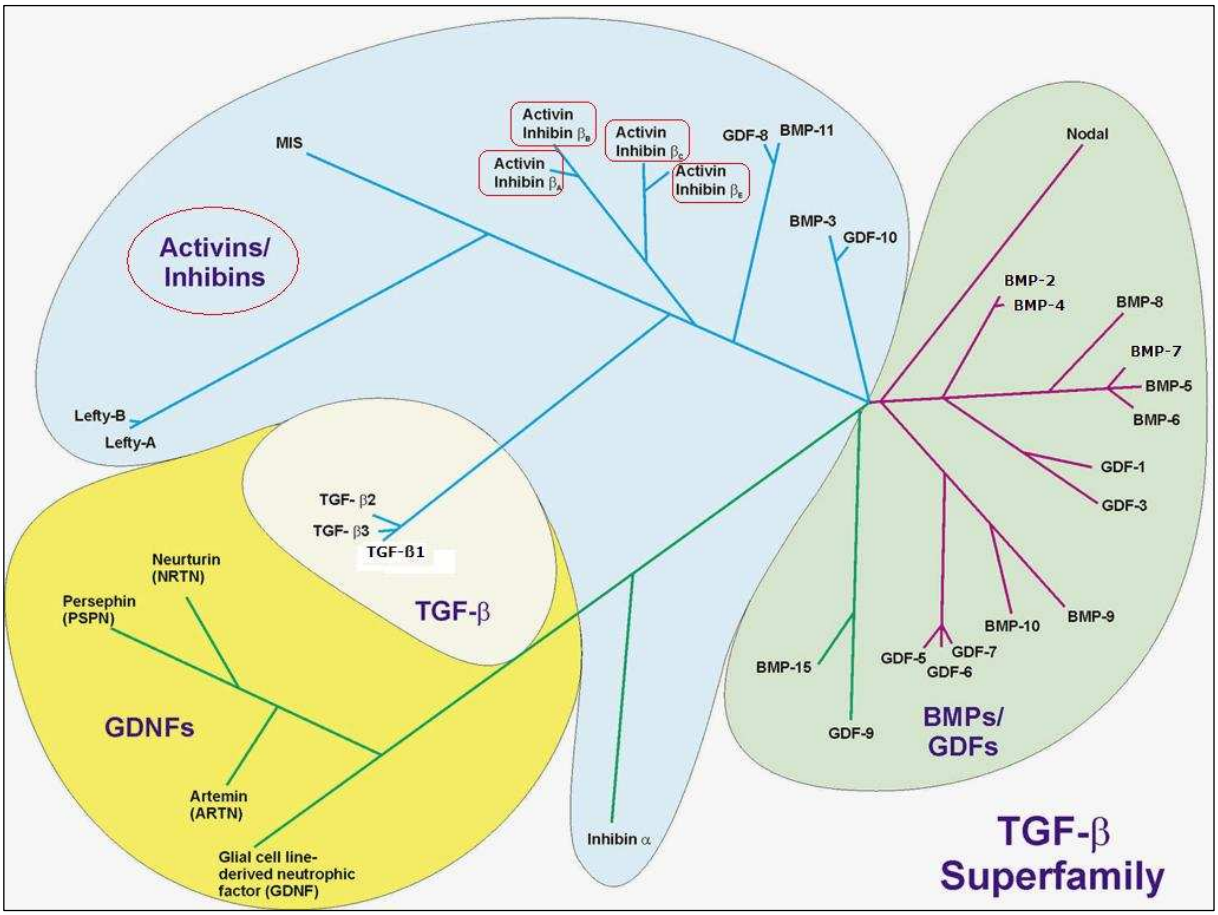

Figure 1. 7 Dendrogram of 35 structurally related members of the TGF-β superfamily. They are subdivided into TGF-βs, activins/inhibins, bone morphogenic proteins (BMPs)/ growth and differentiation factors (GDFs), and the related group of GDNF ligands (Weiskirchen, Meurer et al. 2009).

1.2.2. The Biology and Structure of Activin Subunits

As members of the TGF-β family activins are secreted polypeptides (Schmierer and Hill 2007) They are homo- or heterodimers of two beta subunits each containing a cysteine knot fold with the monomers covalently linked by a single disulphide bond (Jhaveri, Erzurumlu et al. 1998; Chen, Wang et al. 2006). Four different beta subunits exist in mammalian cells termed beta A, beta B, beta C and beta E. Homodimers are denoted by a single letter as activins A, B, C and E, whereas heterodimers by two letters according to their subunit composition (see Figure 1.8) (Tuuri, Eramaa et al. 1994). The activin beta A subunit is synthesized as a 426 amino acid (aa) pro-protein (Deli, Kreidl et al. 2008), containing an N-terminal 28 amino acid signal peptide followed by a pro-region (Mason, Niall et al. 1986). The C-terminal mature 116 aa polypeptide is enzymatically cleaved from the pro-region at an arginine rich processing site (Mason, Niall et al. 1986),(Schwall, Nikolics et al. 1988). The pro-region of the activin beta A subunit plays a key role in the correct folding, secretion and dimerization of the activin protein (Gray and Mason 1990). All of the nine conserved cysteine residues in the mature part of the activin beta A subunit are required for the biosynthesis and biological activity of activin A (Mason 1994). The human activin beta A subunit gene is located on chromosome 7 locus 7p15-p14 (Barton, Yang-Feng et al. 1989). In addition, human (Mason, Niall et al. 1986), porcine (Mason, Hayflick et al. 1985), bovine (Forage, Ring et al. 1986), rat (Esch, Shimasaki et al. 1987; Woodruff, Meunier et al. 1987) and mouse (Albano, Groome et al. 1993; Ritvos, Tuuri et al. 1995) activin beta A subunits are highly conserved and the mature parts of the proteins are identical between these species.

The activin beta A and activin beta B subunits are the most widely expressed throughout the body and have high sequence homology (McDowall, Edwards et al. 2008). The beta B subunit of activin encodes a

precursor protein (407 aa) which contains a hydrophobic signal sequence as well as a pro-region of 292 aa separated from the mature C-terminal part (115 aa) by basic amino acids (Mason, Berkemeier et al. 1989). The activin beta B subunit lacks also putative glycosylation sites like the mature part of activin beta A. The human activin beta B subunit gene is localized on chromosome 2qcen-q13 (Barton, Yang-Feng et al. 1989) and consists of two exons separated by a 2.5 kb intron (Mason, Berkemeier et al. 1989).

The activin C subunit is mostly expressed in the liver. The activin beta D subunit has only been found in Xenopus and the activin beta E subunit was isolated from mouse cDNA libraries but is present in all mammals. The activin beta E subunit shows close similarity to the activin beta C subunit in homology and expression patterns (McDowall, Edwards et al. 2008).

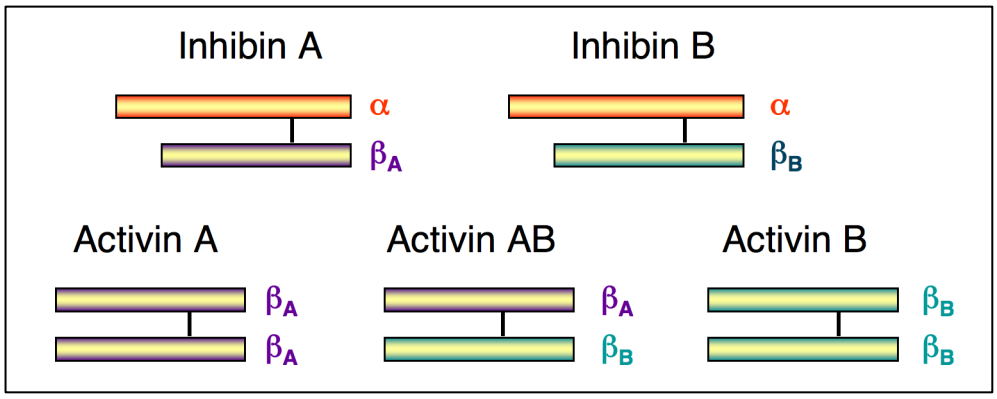

Figure 1. 8 Structural features of activin and inhibin. The mature activins (receptor binding active cytokine) are dimers consisting of two beta subunits joined by a disulphide bond, whereas inhibins are heterodimers of one alpha and one beta subunit. (http://en.wikipedia.org/wiki/Activin_and_inhibin, accessed on 2011-02-08)

1.2.4. Activin Receptors and Activin Mediated Receptor Signaling

The members of the TGF-β superfamily like activins A, B and AB exert their actions in signaling by interacting with two types of transmembrane receptors containing a serine/theronin kinase domain. For activin A it has been demonstrated that the signaling is started by ligand binding and activation of ActR-II (aka ACVR2) or ActR-IIB (aka ACVR2B). Type-II receptors activate the Type-I receptors ALK4 (aka ActR-IB / ACVR1B). (Wrana, Attisano et al. 1994; Vivien, Attisano et al. 1995; Vivien and Wrana 1995; Schmierer and Hill 2007). The activitated type-I receptor allows to phosphorylate intracellular smad proteins (McDowall, Edwards et al. 2008). There are three classes of smad proteins. Receptor regulated smads, known as R-smads, comprising smads1, 2, 3, 5 and 8; inhibitory smads (I-smads) are smad6 and smad7 and the last class of smads is the co-smad4 (McDowall, Edwards et al. 2008). smad2 and smad3 are phosphorylated by the receptors ALK4, ALK5, and ALK7, whereas smad1, smad5, and smad8 are phosphorylated by ALK1, ALK2, ALK3, and ALK6 receptors. All R-smads bind to a smad4, allowing for nuclear translocation, where they regulate transcription of target genes, trough physical contact and functional cooperation with DNA-binding transcription factors and p300 or CBP co-activators (see Figure 1.9.) (Lagna, Hata et al. 1996; Heldin, Miyazono et al. 1997; Derynck and Zhang 2003; Schmierer and Hill 2007; McDowall, Edwards et al. 2008). Activins have only been shown to lead to phosphoryltation of smad2 and smad3, whereas stimulation with BMPs resulted in phosphorylted smad1, smad5 and smad8 (Harrison, Wiater et al. 2004).

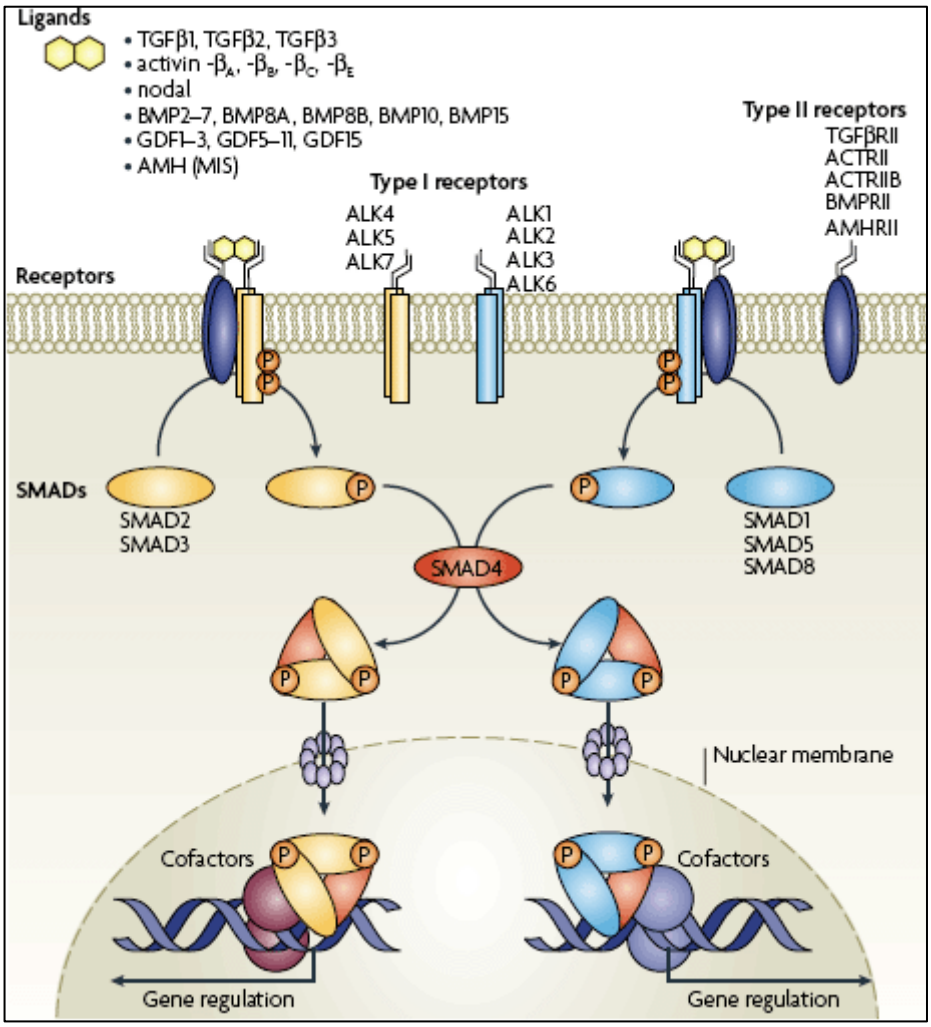

Figure 1. 9 General mechanism of smad dependent TGF-ß signalling pathway. The ligand binds transmembrane receptor serine/threonine kinases (types I and II) and type-II receptor kinases activate the type-I receptor. Activated Type-I receptors phosphorylate R-smads, which then form a complex with smad4 and translocate into the nucleus, where they regulate transcription of target genes (Schmierer and Hill 2007).

In addition to smad-dependend signaling, activins can activate also other signaling cascades such as p38 MAPK, Erk and JNK kinase pathways (Derynck and Zhang 2003). Activation with rapid kinetics in some cases results from smad-independend signaling, whereas the slow activation (>15min) suggests dependence from transcription (Massague 2000).

1.2.5. Biological Function of Activin

The biological activity of activins on cell growth and differentiation are basically mediated through the smad-dependent signalling pathways. Hep3B cells treated with activin A showed growth inhibition by downregulating Bcl-xL (anti-apoptotic) expression through smad2 or smad3 (Kanamaru, Yasuda et al. 2002). Besides treatment of HepG2 cells with activin induced cell cycle arrest and increased gene expression of the cyclin-dependent kinase inhibitor p15INK4B (Ho, de Guise et al. 2004). It has been demonstrated that activin is overexpressed in wounded skin and the overexpression of activin in the epidermis of transgenic mice helps wound healing and enhances scar formation (Munz, Smola et al. 1999; Chen, Wang et al. 2006). Inhibition of activin activities delays wound healing in the skin but improves the quality of the healed wound (Chen, Wang et al. 2006). In addition to the regulation of cell growth, activin plays a key role in morphogenesis and regulation of branching organs such as the prostate, lung, and kidney (Ball and Risbridger 2001) and it regulates the differentiation of human trophoblast tissues during early pregnancy (Caniggia, Lye et al. 1997).

Activin A induces apoptosis in numerous types of cells and tissues. In hematopoietic cells, activin/TGF-β induced expression of SHIP (SH2 domain-containing inositol phosphatase). This phosphotase is a regulator of phospholipid metabolism and mediates changes in the pool of intracellular phospholipids, downregulation of Akt/PKB phosphorylation and cell survival (Valderrama-Carvajal, Cocolakis et al. 2002). In addition, activin-C and activin-E have also been demonstrated to induce apoptosis in either human or rat hepatoma cells (Vejda, Erlach et al. 2003).

In addition to effecting apoptosis, activin may have other anti-tumorigenic functions. The proliferation of human tumor cells derived from gall bladder

(Yokomuro, Tsuji et al. 2000) , prostate (McPherson, Mellor et al. 1999), and pituitary gland (Danila, Inder et al. 2000) is decreased by activin. Activin inhibits the proliferation of T47D breast cancer cells by upregulating of p15 cyclin-dependent kinase inhibitors and phosphorylating the retinoblastoma protein (Burdette, Jeruss et al. 2005). In this respect, biological function of activin seems to be similar to the TGF-β effect on epithelial cells. Besides. activin A also indicates its tumor suppressor function as an inhibitor of angiogenesis (Chen, Wang et al. 2006).

Finally, to find out the complicated biological functions of activin in cell proliferation, differentiation as well as in carcinogenesis is not simple. For instance, activin can be tumorgenic or antitumorigenic as described above, depending on the setting (Chen, Wang et al. 2006). Increased expression levels of activin A in human endometrial adenocarcinoma tissues have been proposed to be involved in carcinogenesis, by reducing activin mediated signals which have inhibitory effect on these tissues (Tanaka, Toujima et al. 2004; Chen, Wang et al. 2006). In addition, activin A has been demonstrated to lead to high expression of N-cadherin on the carcinoma cell surface that is associated with tumor aggressiveness (Yoshinaga, Inoue et al. 2004).

1.3. Activin Binding Proteins

1.3.1. Activin Antagonist Structures

The actions of activin are cell type specific. In some tissues or cell types, it induces proliferation, whereas in others it causes differentiation or apoptosis (Stove, Vanrobaeys et al. 2004). In addition, both overexpression as well as inhibition of activin signaling is correlated with multiple pathological states, including reproductive disorders, inflammation and carcinogenesis (Risbridger, Schmitt et al. 2001; Chen, Lui et al. 2002; Cho, Yao et al. 2003). Negative regulation of activin signaling may occur by mutation or downregulation of activin response genes or inhibitory interaction at the level of smad family members (Chen, Lui et al. 2002). In addition, several activin binding proteins such as follistatin, cripto, and FLRG regulate the activity of activin and block its interaction with activin receptors (Gumienny and Padgett 2002; Gray, Harrison et al. 2003).

Fst was first purified from ovarian follicular fluid and identified as a follicle stimulating hormone suppressing substance (Esch, Shimasaki et al. 1987; Robertson, Klein et al. 1987). It has been demonstrated that alternative splicing events at the 3`end of the mRNA cause production of different isoforms, which undergo post-translational proteolytic processing (Sugino, Sugino et al. 1997; Patel 1998). The most common isoforms of follistatin are Fst-315 and Fst-288. Fst-288 has a higher affinity for heparin sulfated proteoglycans located on the cell surface. This helps Fst-288 to be concentrated on the cell surface and create a barrier that prevents activin from accessing its transmembrane receptors (Delbaere, Sidis et al. 1999; Xia and Schneyer 2009) and leads to endocytosis and degradation of surface bound activin (Hashimoto, Nakamura et al. 1997). Fst-315 encodes an additional acidic C-terminal tail that interferes to some extent

with heparin binding. Therefore Fst-315 is the major isoform in solution (Schneyer, Hall et al. 1996). Both isoforms contain an N-Terminal activin binding and three sequential 10-cysteine follistatin domains, the first one (Wang, Keutmann et al. 2000) contains a lysine rich heparin binding sequence (Wang, Keutmann et al. 2000; Sidis, Schneyer et al. 2001; Innis and Hyvonen 2003).

Follistatin-like-1 (Fstl1) is a 308 amino acid extracellular glycoprotein. It has been grouped into the follistatin family but has only limited functional and structural similarity to Fst. It was first cloned from a mouse osteoblastic cell line as a TGF-β inducible gene (Shibanuma, Mashimo et al. 1993; Hayette, Gadoux et al. 1998).

Follistatin related gene (FLRG) encodes an extracellular matrix protein that has been also referred to as Fst-like-3 (Fstl3) (Schneyer, Tortoriello et al. 2001). The overall structure of FLRG is quite similar to Fst and the domains such as the N-terminus, the follistatin domains and the tail domain are each encoded on separated exons. FLRG has one follistatin domain less than Fst and does not contain a heparin binding sequence (Schneyer, Tortoriello et al. 2001).

Another activin-antagonistic protein, cripto is a 36 kD protein containing two cysteine rich domains, an epidermal growth factor-like domain that takes part in Nodal signaling, and a CFC (cripto-FRL-criptic) domain that binds ALK4. Cripto makes complexes with activin A in the presence of ActRII/IIB receptor and so reduces activin A cross linking to ALK4 (Gray, Harrison et al. 2003; Strizzi, Bianco et al. 2005; Kelber, Shani et al. 2008).

1.3.2. Interaction between Follistatin and Activin

The mature follistatin protein described above consists of an N-terminal unique domain, called Fs0 and three follistatin domains Fs1, Fs2 and Fs3. (Shimasaki et al, 1989; Ullman and Perkins, 1997).

The activin A–Fs1 and Fs2 complex structure was shown to have a 1:2 stoichiometry with binding of follistatin to the outside surfaces of the activin subunits. The flexibility of the domain structure of follistatin allows it to bend and follow the curvature of the activin subunits (Figure 1.10) (Harrington, Morris-Triggs et al. 2006).

The dimeric structure of activin A has been shown to be in a more closed conformation in comparison to BMPs and TGF-βs, with the follistatins binding from the side in the complex (Figure 1.10B) (Harrington, Morris-Triggs et al. 2006). Each follistatin fragment in the structure interacts with only one of the activin subunits via its Kazal-like subdomain, while the heparan sulphate binding EGF-like N-terminal part points away from activin, and is poorly resolved in the structure. On the other hand, the Fs2 domain interacts with activin using both EGF- and Kazal-like subdomains with different orientation (Hohenester, Maurer et al. 1997; Harrington, Morris-Triggs et al. 2006)

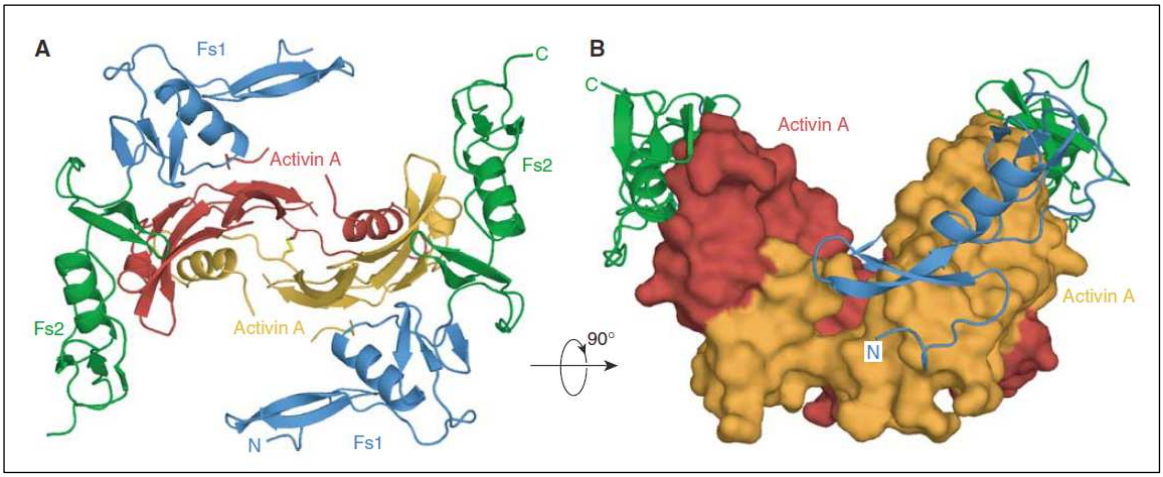

Figure 1. 10 Model of activin–Fs1-2 complex. (A) Binding of two follistatin fragments to the back of the activin A fingers. Activin protomers are painted red and orange, the interchain disulphide is coloured in yellow. Follistatin domains Fs1 and Fs2 are shown in blue and green, respectively. (B) Model of the closed conformation of activin and Fs1-2 fragments wrapping along activin A. (Modified from EMBO Journal,(Harrington, Morris-Triggs et al. 2006).

1.3.3. Regulation of Activin Activity by Antagonists

It has been shown that follistatin plays a significant role in the regulation of various actions of activin. The interactions between follistatin and activin can be occurring by two mechanisms; (i) follistatin binds circulating activin thereby preventing activation of its receptors (Figure 1.11), (ii) follistatin bound by heparin sulfate at the cell surface forms a complex with activin causing its endocytosis and lysosomal degradation (Figure 1.11) (McDowall, Edwards et al. 2008).

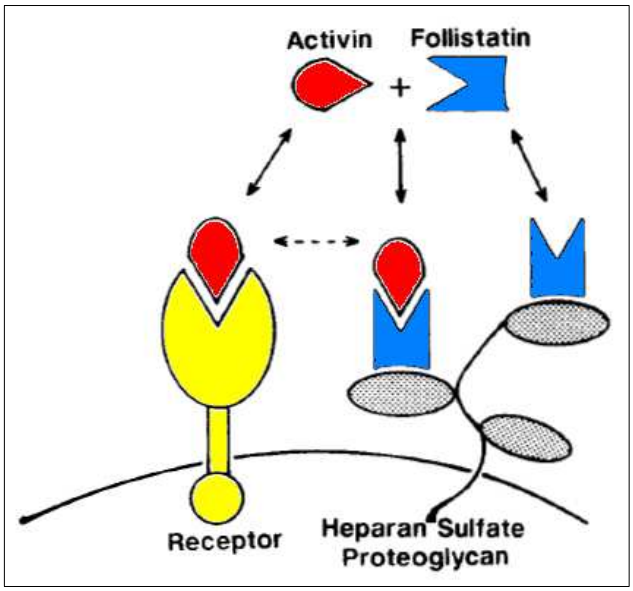

Figure 1. 11 Mechanisms of activin antagonism by follistatin. Follistatin either binds activin in the circulation or it attaches to heparin sulfate proteoglycans on the cell surface and captures activin there. (Modified from The Journal of Biological chemistry, Nakamura, Assosiation of Follistatin on Cell Surface, s19436, 1991)

These two hypotheses were tested by producing different isoforms of recombinant follistatin and FLRG studying their binding kinetics for various TGF-β ligands as well as their capability to block biological activity when transfected into cells or when added as proteins. The Fst isoforms and FLRG possess comparable activin binding kinetics but their cell surface binding significantly differed (Sidis, Mukherjee et al. 2006). Surprisingly, the degree of inhibition of endogenous activin bioactivity depended on surface binding activity, whereas inhibition of exogenous activin activity was not markedly different among the Fst isoforms (Sidis, Mukherjee et al. 2006). This supports the hypothesis that memrane localization via heparin sulfate proteoglycans markedly increases the inhibition of autocrine activin (Xia and Schneyer 2009).

In support of the hypothesis, that follistatin isoforms show different actions *in vivo*, cDNAs of two isoform were expressed transgenically under the

control of the human Fst promoter and crossed into the Fst null mouse line (Xia and Schneyer 2009) that had been reported to be neonatally lethal (Matzuk, Lu et al. 1995). The Fst-288 protein expressing transgenic mice did not rescue this lethality, whereas Fst-315 expression allowed mice to survive with an - albeit partial - reversal of the skeletal abnormalities seen in Fst null mice (Lin, Craythorn et al. 2008). Nevertheless, these mice showed neonatal growth retardation, decreased tail growth and female infertility. These results suggest that the shorter Fst-288 isoform may be necessary for ovarian function and normal vascularization. On the other hand, the Fst-315 isoform is sufficient for other regulatory systems that when absent or deficient, result in neonatal death (Lin, Craythorn et al. 2008).

FLRG does not contain a heparin binding sequence and thus does not bind cell surface proteoglycans (Sidis, Mukherjee et al. 2006). When comparing its secretion with that of the Fst isoforms using 35S pulse-chase labeling, FLRG was found to be secreted slowest with a fraction of newly synthesized proteins being transported to the nucleus, whereas Fst-288 was secreted faster and Fst-315 was secreted and synthesized fastest (Saito, Sidis et al. 2005). Interestingly, the nuclear FLRG protein was glycosylated but not to the same degree as secreted FLRG, suggesting it may be acquired from the endoplasmic reticulum resident pool that means it is transported directly to the nucleus without being secreted (Saito, Sidis et al. 2005). These results suggest that FLRG and Fst may have extracellular as well as intracellular roles that are governed to some degree by heparin binding affinity (Saito, Sidis et al. 2005; Xia and Schneyer 2009).

1.4. Aims of the study

Previous data from the literature demonstrate that Fst is frequently overexpressed in melanoma cells (Stove, Vanrobaeys et al. 2004). Unpublished observations from our group suggested, however, that melanoma cells can be growth inhibited by treatment with exogenous activin A. The aim of this study was therefore to further investigate the role of activin signals in melanoma growth and progression.

In the first part of the thesis the objective was to analyse the expression of activins and their functional antagonists in a panel of melanoma cell lines established from primary melanoma or metastatic lesions in comparison to primary melanocytes.

The goal of the second part of the thesis was to engineer melanoma cells to stably overexpress Fst-315. These sublines were then characterized with respect to proliferation, migration and tumor growth by various in vitro assays as well as in a xenotransplant model in vivo.

The third aim of this thesis was to establish the impact of the activin beta E subunit on activin A-mediated signaling mechanisms.

2. Materials and Methods

2.1. Cell lines and Media

All melanoma cell lines (Table 2.1) were supplied by the cell culture department of the Institute of Cancer Research. Roswell Park Memorial Institute (RPMI) medium with 10% fetal bovine serum (FBS) was used for all melanoma cell lines. RPMI medium and FBS were purchased from Sigma Aldrich. Penecilin/Streptomycin was bought from PAA and added to the medium only for selection of stable transfectants.

Table 2. 1 Used cell lines. PT: Primary tumor; MT: Metastases; SC: Subcutaneous; LM: Lymph node; BN: Bone; BR: Brain; ME: Malignant effusions; SMM: Superficially spreading melanoma; NM: Nodular Melanoma; PM: Primary Melanocytes.

Melanoma Cell Lines			
VM No.	**Cell Line**	**Origin**	**Histology**
VM-7	GTBS	PT	NM
VM-10	JMUM	PT	SSM
VM-21	RHTP	PT	NM
VM-23	RKTJ	PT	NM
VM-30	WCRE	PT	SSM
VM-1	FTSL/A	LN	SSM
VM-8	GUBS/A	LN	NM
VM-24	STHJ/A	LN	Unknown
VM-28	TMFI	BR	Unknown
VM-47	HOST	BR	NM
VM-48	KAKA	BR	NM
VM-31	WLTJ	ME	NM
Other Cell Lines			
HepG2	Human hepatoma cell line (Knowles, Howe et al. 1980)		
MGC	Human glioblastoma cell line (Allerstorfer, Sonvilla et al. 2008)		
HEK293	Cell line derived from human embryo renal cortical cells (Graham, Smiley et al. 1977)		

Table 2. 2 Used Media in cell culture.

DMEM	Dulbecco's modified Eagle's medium	for Hek293 cells with 10% FBS; 1% P/S
RPMI	Roswell Park Memorial Institute medium	for all melanoma cell lines with 10% FBS; 1% P/S
MNP	MEME; 1 mM sodium pyruvate (Sigma); 1% non-essential amino acids (PAA)	for HepG2 and MGC cells with 10% FBS; 1% P/S

2.2. Standard Growth Conditions

All melanoma cell lines were grown in a humidified incubator (Forma Scientific) at 37°C and 5% CO_2. Tissue culture flasks (T25 (=25cm^2), T75 (=75cm^2) and T175 (=175cm^2)) were bought from Greiner Bio-One as well as from BD Falcon. 6-well, 12-well and 24-well plates were bought from IWAKI. 15 cm tissue culture dishes were bought from Sarstedt and 96-well-plates were from TPP.

2.3. Splitting

To split the adherent cells, medium was aspirated and cells were washed once with PBS (2.7 mM EDTA (Merck); 137 mM NaCl (Merck) and 10 mM Na_2HPO_4 (Merck) pH 7.4). Trypsin/EDTA (T/E; 0.01% EDTA (Fluka); 0.1% Trypsin (Difco)) was added and incubated at 37°C for about 5 minutes depending on the cell line. Cells were resuspended in medium and the desired number of cells was seeded into a new dish or tissue culture flask, containing full medium.

2.4. Cell Counting

Cells were counted by using either a Casy Cell Counter (Schärfe) or a Neubauer Chamber (Phelan, 2007; Paul Marienfeld GmbH).

2.5. Freezing

Cells were grown in T75 culture flask in full medium until they were nearly confluent, were trypsinized with tyrypsin (T/E) and resuspended in 5 ml medium with 10% FBS. 1 ml of cell suspension was transferred into cryo tubes (Greiner) and 50 µg/ml DMSO (dimethylsulfoxid) were added. The suspension was immediately put on ice and cooled down in a styrofoam container at –80°C for at least 24 hours. Afterwards, cells were transferred into a liquid nitrogen tank for long term storage.

2.6. Miniprep

Plasmid minipreps were performed by the Wizard Plus SV Miniprep DNA Purification System (Promega). 50 ml of bacterial over night culture were pelleted, centrifugation was done at 15 000 g for 1 min. Supernatant was removed and cells were solubilized by 250 µl Cell Resuspension Solution, then 250 µl Cell Lysis Solution were added and the reaction was incubated for 3 min at RT. 10 µl Alkaline Protease Solution were pipetted into the solution and incubated again for 5 min at RT. This step was followed by the adding of 350 µl Neutralization Solution and centrifugation at 16 100 g for 10 min. Supernatant was transferred to a Spin Column which had been inserted into a Collection Tube and centrifuged for 1 min at 16 100 g. Flowthrough was removed and 750 µl Column Wash Solution were added to the column. Centrifugation was done at 16 100 g for 1 min and flowthrough was removed again and another 250 µl of fresh Wash Solution were added to the column and passed through by another step of centrifugation for 2 min. 50 µL of nucleuase free water were added to the column and DNA was eluted by a final centrifugation step at 16 100 g for 1 min into an Eppendorf tube.

2.7. Lipofection

3×10^5 cells were seeded into 6-well plates with 2 ml 10% FBS containing appropriate medium and incubated overnight. Next day, cells achieved about 50-80% confluence. Either FuGENE 6 (Roche) or Turbofect (Fermantas) was used as lipofection reagent. 97 µl Serum Free Medium (SFM) were transferred into Eppendorf tubes, 3 µl transfection reagent were added directly into the medium, mixed in a total volume of 100 µl and incubated for 5 minutes. Then 1 µg plasmid was added, mixed and incubated for another 25 minutes at room temperature. If the plasmid did not contain a desired selection marker, a second plasmid possessing the specific marker was added, thereby performing a co-transfection.
The total volume of transfection suspension was dropped onto the medium containing adherent cells and gently shaken (2 ml/well of a 6-well plate). The following day, lipofected cells were expanded into 10 cm tissue culture dishes, thereby medium was changed, and incubated overnight. Then the standard growth medium was changed to selection medium containing antibiotics, according to the used resistance gene.

2.8. Selection of Stable Transfectants

As antibiotics neomycin/G418 or puromycin were used to select for cells that had integrated the plasmid coding for a selection marker and the gene of interest into their genome. To reduce the fake clone phenomenon, where only selection markers integrate into the genome but not the gene of interest, some plasmids coded for bicistronic mRNAs with gene of interest and selection marker seperated only by an IRES (Internal Ribosomal Entry Site) sequence (Gurtu, Yan et al. 1996).
During post-transfection, either G418 or puromycin was added to the cells and selection medium was changed several times to remove the dead cells. When resistent clones appeared, usually after 1-2 weeks, clones

were picked and reseeded to facilitate growth. New cell lines were frozen down and tested for expression, as soon as possible, to reduce the risk of losing clones due to contamination. However, all selection media contained also penicillin and streptomycin to prevent bacterial infections. All generated cell lines were tested for expression of the respective genes of interest by Western Blot analysis, ELISA and qRT-PCR.

Table 2. 3 Stable transfectants and their selection

Gene of interest	Vectors	Selection
Activin-A	pHs INHBA-IRESpuro	Puromycin
Follistatin-315	pFst315/EGFPe	Neomycin
Follistatin-288	pFst288/EGFPe	Neomycin
Follistatin-like-1	pHsFstl-1-IRESpuro	Puromycin
FLRG	pIRESpuro-Fstl-3	Puromycin
Control GFP	EGFP-IRESpuro	Puromycin
Control GFP	EGFP-IRESneo	Neomycin
Control GFP	pLuc-IRESe-EGFPneo	Neomycin

2.9. Microscopy

All microscopy methods (Light, fluorescence and fluorescence time-lapse microscopy) were performed using an Eclipse TE300 microscope (Nikon), a Digital Sight DS-5M (Nikon) or SPOT INSIGHT (Diagnostic Instruments) CCD camera and MetaMorph software (Molecular Devices). All microscopy was performed at room temperature.

2.10. Isolation of RNA

Cells were grown in T25 cell culture flasks. When they were almost completely confluent, medium was discarded and cells were washed with cold PBS (2.7 mM EDTA (Merck), 137 mM NaCl (Merck) and 10 mM Na2HPO4 (Merck); pH 7.4). Following steps were performed on ice. Trizol (Invitrogen) was added onto the cell layer (for 25cm^2 confluent cell layer 1.5 ml volume was used). The cells were scraped in Trizol reagent and

were transferred into 2 ml Eppendorf tubes. Lysating cells were vortexed and incubated for about 5 minutes. For RNA extraction, chlorophorm/isomylalcohol (50:1; 0.2 x vol. Trizol) was added, vortexed and incubated again for approximately 5 min. Cell waste was centrifuged down at 11 000 g at 4°C for 10 min. Aqueous supernatant, containing resuspended RNA was transferred into new Eppendorf tubes and isopropanol (0.5 times vol. Trizol) was added, vortexed and again centrifuged at 11 000 g at 4°C for 10 min. Supernatant was discarded and about 0.5 ml of 70% EtOH/DEPC was added onto the pellet, vortexed and centrifuged at 7 500 g at 4°C for 7 min. The alcohol was removed, the pellet was air dried and taken up in DEPC/EDTA (3 mM; 10-30 µl volume) water. As quality control, purified RNA was loaded onto agarose gel (1 µg RNA; 1% agarose gel) and separated. RNA was frozen at –20°C or at –80°C for long term storage.

2.11. Determination of RNA Concentration

The concentration of purified RNA was determined by a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific).

2.12. cDNA Syntesis for qRT-PCR

Total RNA was extracted from approximately 5 x 10^5 cells and 1 µg RNA was diluted with DEPC water to 11 µl and incubated for denaturation at 70°C for 10min. Afterward it was put on ice. Final volume was brought to 20 µl by addition of 9 µl cDNA Synthesis Master Mix (Table 2.4). Then it was incubated for 1.5 hours at 37°C. The quality of cDNA was tested by performing standard RT-PCR of a "housekeeping gene" and agarose gel electrophoresis. cDNA was stored frozen at –20°C.

Table 2. 4 cDNA synthesis master mix

Stock	for 1X; 20µl
RNA (1µg in 11µl DEPC water)	11µl
M-MLV RT buffer (5X; Promega) dNTPs(10mM) DTT(100mM) Hexanucleotids (20ng/µl) MMLV-RT (200 u/µl; Promega)	4µl 1µl 2µl 1µl 1µl
	20µl total volume

2.13. Reverse Transcriptase PCR

The RT-PCR Master Mix was prepared on ice. 1 µl cDNA solution was pipetted into 0.2 ml PCR Tube Strips (Bio-Rad) and then the Master Mix was added and mixed. Reaction was carried out in a MyCyclerTM Thermal Cycler (Bio-Rad). For PCR conditions and primer sequence see tables 2.5 – 2.7 below.

Table 2. 5 General reaction mixes for RT-PCR

17.375 µL nuclease free water (Fermentas)
5 µL 5X Colorless GoTaq Reaction Buffer (Promega)
0.5 µL dNTPs (10mM; Fermentas)
0.5 µL upstream primer (20 µM)
0.5 µL downstream primer (20 µM)
0.125 µL GoTaq DNA polymerase (5 units / µL)
1 µL template DNA

Table 2. 6 Primer used for RT-PCR

Target cDNA	Sense primer (5´-3`)	Antisense primer (5`-3`)
Follistatin	Ccagcgagtgtgccatgaag	tcatcttcctcctcttcctcg

Table 2. 7 RT-PCR Conditions

Gene of Interest Follistatin	Dissociation	Annealing	Elongation
Temperature (°C)	94	61	72

Number of Cycles	Time		
1x	3´		
35x	30´´	50´´	50´´
1x			2

2.14. Quantitative Real Time-PCR (qRT-PCR)-Taqman

The TaqMan Master Mix was prepared on ice. 2 µl of serial 10-fold dilutions of original cDNA template were layered into two wells (per sample) of 96-Well Reaction Plates (Micro Amp^{R}; Applied Biosystems), in case of 18S-rRNA for normalization. 10 µl Master Mix was added to a total volume of 12 µl. Fluorescence was measured using an ABI Prism 7000 SDS fluorometer (ABI), according to ABI manuals. Following amplification, CT values, ΔCT values and Log2 ratios were recorded for target genes and internal standards.

Table 2. 8 Real time PCR master mix

Reagent	Volume (for 1 sample)
Taqman universal PCR mix	6.25µl
Probe	0.625µl
cDNA	2.00µl
H2O	3.125µl
	12µl total volume

2.15. Restriction Digest

Master Mix, including restriction enzyme and corresponding buffer (Fermentas), was prepared on ice. 15 µl of the PCR product was resuspended with 5 µl Master Mix and so digestion was performed in a

total volume of 20 µl. Incubation time was usually 60 min at the suggested incubation temperature (usually 37°C), unless different conditions were required for the enzyme as suggested by Fermentas.

Table 2. 9 Reagents of restriction digest

Master Mix	1µl Restriction Enzyme
	2µl Buffer
	2µl Water Mix
Restriction Digestion	15µl PCR Product
	5µl Master mix
	20µl (Tot.Vol.)

2.16. Gel Electrophoresis

DNA and RNA fragments were seperated on gels consisting of 0.5-1% (w/v) agarose (Bioenzyme) in 0.5 x TBE buffer (5.4 g/l Tris (Fluka), 2.75 g/l boric acid (Sigma) and 1 mM EDTA). Samples were mixed with 6 x loading buffer (333 µl/ml 6 x loading dye (Fermentas), 250 µl/ml 80% glycerol (Merck), 66.5 µl/ml 0.5 M EDTA, 0.5 µl/ml 10 000 x Vistra Green (GE Healthcare)) and loaded onto the gel. Depending on expected fragment sizes 2 µL GeneRuler 1 kb DNA Ladder or 100 bp DNA Ladder (Fermentas) markers mixed with appropriate amounts of water and loading buffer were used. As running buffer for agarose gels 0.5 x TBE was used and the run was initiated with constant voltage of 50 V for 10 min followed by 120 V until the marker dye indicated sufficient separation. The gel was stopped and bands were visualized using a FluorImager 595 Scanner from Molecular Dynamics.

2.17 Precipitation of Protein from Cell Supernatants and Preparation for Gel Electrophoresis

Serum free cell supernatant was transferred to a SS34 centrifugation tube and 4 volumes of 100% EtOH were added. The solution containing cell supernatant and 100% EtOH was incubated at –20°C overnight. The following day precipitate was harvested by centrifugation at 4°C for 10 min at 15 000 g. Supernatant was removed and the precipitate was washed in cold 70% EtOH. It was pelleted again at 16 100 g for 5 min. Supernatant was removed completely and the pellet was briefly left to dry. It was then resolubilized in a minimal volume (typically 25 µl for the precipitate from a T25 bottle) of sample buffer (7.5 M urea (Merck); 1.5 M thiourea (Sigma); 4% CHAPS (Merck); 0.05% SDS (Fluka)). Insoluble precipitate was removed by centrifugation at 16 100 g for 5 min. Finally 6 µl 5 x SDS-PAGE loading buffer were added and the mixture was loaded onto the gel without prior heating to prevent carbamylation of the proteins in the sample (McCarthy, Hopwood et al. 2003).

2.18. Isolation of Proteins from Cells

To isolate protein from cells for SDS-PAGE and Western blot analysis, around 5 x 10^5 cells were seeded in a 6-well and incubated overnight. The next day, growth medium was removed and cells were firstly washed two times with cold PBS and then were scraped (Cornig) into PBS. Cells were pelleted by centrifugation at 1 000 g for 2 min. All successive steps were done on ice. Supernatant was removed and pellet was lysed by adding 2 x Laemmlie buffer (4% SDS; 24% glycerol; 120 mM Tris/HCl pH 6.8; 2,8% 2-mercaptoethanol (Aldrich); 0.01% bromophenol blue (Koch-Light Laboratories))and sonification 3 x for 3 sec (Bandelin). Amounts of protein were analyzed by SDS-PAGE.

2.19. Polyacrylamide Gel Electrophoresis and Western Blot

For detection of a protein of interest in cell lysate or supernatant, samples were separated by SDS-PAGE (Gallagher 2007) and immunoblotted. The Mini Protean 3 system (Bio-Rad) was used for blotting and transfer of protein to a PVDF membrane (Hypond-P; GE Healthcare) for immunodetection with specific antibodies. Denaturing, discontinous polyacrylamide gels with gel densities of the separation between 10 and 15% were typically used. Occasionally PAGEr Gold Precast Gels 4-20% (Lonza) were used. The separation gel was cast and topped with isopropanol. Following polymerization (15-30 min), isopropanol was rinsed off with water which was removed with filter paper. The stacking gel was then cast on top of the separating gel. Prior to loading, the gel pockets were cleaned with SDS running buffer (25 mM tris; 192 mM glycine; 0.1% SDS) which was also used as running buffer for all SDS-PAGE gels. For each pocket 5-25 µg of protein lysate were mixed with 5 x SDS-PAGE loading buffer and heated for 5 min to 95°C before being loaded. 5 µL of Page Ruler Plus Prestained Protein Ladder (Fermentas) were used as a marker. The gel was run at a voltage of 60 V for 30 min, followed by 120 V until the the bromphenol blue tracking dye had left the gel.

Table 2. 10 Preparing of PAGE gels

	Seperation gel	10%	15%	Stacking gel	
ml	Water	1960	1122	Water	1.5
ml	1.6 M Tris pH 8.8	1250	1250	0.5 M Tris pH 6.8	0.625
µL	10% SDS	100	100	10% SDS	25
ml	30% Acrylamid/Bis; 29:1 (Bio-Rad)	1675	2513	30% Acrylamid/Bis; 29:1	325
µl	10% APS (Merck)	25	25	APS	25
µl	TEMED (Amresco)	5	5	TEMED	2.5

For Western blotting, separated proteins were transferred to a PVDF membrane using the tank transfer system (Gallagher, Winston et al. 2008). Before assembly of the blotting sandwich, the PVDF membrane was activated with methanol, rinsed thoroughly with tap water and equilibrated in Towbin buffer (25 mM Tris; 192 mM Glycin; 5% MeOH) which was also used as transfer buffer. Transfer was performed at a current of 200 mA for 2 h at 4°C with a –20°C cooling pack in the tank.

Following transfer, the membrane was rinsed with distilled water and protein bands were visualized using Ponceau S staining. The membrane was incubated with Ponceau S solution (0.5 g/L Ponceau S (Sigma); 1 ml/l glacial acetic acid (Gallagher, Winston et al. 2008) for 5 min. Background staining was removed by washing several times with distilled water. Staining was documented using a photocopier.

Following incubation, membranes were blocked with TBST-M (TBS: 8 g/L NaCl; 0.2 g/L KCl; 3 g/L tris; pH 7.4; T: 0.1% tween 20 (Sigma); M: 5% skim milk powder (Fluka)) for 1 h on a shaker at RT. Membranes were then washed 3 x 5 min and incubated in primary antibody solution over night in a 15 ml Falcon tube on a shaker at 4°C. See Table 2.11 for antibodies used. The next day, membranes were washed 3 x with TBST and incubated in secondary antibody solution for 1 h on a shaker at RT. This was followed by washing 3 x with TBST and 2 x with TBS before

incubation upside-down on a parafilm with Immun-Star WesternC reagent (Bio-Rad) and visualization of the signal with Hyperfilm ECL (GE Healthcare).

Table 2. 11 Antibodies used for western blot

Antibody	Dilution	Supplier	diluent	size of target
Mouse-anti-GFP	1:2000	Roche; 11814460001	TBST-M	27kDa
Rabbit-anti-Phospho-smad2	1:1000	Cell Signaling; 3101	TBST-M	60kDa
Rabbit-anti-smad2/3	1:1000	Cell Signaling; 3102	TBST-5%BSA	60kDa

2.20. Smad Phosphorylation Assay

HepG2 cells were seeded and incubated in full medium overnight. Medium of HepG2 cells was removed and cells were washed 2 x with PBS. 1 ml of conditioned media from transfected HEK293 cells or purified recombinant cytokines were added, and the cells were incubated for 30 min in the incubator. Cells were then harvested as described under 2.18 and cell lysates were subjected to gel electrophoresis and western blot.

2.21. ELISA

The Quantikine Elisa kit was used for the quantitative determination of human follistatin concentrations in cell culture supernatants. A suggested 10-fold dilution of 25 µl cell culture supernatants were mixed with 225 µl Calibrator Diluent RD5-21. Standards for calibration were used according to the manufacturer's instructions (16.000 pg/ml-4000 pg/ml-1000 pg/ml-250 pg/ml). After 100 µl of Assay Diluent RD1-8 were added to each well, 100 µl of control, sample and standard were also added and the wells were coverd with the adhesive strip provided. They were incubated for 3 h

at 4° C. A plate layout is provided to record standards and samples assayed. Each well was aspirated and washed, the process was repeated three times for a total of four washes. After the last wash, remaining Wash Buffer was removed by decanting. The plate was inverted and blotted against clean paper towels. 200 µl of cold follistatin conjugate were added to each well and they were coverd with a new adhesive strip and incubated for 2 h at 4°C. Wash and aspiration steps were performed as described before. Afterward 200 µl of Substrate Solution were added to each well and protected from light. They were incubated 20 min at RT. By adding of 50 µl Stop Solution the colour in the wells changed from blue to yellow. The optical density of each well was measured at 450 nm and correction was set to 540 nm or 570 nm using a Synergy spectrometer.

2.22. Growth Curve Assay

Cell lines stably transfected with the desired construct were counted under the microscopy and then seeded to 12-well plates at a density of $1x10^5$ cells/well. Number of viable cells was measured every second day until the eighth day, using the Casy Cell Counter.

2.23. Clonogenic Assay

Cells were washed with PBS, trypsinized and counted. 100 and 1000 cells were seeded into six well plates containing 2 ml RPMI medium (10% FCS) to form colonies in 1–3 weeks. After the desired clone sizes (>1 mm) were reached, depending on specific clonogenicity of the cell line used, cells were stained with crystal violet. Numbers of clones larger then 1mm in diameter were counted.

2.24. Crystal Violet Staining

Supernatant of cells was removed, cells were washed (PBS) and fixed by incubation in Methanol:Aceton (1:1) for about 20 minutes. Then fixation solution was changed to crystal violet solution, diluted 1:1000 in PBS (stock: crystal violet in PBS and 10% ethanol) and incubated for another 20 minutes. Staining solution was discarded and cells were air dried over night.

2.25. Soft Agar Assay

For the anchorage independent growth assay, the bottom agar layer consisted of soft agar medium and 1% agar solution. Soft agar medium (2ml $NaHCO_3$ (110 mg/ml, in water bidest.), 1 ml glutamin, 17 ml water (bidest.) and 50 µl folic acid in 10 ml 10 x RPMI medium (Sigma)) was prepared the day before seeding of the cells. The solution was brought to a pH value of 8 and filtered. Afterwards, 20 ml FCS and 1ml penecilin-streptomycin were. To form the bottom layer, the solution was brought to a temperature of 37-40°C. For the 1% agar solution, 1.2 g agar was added to 80 ml water (bidest.) and solubilized by cooking. Simultaneously another 30 ml of water (bidest.) were cooked up and used to fill up the 80 ml suspension to 100 ml and finally kept at 40°C in the water bath.

The next day, 30.000 cells were pelleted at 800 rpm for 5 min and gently resuspended in 750 µl soft agar medium, pre-warmed to 37°C. The 750 µl 1% agar solution, which was kept at 41°C in the water bath was added to the cell suspension and mixed 1:1. The total volume of 1.5 ml was added on top of the bottom agar layer and gently shaken, thus resulting in a total volume of 3 ml per well of a six well plate. Clone size was quantified

using metamorph software and after the desired clone size was reached (>30 µm), the number of clones was counted under the microscope.

2.26. MTT Assay

For determination of cell viability, the MTT kit EZ4U from Biomedica was used according to the manufacturer's protocol. Approximately 2000 stably transfected cells were seeded in 100 µl 4% and 10% serum containing RPMI medium into each well of a 96 well-plate. After five days incubation, 20 µl of MTT tetrazolium salt solution were added in each well and incubated for 2-3 h at 37°C. Absorption was measured at 450 nm and 620 nm as reference using a Synergy spectrometer.

2.27. Transwell Migration Assay

For investigating cell migration, transwell chambers of 24 well-plate format were used. 40 000 sample and untreated control cells in 200 µl medium were seeded into porous chambers, 800 µl medium were pipetted into the well, resulting in a total volume of 1 ml. RPMI medium containing 4% and 10% FCS was used. Migration through porous chambers was visualized by crystal violet staining. Incubation times ranged from 24-72 hours.

2.28. Tumor Growth in SCID Mice

For xenotransplantation experiments, cells were seeded in 10% RPMI and incubated, until they were nearly confluent. Afterwards cells were counted by Casy. A final number of $1.2x10^7$ cells was pelleted and resuspended in

300 µl serum free RPMI medium (for 4 mice). All successive steps were done on ice. $1.5x10^6$ cells per mouse were injected subcutaneously. Tumor growth was checked every two to three days by palpation and measured with a calliper. Tumor volume was calculated according to the formula: shorter diameter^2 x longer diameter x 0.5. All animal experiments followed FELASA guidelines and were approved by the local ethics committee.

3. Results

3.1. Expression Analysis of Activin Family Members and Activin-Antagonizing Proteins in Melanoma Cell Models by Real-Time PCR

To verify the potential involvement of activin signals in melanoma, we have investigated the expression of all four human activin subunits and antagonistic factors, such as follistatins and cripto, in a panel of human melanoma cell lines (Figure 3.1, 3.2.).

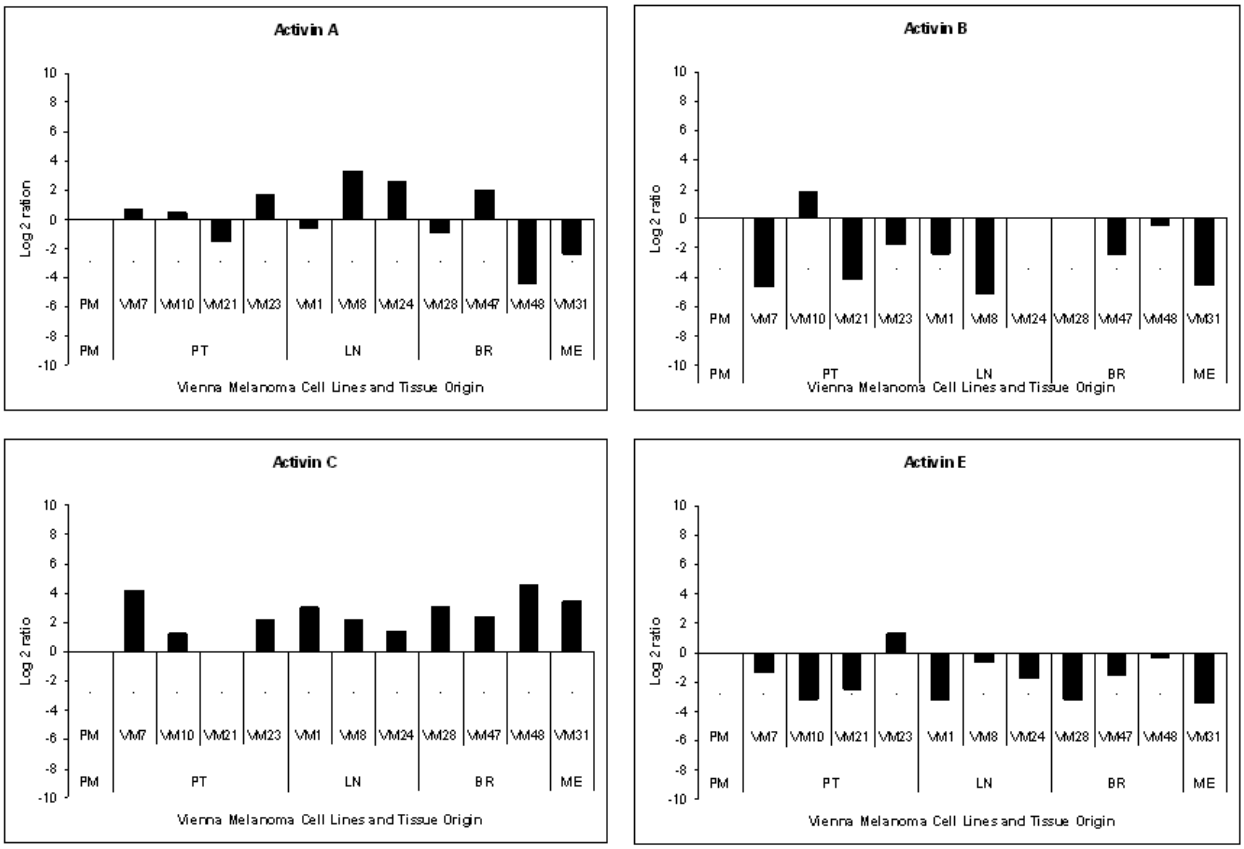

Figure 3. 1 Expression levels of activin subunits beta A, B, C and E in melanoma cell models established from primary tumors (PT) or metastatic lesions from lymph nodes (LN) or brain (BR) or from malignant effusions (ME). Each bar represents relative expression of the individual cell lines compared to primary melanocytes (PM). All detectable Ct-values were normalized to 18S-rRNA expression. Primary melanocytes were used as calibrator (set as 1). Fold expression values were calculated using the formula: 2^(-ddCT) and results were Log2 transformed.

Expression levels were investigated by real-time PCR in 12 human melanoma cell lines and normal melanocytes (PM).The activin subunits beta A, beta B, beta C and beta E were detectable in all cell lines. Elevated expression levels of activin beta C (10/12) were found in melanoma cell lines, compared to primary melanocytes. The expression of activin beta B (9/12) and activin beta E (11/12) in contrast, was clearly downregulated, whereas activin beta A showed up- or down-regulation depending on the cell line. Overall activin beta A had the lowest Ct values (range 25 – 35) corresponding to highest expression levels, whereas activin beta C was least expressed with most CT values above 35 (Figure 3.2).

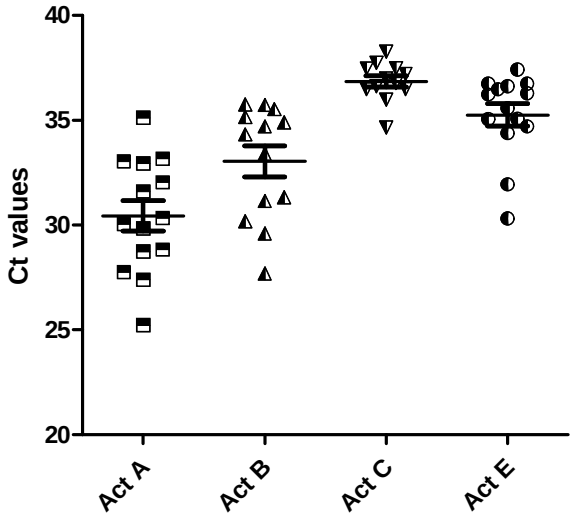

Figure 3. 2 The scatter plots show the expression levels of activin subunits in the melanoma cell lines. Values are given as cycle threshold numbers (Ct values). Each dot represents the mean of two determinations.

Activin antagonists were also detectable in all cell lines. Expression levels of follistatin (9/12) and cripto (12/12) showed mostly upregulation compared to primary melanocytes. On the other hand follistatin-like-1 and FLRG were up- or down-regulated, depending on the cell line (Figure 3.3). The lowest CT values (highest expression) were found for follistatin-like-1 (Figure 3.4).

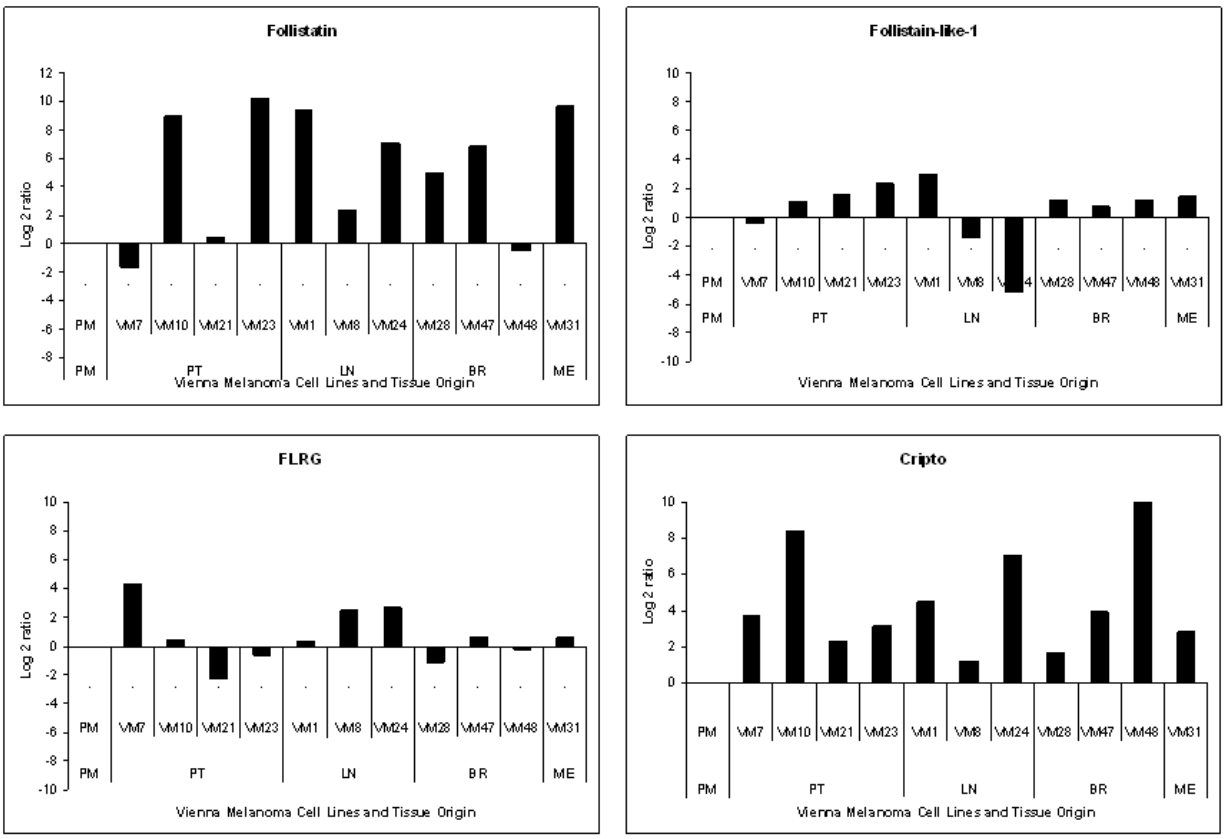

Figure 3. 3 The expression patterns of the activin-antagonizing proteins were determined by real-time PCR. Each bar represents the log2 transformed fold expression value compared to PM. PM: Primary Melanocytes; PT: Primary Tumor; LN: Lymph Node Metastases; BR: Brain Metastases; ME: Malignant Effusions.

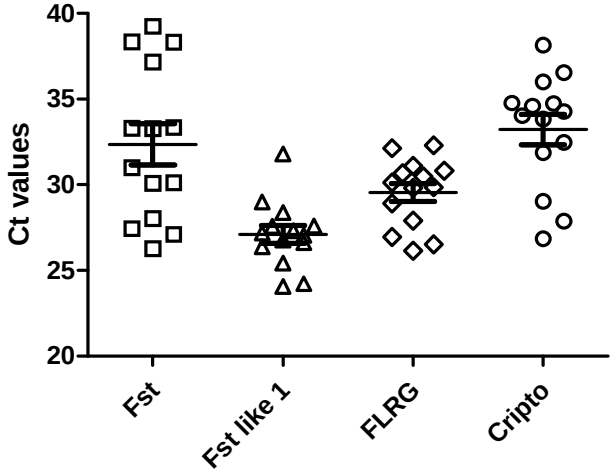

Figure 3. 4 Cycle threshold values plotted for follistatin, follistatin like-1, FLRG and cripto as means of duplicate samples.

3.2. Determination of Follistatin Isoform Expression by RT-PCR

Follistatin is produced by all melanomas according to quantitative expression analysis (Figure 3.3.). As follistatin can be expressed as two different isoforms termed Fst-315 and Fst-288, we tested, which isoform is predominantly expressed in melanoma models. Only one of the two isoforms of follistatin, encoding a 27 amino acid extention at the C-terminus, Fst-315 was found in nearly all melanoma cells, including primary melanocytes (Figure 3.5). Therefore, it appears that Fst-288 is not produced by most melanoma cells.

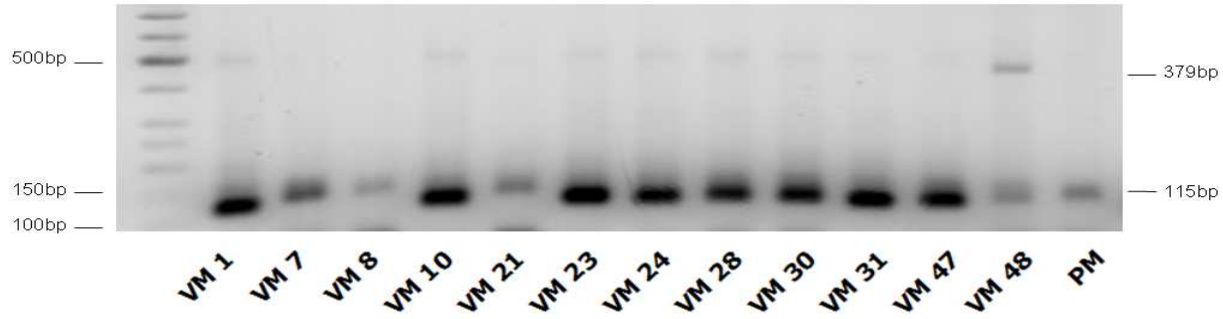

Figure 3. 5 RT-PCR analysis of Fst-315 and Fst-288 cDNA in the indicated cells. The long isoform-Fst315 (corresponding to the 115bp PCR fragment) is detectable in all cell lines, whereas the short form of follistatin (379bp) is produced at a significant level only in VM48 cells.

3.3. Generation of Stable Cell Lines

For further analysis of the role of activin family members or activin antagonists in melanoma, we focused on cell models with low endogenous expression levels to generate stable cell lines, which overexpress various activin or activin antagonizing proteins.

VM1, VM48 and VM21 cells were transfected with expression vectors coding for various variants of different activins and follistatins. As described in the materials and methods section, melanoma cells were transfected using FuGene 6 transfection reagent with plasmids expressing besides the gene of interest also GFP and/or a selection marker. Stable transfectants were successfully selected for resistance to G418 or puromycin antibiotic drugs. See table 3.1 for a list of stable cell lines, generated during this diploma thesis. Expression levels of stable cell lines transgenic for Fst were functionally verified by Western Blot analysis and by ELISA.

Table 3. 1 List of the generated stably transfected cell lines

Cell line	Plasmid used for transfection	Gene of interest to be overexpressed and function
VM48*	pfst315/EGFPe	Follistatin isoform 315
VM48	pfst288/EGFPe	Follistatin isoform 288
VM48	pIRESpuro-fstl-3	FLRG
VM48	pHsINHBA-IRESpuro	Activin A
VM48	EGFP-IRESpuro	EGFP/control
VM48	pEGFP-IRESneo3	EGFP/control
VM48*	pLuc-IRESe-EGFPneo	GFP-Luciferase/control
VM21*	pfst315/EGFPe	Follistatin isoform 315
VM21*	pLuc-IRESe-EGFPneo	GFP-Luciferase/control
VM21*	EGFP-IRESpuro	EGFP/control
VM1*	pfst315/EGFPe	Follistatin isoform 315
VM1*	pLuc-IRESe-EGFPneo	GFP-Luciferase/control
VM1*	EGFP-IRESpuro	EGFP/control

Cells, labeled with (*) in the table, were checked by FACS (Fluorescence Activated Cell Sorter) analysis for accurate quantification of fluorescent cells. The ell lines ectopically expressing follistatin 315 and respective GFP controls were then used for further characterization and investigations.

3.4. Confirmation of Overexpression of Follistatin in Follistatin Transfected Melanoma Cell Lines with Different Methods.

3.4.1 Smad Phosphorylation Western Blot

The Western blot is a reliable method used to detect specific proteins. Follistatin and GFP overexpressing melanoma cell lines were cultured for two days and supernatants were collected. HepG2 cells were treated with supernatants of these cells mixed with 5 ng/ml recombinant activin A and incubated for 30 minutes, in order to verify whether follistatin-315 transfected melanoma cells produce sufficient follistatin protein to inhibit

activin-mediated smad2 phosphorylation. Untreated HepG2 cells and HepG2 cells which were treated with 5ng/ml recombinant activin A or 5ng/ml recombinant activin A + 20ng/ml recombinant follistatin were used as positive and negative controls. Cells were harvested, lysed by adding Laemmli buffer and separated using SDS-PAGE gel. Western blotting was done using an anti-phospho-smad2 antibody and a total smad2/3 antibody as loading control.

When 5 ng activin A was added to the medium, smad2 phosphorylation was decreased in all melanoma cell lines overexpressing follistatin compared to those overexpressing GFP (Figure 3.6), implying that follistatin blocks activin activity. Of note, although stimulation using recombinant activin and follistatin reduces smad phosphorylation only about 20%, ectopically expressed follistatin can inhibit up to 75%. This means that the generated follistatin expressing melanoma cell lines contain enough follistatin to block activin activity. Follistatin expressing VM48 cell supernatants, however, inhibit smad phosphorylation to a much lesser degree than those from the other other two follistatin overexpressing cell lines.

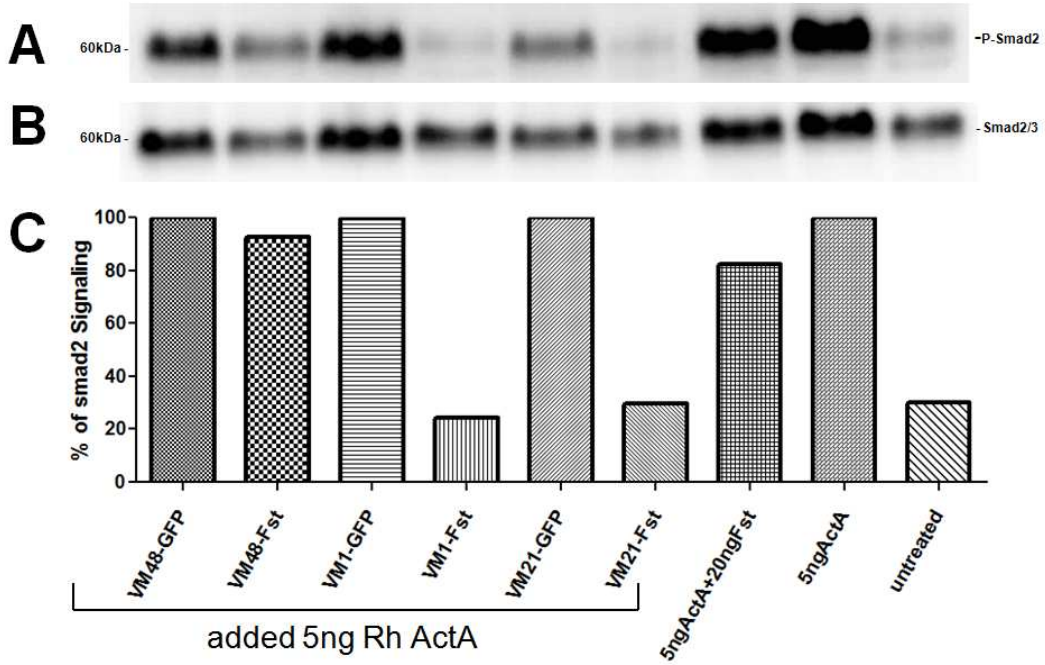

Figure 3. 6 Inhibition of activin A-induced smad signaling by follistatin overexpression. Cell lysates of HepG2 which had been incubated with supernatants of the indicated melanoma cells + 5 ng/ml recombinant activin A , were analysed using an anti-phospho-smad2 antibody (A) and a total smad2/3 antibody (B) and signal intensity quantified by ChemiCapt and Bio1D software (C). Smad2/smad2/3 ratios were calculate and those derived from the respective GFP control supernatans were set as 100%.

3.4.2. Analysis of Follistatin Overexpression by ELISA

Before analysing the influence of follistatin overexpression on cell proliferation, differentiation, or migration in melanoma cell lines, we first determined the amount of follistatin secreted by the cells, into the culture media by immunoassay.

ELISA is an effective method for quantitative determination of human follistatin concentration in cell culture supernatants from serum free media containing secreted follistatin proteins. The assay was performed according to the Quantikine-Human Follistatin Immunoassay protocol.

This immunoassay is calibrated with recombinant human follistatin, and detects all isoforms of follistatin. All melanoma cells were grown to about 80% confluence in serum containing medium in 6 well plates, prior to 48h incubation in serum free medium, which was then harvested and used for the analysis. For standard curve generation, recombinant human follistatin proteins were added to RPMI media at a concentration ranging from 250 pg/ml to 16.000 pg/ml.

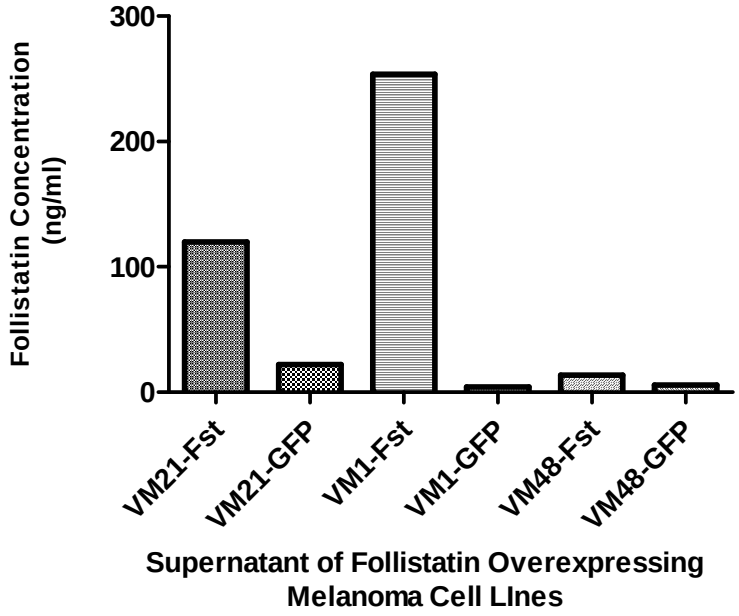

Figure 3. 7 Expression of follistatin was confirmed and quantified by detecting follistatin in the supernatant of transfected VM1, VM21 and VM48 cells by ELISA using different concentrations of human recombinant follistatin protein as standard.

In all cases, except follistatin transfected VM48 cells, overproduction of follistatin compared to GFP controls was confirmed. Supernatants of follistatin transfected VM21 cells contained approximately 120 ng/ml follistatin, and those of VM1 cells even 250 ng/ml (Figure 3.7).

Together with the smad2 Western analysis, these results show that follistatin transfected cells secrete enough follistatin to regulate activin activity via the smad signaling pathway.

3.5. Functional Analysis of Follistatin Overexpressing Melanoma Cells

3.5.1. Cell Proliferation / MTT Assay

In order to test the impact of follistatin on cell proliferation, follistatin overexpressing VM1, VM21, and VM48 cells and their respective GFP containing controls were seeded into 96-well plates at low densities. After 5 days, cell number was measured by MTT assay using staining and spectroscopic methods as described under 2.24.

The results clearly show that follistatin overexpression has a different effect on cell proliferation depending on the cell lines (Figure 3.8). Apparently, follistatin mediated blocking of activin bioactivity leads to a decreased MTT-reducing activity of about 60% in VM21 cells, compared to the GFP control. In VM48-Fst cells proliferation was decreased also by around 20% compared to VM48-GFP when cells were grown in medium with 4% FCS, despite the low ectopic expression level of follistatin according to the ELISA analysis. On the other hand proliferative activitiy of VM1 cells, which expressed a high level of ectopic follistatin, was not reduced. Additionally, we observed that the effect of follistatin on proliferation capability was significantly influenced by adding different concentration of FCS to the cell culture media. In medium with 10% FCS neither VM1-Fst nor VM48-Fst cells showed any difference in growth compared to GFP controls, whereas the growth reduction of VM21-Fst cells was even more pronounced.

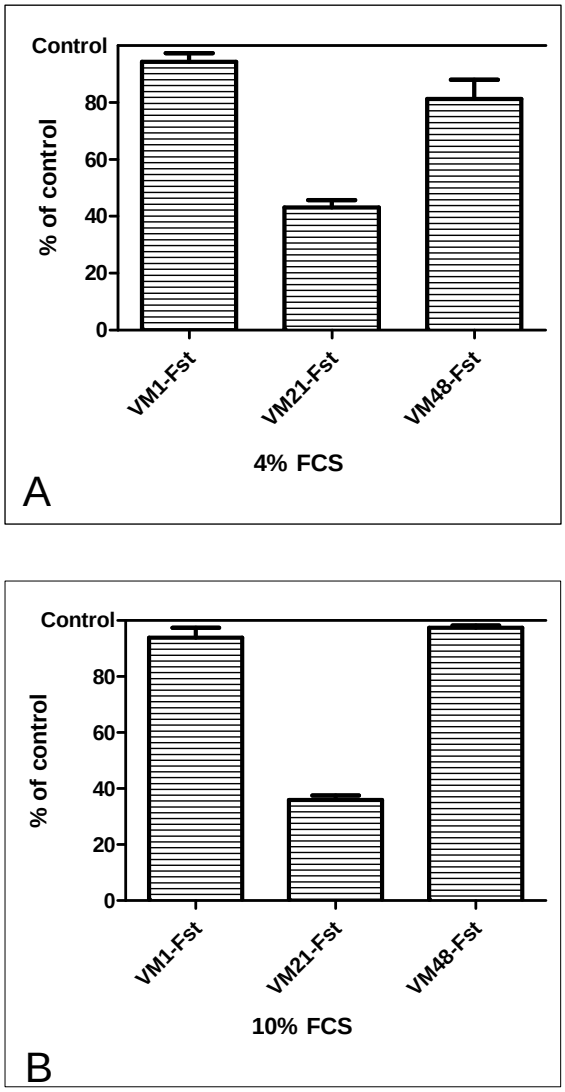

Figure 3. 8 Cell proliferative activity of follistatin overexpressing melanoma cells by MTT assay. Melanoma cells stably transfected with follistatin or GFP (as control) were incubated in medium with 4% (A) as well as 10% (B) FCS for five days. Two independent experiments were performed in duplicates for three different VM cell lines.

3.5.2. Growth Curve Assay

Next, growth curves of the follistatin overexpressing cell lines were determined. Cell lines, which were stable transfected with follistatin and GFP constructs, were seeded at low density and cell numbers were measured every two days until the eighth day, using a Casy Cell Counter (Figure 3.9). In agreement with the proliferation assay (3.8.), cell VM21-Fst showed also a reduced growth, whereas no significant differences between follistatin and GFP transfected cells was seen in VM1 and VM48 cells.

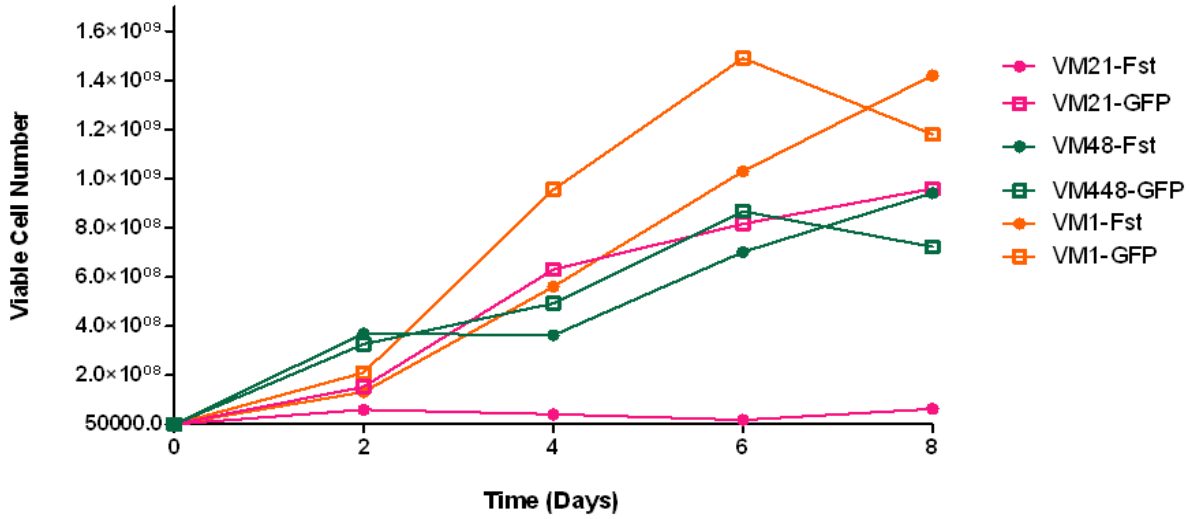

Figure 3. 9 Growth curve assay for three VM cell lines. Cells were incubated in 10% FCS containing medium. Viable cell number was measured every two days after seeding until the eighth day using a Casy Cell Counter.

3.5.3. Clonogenic Assay

Clonogenic assays were performed to investigate the role of overexpresed follistatin in colony formation ability of human melanoma cells. Follistatin transfected cells were seeded at densities resulting in formation of visible colonies within 1–3 weeks. Colonies were fixed with methanol and stained with crystal violet (1%). They were quantified manually under a microscope (Figure 3.10).

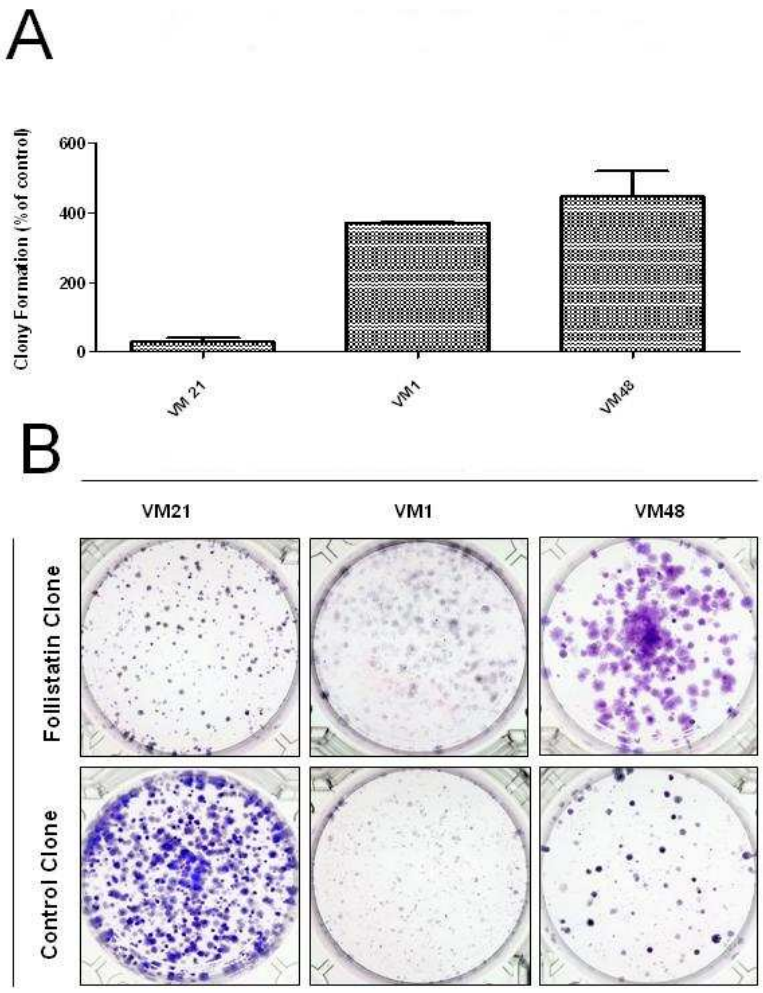

Figure 3. 10 Effect of follistatin overexpression on colony formation. (A) Number of clones in follistatin transfected cells (compared to GFP controls) (B) Representative examples.

The results showed that overexpression of follistatin caused a significant inhibition of number and size of surviving colonies from the VM21 cell line when compared with GFP transfected control cells, whereas VM1 and VM48 cells showed a clearly increased colony formation compared with the controls.

3.5.4. Soft Agar Assay

To test whether follistatin influences the ability of melanoma cell lines to grow in soft agar and become anchorage independent VM1-Fst and VM21-FST cells and respective GFP controls were seeded in 10% RPMI medium containing agar. They were incubated for four weeks and observed for colony formation. Quantification was performed by counting the number of clones with a clone size >30µm (Figure 3.11 and 3.12).

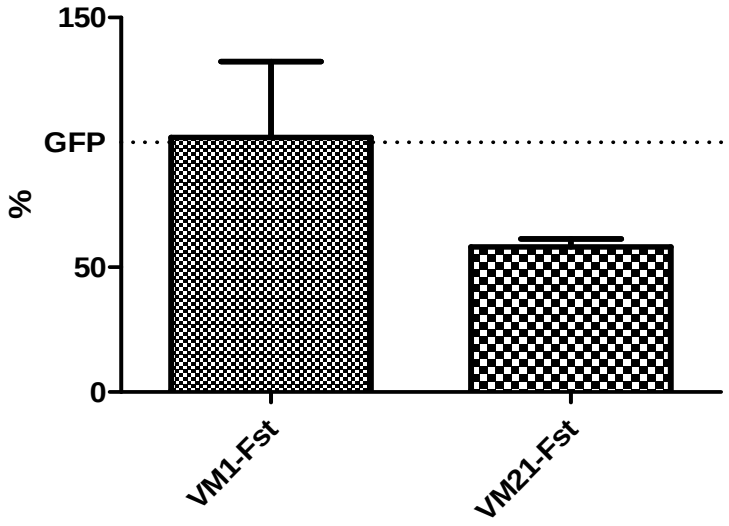

Figure 3. 11 Soft agar colony formation for follistatin expressing VM1 and VM21 cells. Bars show the averages of the two independent wells. GFP expressing controls for each melanoma cell line were used as reference and set as 100%

Results indicated that anchorage independent growth was reduced by about 45% in follistatin overexpressing VM21 cells compared to GFP expressing VM21 cells, whereas anchorage-independent growth in VM1 cells was not different.

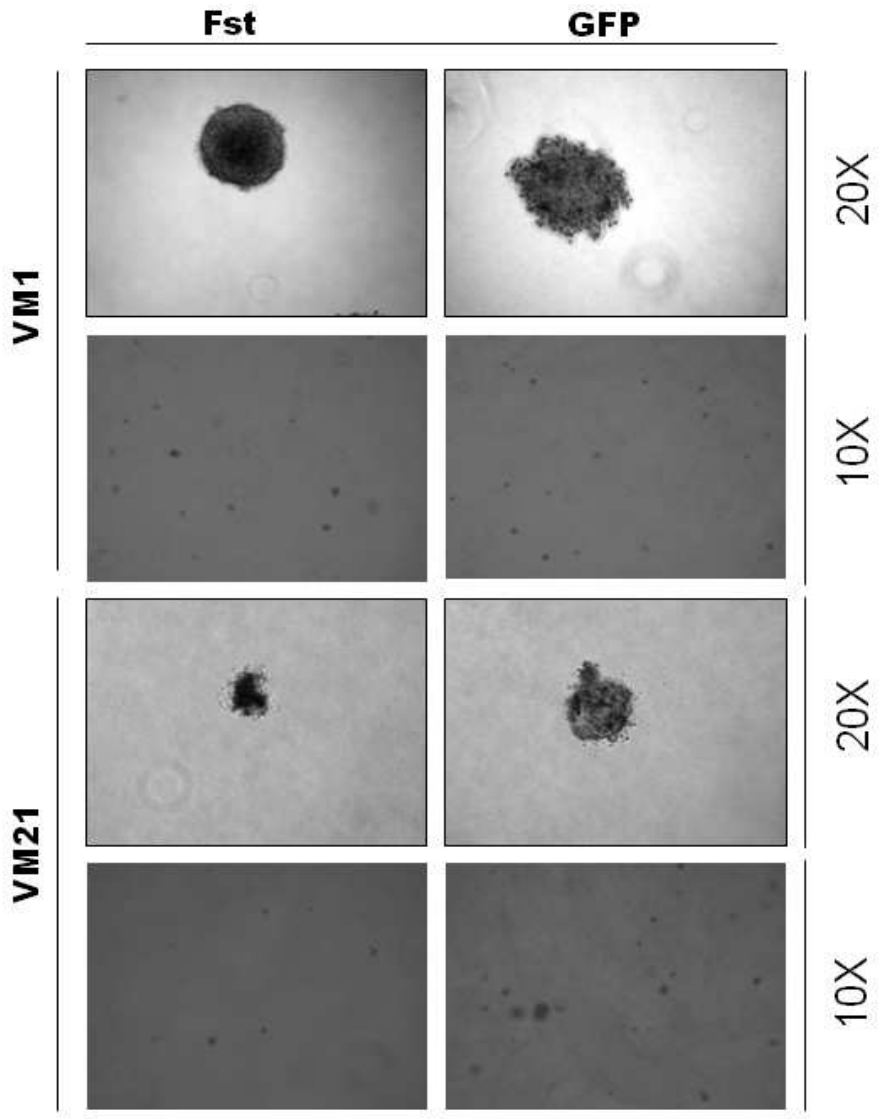

Figure 3. 12 Examples of melanoma cell clones growing in soft agar. Follistatin and GFP expressing melanoma cells were plated into soft agar and resulting colonies counted after four weeks. The number of colonies was counted at four different areas per well.

3.5.5. Migration Assay

Transwell assays were used to investigate, whether overexpression of follistatin and accordingly activin blocking in melanoma cells affects their migration potential.

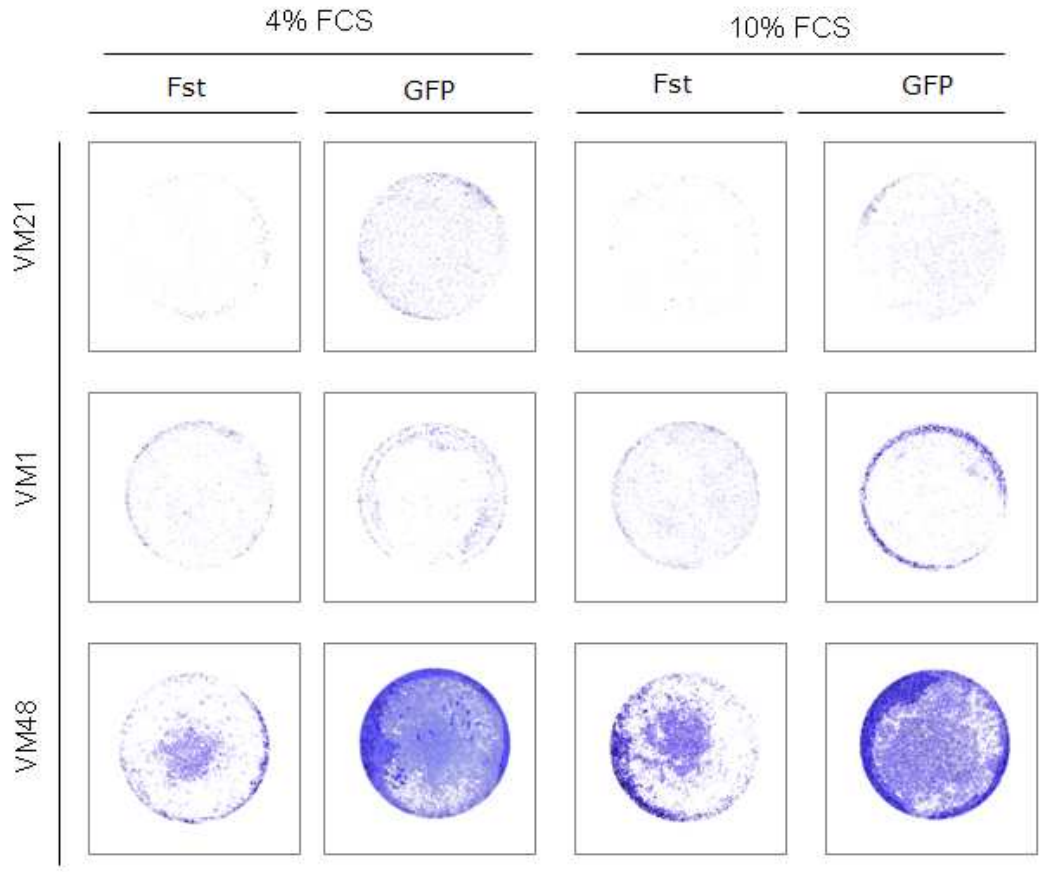

Figure 3. 13 In vitro migration of follistatin-expressing melanoma cells. Cell migration was measured using transwell chamber migration assays. 40000 cells were seeded into a transwell chamber in media containing 4% FCS or 10% FCS and incubate for 72 hours. Cells which migrated through the porous membrane to the bottom surface of the membrane were stained with crystal violet and staining was quantified by Lucia Software Representative examples of two independent experiments done in duplicates.

In the transwell migration assay, cells in medium containing 4% and 10% serum were added to the upper chamber and allowed to migrate through a porous filter. Filters were stained with crystal violet and then cells, which had not migrated through the filter, were scratched off the upper surface,

to detect only those cells that had migrated to the lower surface (Figure 3.13).

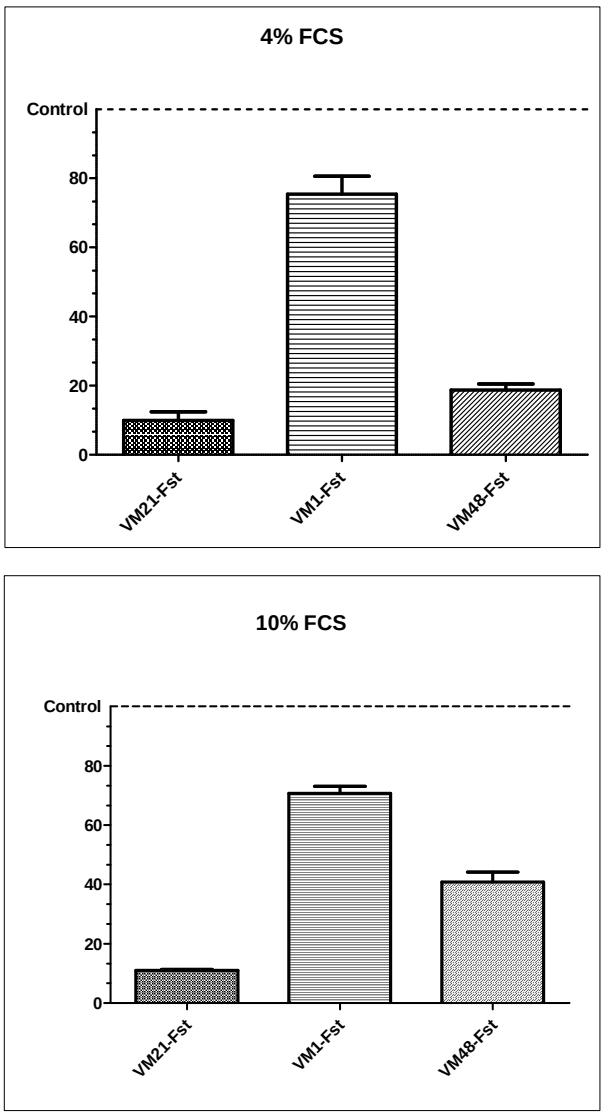

Figure 3. 14 Quantification of transwell assay results. Cells were seeded and stained as described in figure 3. 13. Bars represent two independent sets of experiments.

VM1 and VM21 melanoma cell lines showed a generally lower migratory activity compared to VM48. In all three cell lines tested, the follistatin expressing cells had a reduced migratory activity compared to the respective GFP controls, suggesting that activin signaling may be necessary for cell migration. Additionally, cell migration ability was similar, when the cells were grown in 4% or 10% FCS.

3.5.6. Tumor Growth in a SCID Mouse Model

Finally, the ability of follistatin-transfected versus GFP-transfected VM21 cells to establish tumors in SCID mice were investigated. The clones of follistatin-expressing VM21 cells as well as GFP-transfected VM21 cells were injected subcutaneously into SCID mice (4 per group). All mice injected with VM21 cells developed tumors. However, there was no obvious difference in tumor growth between VM21-GFP and VM21-Fst cells with respect to tumor volume. Mice with VM21-GFP tumors seemed to survive a little bit longer than those with tumors from VM21-Fst cells (Figure 3.15, 3.16). Upon dissection of the mice it was noted that two of the VM21-GFP tumors had developed metastatic lesions adjacent to the main tumor and in the lymph nodes. The tumors as well as lungs, brains, livers, and bones (femurs) of the mice were fixed in formalin and embedded in paraffin for further analysis in subsequent projects.

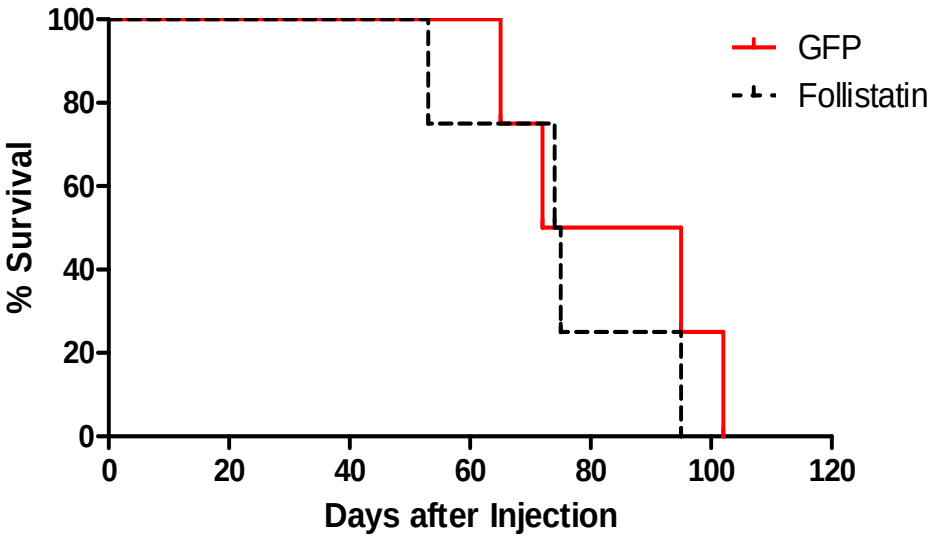

Figure 3. 15 Percent survival of SCID mice bearing tumors from follistatin-expressing and GFP-expressing VM21 cells.

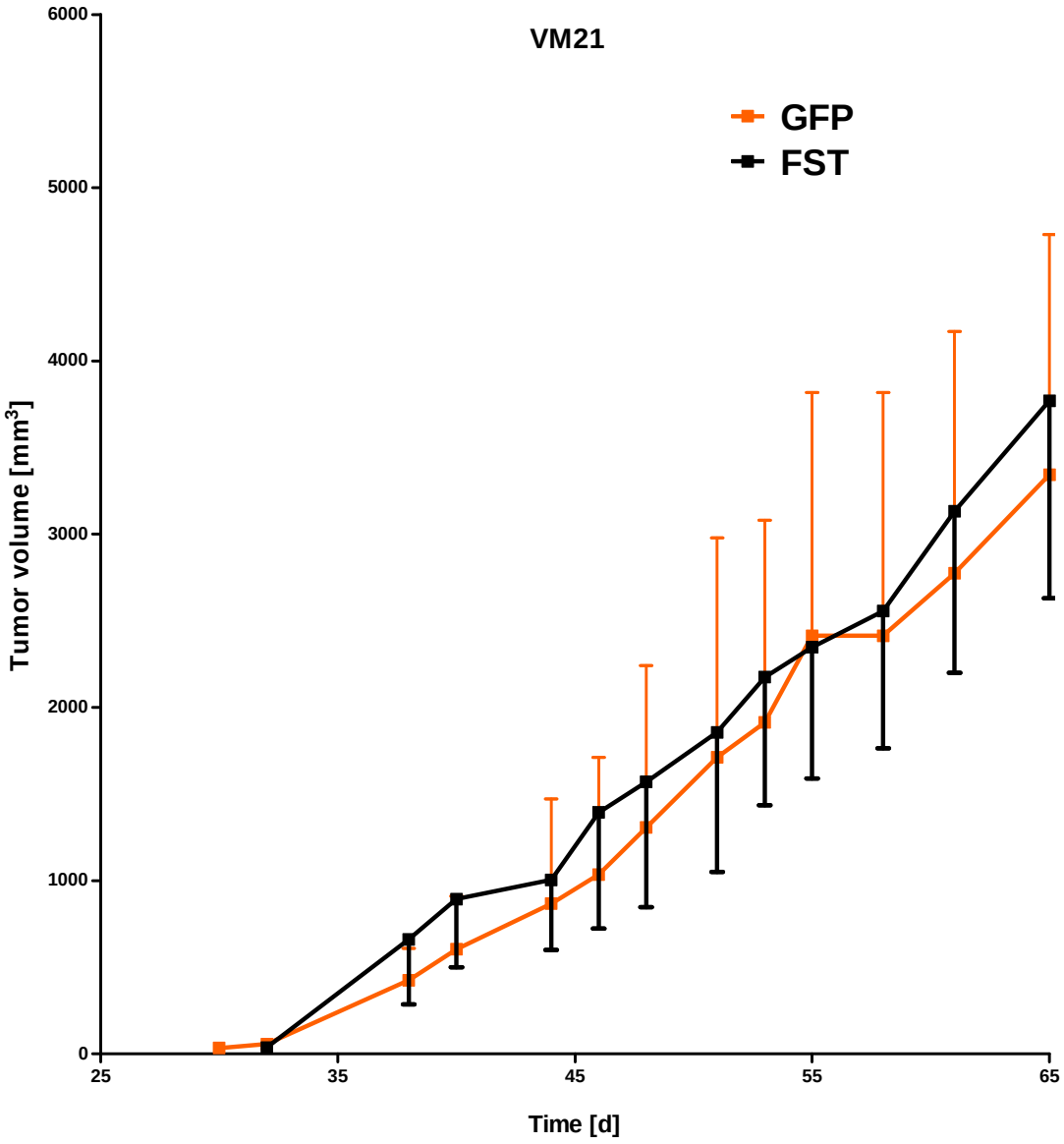

Figure 3. 16 VM21 cells ($3x10^6$ in 50 µl PBS) were injected subcutaneously into SCID mice. The tumor size was measured in two dimensions (a,b) and the tumor volume (mm^3) was calculated by using the formula: $V=(a^2xb)/2$; b>a. Mice were sacrificed when the tumor volume reached a size of about 5000 mm^3.

3.6. Impact of the Activin Beta E Subunit on Signal Transduction via the Smad Pathway

The signaling mechanisms of activin E (as homodimer of two beta E subunits) or of heterodimeric activins containing a beta E subunit are currently unknown. To elucidate the impact of the beta E subunit on signal transduction via smad2, 293 cells (which have a very high transient transfection rate) were transfected with various combinations of cDNAs leading to expression of activin beta E, activin beta A, activin antagonists and yellow or cyan fluorescent protein (YFP, CFP). Then we treated hepatoma cells (HepG2) with these conditioned media and analyzed smad2 phosphorylation by Western blot.

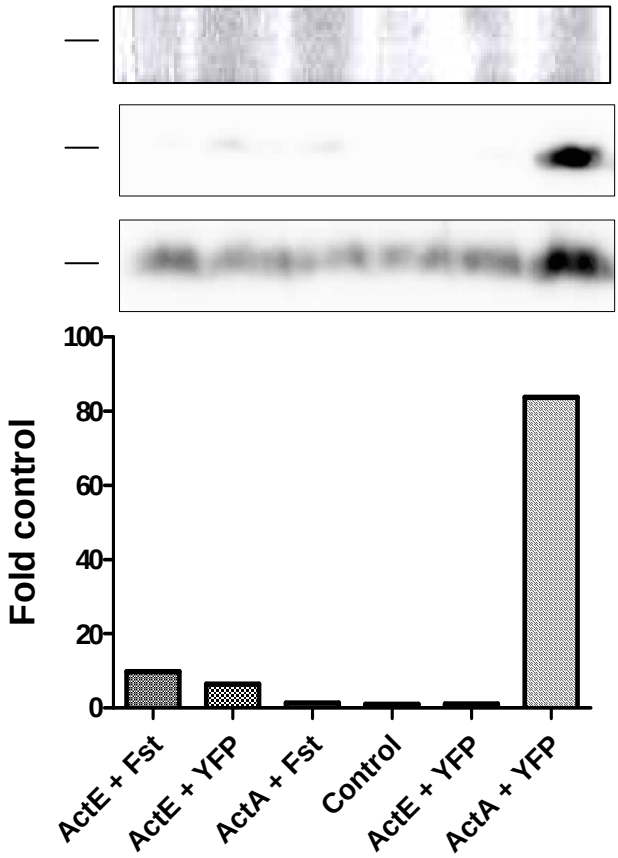

Figure 3. 17 Efect of conditioned media from 293 cells transfected with activin beta A, activin beta E, follistatin-315 and YFP on smad signaling in HepG2 cells. Bars represent the ratios of the psmad2/smad2/3 signals. Unstimulated HepG2 cells (control) were set as 1. Ponceau staining is shown as additional loading control.

Activin A-conditioned medium induced smad2 phosphorylation in HepG2 cells, whereas the activin E-conditioned medium had no effect (Figure 3.17). Follistatin binds to activin A with high affinity and blocks interaction of activin receptor with its ligand (Nakamura, Takio et al. 1990). In consequence, conditioned media from 293 cells co-transfected with activin beta A and follistatin did not lead to smad2 phosphorylation confirming that follistatin blocks specifically the activity of activin A but not activin E in HepG2 cells.

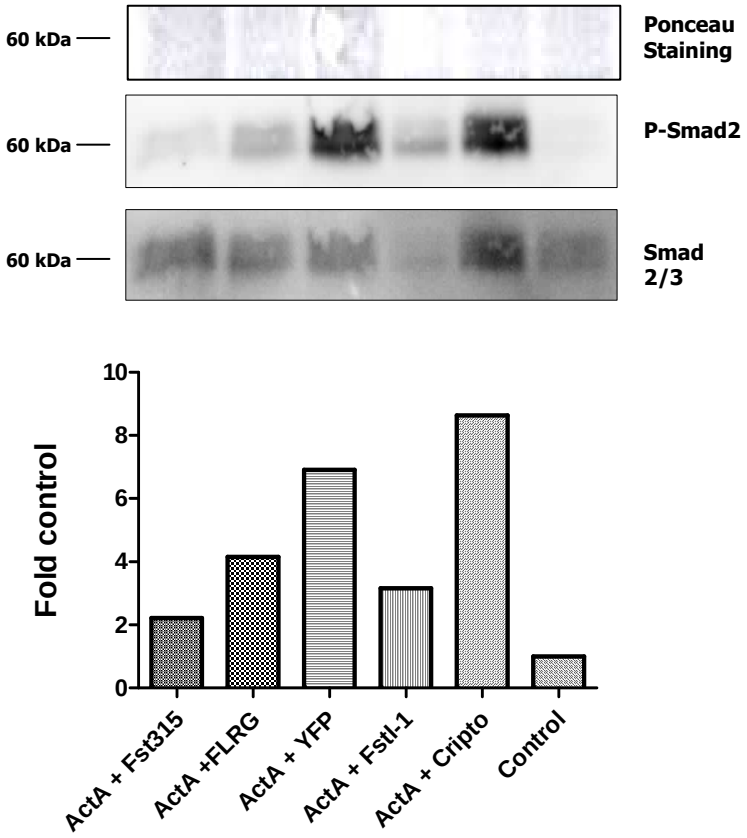

Figure 3. 18 Stimulation of smad2 phosphoryation by treatment with activin A-conditioned medium in VM 48 cells and effect of different inhibitors. Activin can be blocked by follistatins, but not by cripto. Fold control was calculated using the formula: (psmad2)/(smad2/3) and untreated controls were set as 1.

Activins interact with various binding proteins, which include antagonists in the extracellular space (follistatin and FLRG) and antagonistic co-receptors at the cell membrane (cripto). Deregulation of activin signals has been found in several tumor types and linked to the malignant growth of tumor cells (Deli, Kreidl et al. 2008). Follistatin is an important and critical regulator of activin in many adult tissues, including melanoma. Compared to follistatin, less information is available about the biological activity and physiological actions of follistatin-like-1, cripto and FLRG. To test the ability of different antagonists to inhibit activin signals in melanoma cell lines, VM48 cells, which expressed only low levels of activin antagonists (see Figure 3.1.), were treated with conditioned media from 293 cells co-transfected with cDNAs coding for acitivn beta A and different activin antagonists.
Our observations indicated that follistatin can block activin-induced smad2 phosphorylation approximately 2 times more efficiently than FLRG or fstl-1, whereas cripto was unable to block smad2 phosphorylation (Figure 3.18).

Next, it was tested, whether co-transfection of activins beta E and A could alter the smad2 phosphorylating activity of conditioned media, and whether the additional presence of follistatin would influence the result (Figure 3.19)

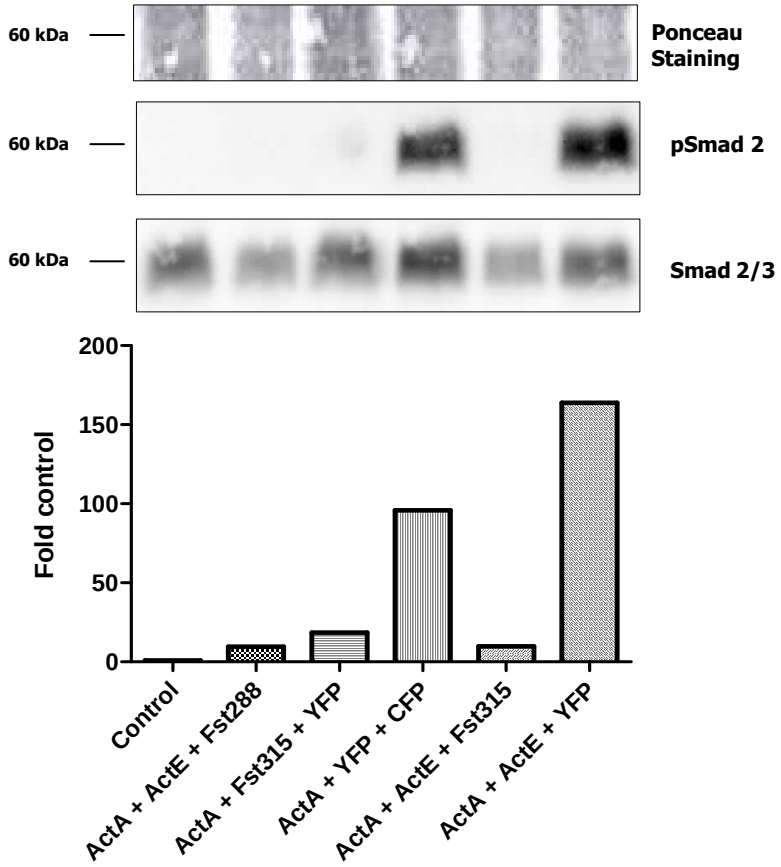

Figure 3. 19 In HepG2 cells, both isoforms of Follistatin (288 and 315) are effective in blocking activin A signals. Expression of the beta E subunit seems to slightly enhance stimulation by activin beta A, but does not influence blocking of activin signals by follistatin. Untreated control was set as 1.

Results indicate that activin beta A and E co-transefected conditioned medium caused significant induction of phosphorylated smad2 similar to conditioned medium from cells transfected with beta A alone. Co-transfection with follistatin (either the 288 or 315 isoform) in all cases abrogated smad2 phosphorylation. The results suggest that activin beta E does not interfere with activin A signaling directly or indirectly via follistatin.

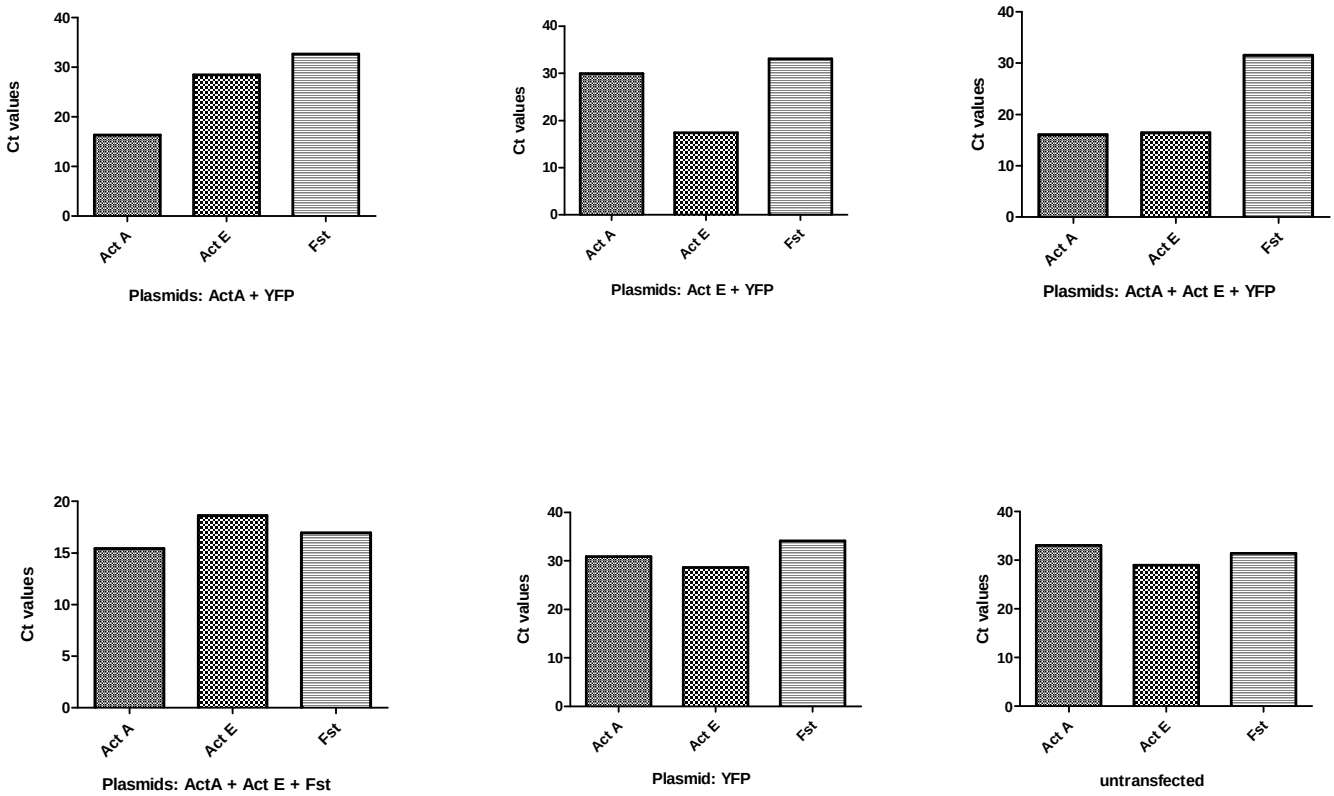

Figure 3. 20 Transcript expression of activin beta A, activin beta E and follistatin in 293 cells after transfection with the indicated transgenes. Delta CT values were calculated by subtracting the CT values of 18S ribosomal RNA from the CT values of the respective analysed gene. Transfections: (A) activin beta A and control vector YFP in co-transfected Hek293 cells; (B) activin beta E and control vector YFP in co-transfected Hek293 cells; (C) activin beta A, activin beta E and control vector YFP in triple-transfected Hek293 cells; (D) activin beta A, activin beta E and follistatin in triple-transfected Hek293 cells. (E) Endogenous expression levels of targeted molecules in YFP transfected and in (F) untransfected 293 cells.

To verify in 293 cells overexpression of the different transgenes, which were used to produce the conditioned media described above, RNA was isolated from 293 cells 48 hours after transfection, reverse transcribed and expression levels of activin beta A, beta E, and follistatin were determined with Taqman assays. The 18S ribosomal RNA was used as reference and delta CT values were determined. Gene expression level and delta CT values have an inverse correlation, because lower CT value reflects increased gene expression, in other words, a high efficiency of transgene expression. These results revealed that both of activin subunits and follistatin were easily detectable and that ectopic expression resulted in a specific decrease of the CT value for the respective transcript of at least 10 cycles compared to the endogenous expression, corresponding to 1000-fold overexpression (Figure 3.20).

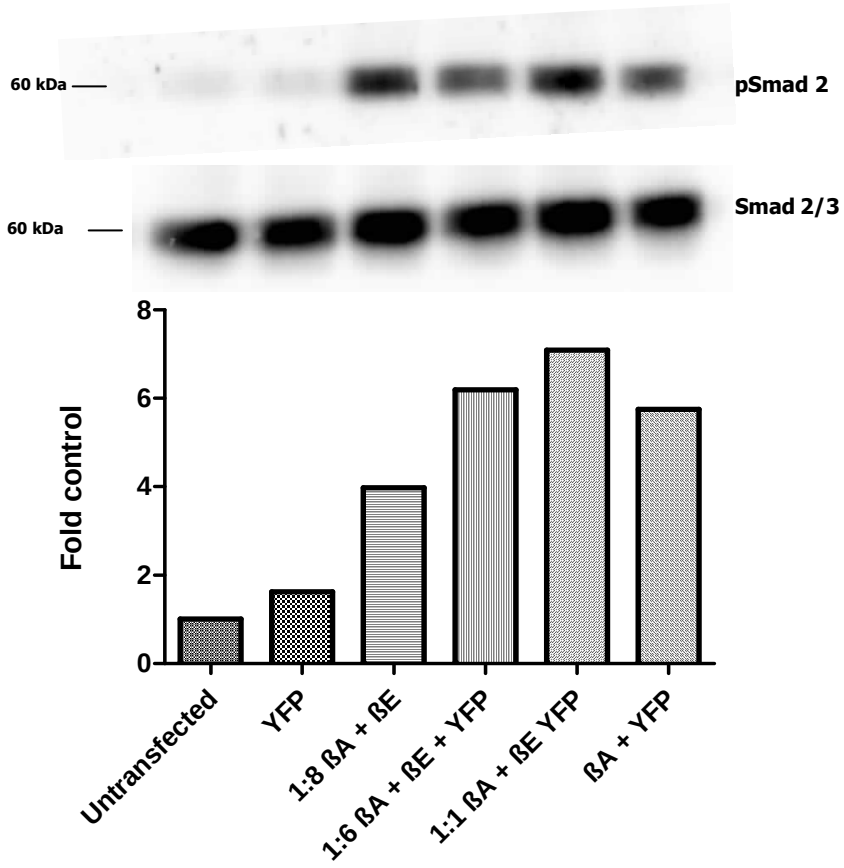

Figure 3. 21 Influence of different ratios of activin beta A and beta E cDNAs on smad2 phosphorylating activity of conditioned media. Conditioned media were generated and smad2 phosphorylation determined in HepG2 cells as described above. Bars represent psmad2/total smad2/3 ratios.

Finally, it was investigated whether the beta E subunit might influence activin A-induced smad2 phosphorylation when expressed in excess of activin beta A, as is the situation in normal hepatocytes. For that purpose, we prepared conditioned media from 293 cells after co-transfection of activin beta A and activin beta at different ratios (1:1 activin beta A + activin beta E; 1:6 activin beta A + activin beta E; 1:8 activin beta A + activin beta E) and treated HepG2 cells as above. Results indicate that conditioned media from 293 cells transfected with activin beta A (500 ng plasmid DNA) induced smad2 phosphorylation independent of the amount of co-transfected activin beta E plasmid.

4. Discussion

4.1. Expression Analysis of Activins and Follistatins

Activins have been found involved in the control of many cellular processes, ranging from the control of hemeostasis to cell growth, differentiation and tissue patterning during embryogenesis in multiple adult tissues (Ball and Risbridger 2001; Luisi, Florio et al. 2001; Chen, Lui et al. 2002; Stove, Vanrobaeys et al. 2004). A previous study has indicated that follistatin is highly expressed in the majority of melanoma cell lines (Stove, Vanrobaeys et al. 2004). The present study has demonstrated that all melanoma cell lines investigated expressed activin subunits and activin antagonistic proteins and showed smad phosphorylation upon treatment with activin. Activin beta A was up- or downregulated depending on the melanoma cell line, whereas activin beta C was upregulated in the majority of cell lines in our panel compared to primary melanocytes. With respect to signal transduction and function, however, activin beta C has also been described as inhibitor of activin A (Butler, Gold et al. 2005; Gold, Jetly et al. 2009). In contrast, activin E and activin B were demonstrated to be mostly downregulated compared to primary melanocytes. Downregulation of activin A has also been observed in high-grade breast cancer and transient upregulation of activin E in rodent liver after partial hepatectomy or portal vein branch ligation (Huang, Li et al. 2002; Jeruss, Sturgis et al. 2003; Takamura, Tsuchida et al. 2005).

Since activin A signals have a pro-apoptotic and growth inhibitory function in many cell types it has been suggested that cancer cells may escape from the effects of activin by several alternative ways; by downregulation or mutation of activin responsive genes or by alterations interfering with the activin signaling pathway (Chen, Lui et al. 2002). Alternatively, several factors such as follistatin, inhibin or cripto inhibit the ability of activin to gain access to its receptor (Gumienny and Padgett 2002; Stove,

Vanrobaeys et al. 2004). In our investigation, follistatin and cripto were upregulated in the majority of melanoma cell lines compared to primary melanocytes, whereas no clear trend towards up- or down-regulation was seen for FLRG and follistatin-like-1. As previously reported, follistatin is expressed in most of the organs, which also express activin related proteins (Michel, Rao et al. 1991; Tuuri, Eramaa et al. 1994) and the highest expression of follistatin was found in testis, ovary and pituitary (Tortoriello, Sidis et al. 2001). However, in some animal models including human liver cancer expression of follistatin levels was increased in about 60% of tumor tissues (Grusch, Drucker et al. 2006). Whereas the highest tissue expression of FLRG was found in placenta (Tsuchida, Arai et al. 2000; Tortoriello, Sidis et al. 2001), in contrast to follistatin, FLRG is expressed at high level in normal liver but downregulated in human liver tumors. In accordance with this finding, the data from this thesis suggest that malignant melanoma cells and primary melanocytes follow a similar pattern as liver tumor cells and normal liver cells with respect to follistatin and FLRG expression. Overall it appears that the balance of activins to activin antagonists is shifted towards the antagonist side in melanoma cells compared to primary melanocytes.

4.2. Effect of Overexpression of Follistatin on Melanoma Cells

As most of the melanoma cell lines showed high expression levels of follistatin, the consequences of high follistatin levels for the malignant behavior of melanoma cells was investigated. For that purpose three different cell lines, two of them (VM21 and VM48) with low endogenous expression) were stably transfected with follistatin and subjected to various growth assays. We could demonstrate that stable transfection with follistatin clearly decreased the number of cells compared with GFP control in VM21 cells. This effect was investigated using two different detection methods in three different cell lines (VM1, VM21 and VM48). The

metabolic activity of cells (MTT assay) and cell number were determined. Stove et al. showed that exogenous activin is able to decrease proliferation and induce apoptosis in primary melanocytes (Stove, Vanrobaeys et al. 2004). Consequently, we speculated that ectopic expression of follistatin might lead to increased proliferation via blockade of endogenously produced activin A, especially in melanoma cells with low endogenous follistatin expression. However, while melanoma cells ectopically overexpressing follistatin showed decreased smad2 phosphorylation when stimulated with exogenous activin A, none of them had a growth advantage and VM21 even showed reduced proliferation. A similar decrease compared to GFP controls was found for VM21 cells also with respect to colony formation, migration in transwell assays, and anchorage-independent growth in soft agar. VM1 and VM48 cells showed increased colony formation but decreased migration. While high expression of endogenous antagonists like cripto could account for lack of response to follistatin overexpression, the growth inhibitory effect of follistatin could either mean that activin signals may be growth stimulatory for melanoma cells as has been reported for several tumor types like esophageal carcinoma (Yoshinaga, Yamashita et al. 2008), or lung cancer (Seder, Hartojo et al. 2009) or that follistatin acts via induction or inhibition of some other genes involved in cell cycle regulation. The importance of aberrant cell cycle regulation by signaling pathways in melanoma has previously been highlighted by the observation that almost 70% of melanomas and primary nevi have kinase-activating mutations in BRAF (Davies, Bignell et al. 2002; Pollock, Harper et al. 2003; Goodall, Wellbrock et al. 2004). Furthermore activating mutations in BRAF inducing activation of MAP kinase signaling pathway are key characteristics of melanoma (Davies, Bignell et al. 2002). This constitutive activation of the MAP kinase cascade might switch activin signals from growth inhibitory to growth promoting and these pro-tumorigenic activities of activin signals might be antagonized by follistatin.

In summary, overexpression of follistatin in human malignant melanoma could decrease their proliferation via two different signaling pathways, blocking of the activation of smad family of transcription factors and interference with TGF-β family receptor-mediated crosstalk with the BRAF/MAP kinase dependent signaling intracellular pathway (Grusch, Petz et al. 2010).

It has been shown that melanoma invasion requires migration through the vascular barrier and is necessary for the adhesion of metastatic cells to the endothelium (Teti, De Giorgi et al. 1997). Results of the migration assays indicate that cell migration levels were reduced, when activin which shares the intracellular signaling pathway with TGF-β was blocked by overexpressed follistatin. This suggests that activin-mediated smad signaling may be necessary for cell migration and activin could be one of the genes involved in the coordinated expression changes associated with migration.

As known, follistatin can regulate also potentially tumor growth. Krneta et. al. demonstrated that activin-expressing mammary carcinomas grow faster than follistatin-expressing tumors, which grow slower than activin-transduced or mock-transduced tumors (Krneta, Kroll et al. 2006). Additionally, follistatin transfectants of small cell lung cancer cells induced fewer metastatic colonies in multiple organs, when compared with their control clones (Ogino, Yano et al. 2008). Deregulation in the activin signalling pathway may also result in disturbances of normal bone metabolism and has been implicated in bone metastasis in prostate cancer (Leto 2010).

In this thesis, VM21 cells overexpressing follistatin showed no difference compared to GFP controls with respect to tumor volume in a SCID mouse xenotransplant model. However, follistatin expressing tumors did not lead to metastasis compared with control tumors. During dissection of the mice, metastatic lesions were observed in the lymph nodes of the triangle

of the neck in two of four mice bearing VM21-GFP cells but not in mice bearing VM21-Fst cells. Tumors and several organs including lungs and bones of the mice have been fixed and paraffin embedded and will be analysed for the presence of metastases in future work.

4.3. Understanding the Signalling Pathway of Activin E

Members of the TGF-β superfamily execute their biological activities by binding serin-threonine kinase receptors followed by phosphorylation of smad proteins. The first branch of the smad signaling pathway uses smad2 and smad3 and is shared by TGF-β activin and nodal, the second branch of the signaling pathway uses smad1, smad5 and smad 8 and is shared by BMPs and GDFs (Ethier and Findlay 2001). To elucidate whether activin E could activate smad2 signaling, a plasmid expressing activin beta E was transiently transfected into 293 cells and supernatants were collected and used for treatment of HepG2 cells. A plasmid expressing activin beta A was used as positive control. Treatment of HepG2 with activin E conditioned media showed no smad2 signal. Thus our investigation of the signaling pathway of activin E suggests that smad2 can not be activated by activin E. However as no receptors for activin E have been identified, it cannot be ruled out at present that failure to activate smad2 is caused by the lack of expression of a suitable combination of receptors for activin E in HepG2 cells.

As mentioned in section 3.1. follistatin is an activin binding protein and antagonizes the binding activity of activin A to its receptor, forming an inactive complex (Nakamura, Takio et al. 1990; de Winter, ten Dijke et al. 1996). It has been previously demonstrated that the endocytotic internalization of activin A was accelerated by follistatin into pituitary cells leading to lysosomal activin degradation. Activin E can also bind to follistatin (Hashimoto, Tsuchida et al. 2002). Therefore we hypothesized

that activin E could sequester follistatin into a complex, which could result in an increased bioavailability of activin A. However, also according to our results even an excess of activin E did not impair the ability of follistatin to block activin A-induced smad phosphorylation in HepG2. One reason for this could be a much higher affinity of follistatin for activin A than for activin E.

According to our western blot results, follistatin blocked the activin A binding affinity to its receptor. We observed also that follistatin-like-1 and FLRG inhibit activin A activity, albeit with lower efficiency. This is surprising for follistatin-like-1 as it contains only one follistatin domain and was therefore not expected to bind activins.

To investigate whether activin beta E has an effect on activin A-induced smad phosphorylation, we performed co-transfection with present of both proteins. It was hypothesized that activin beta E might alter activin signals via formation of a heterodimer with the beta A subunit. It was previously suggested that activin beta C might form an inactive heterodimer and thereby reduce the amount of dimeric activin A, B and AB (Mellor, Cranfield et al. 2000; Hashimoto, Tsuchida et al. 2002). In this case the activin beta E subunit also could act as an inhibitor of activins. Another study showed that activin C as a secreted dimer could inhibit activn A via competition for activin receptors (Gold, Jetly et al. 2009). However the western blots performed with conditioned media in this thesis seem to suggest that transfection of the beta A subunit into 293 cells leads to smad2 phosphorylatin activity in conditioned media also when the activin beta E subunit is co-transfected at a 1:1 ratio and even at an 8-fold increased ratio of beta A over beta E. In addition, follistatin showed its antagonizing effect not only in activin A conditioned medium, but also in activin A plus E conditioned medium. Taken together, these data argue against a major impact of beta E expression on activin A signaling. Thus, while these data can disprove some hypotheses, the actual mechansism, how the beta E subunit could function, remains enigmatic.

5. List of Abbreviations

Act - Activin

ActR - Activin Receptor

ACV - activin receptor

ALK - Activin Receptor–like Kinase

APS - Ammonium Persulfate

BMP - Bone morphogenetic proteins

BSA - Bovine Serum Albumin

CDK - Cyclin Dependend Kinase

CFP - Cyan Fluorescent Protein

Cx - Connexin

DEPC - Diethylpyrocarbonate

DMSO - Dimethylsulfoxid

DTT - Dithiothreitol

ECM - Extra Cellular Matrix

EDTA - Ethylendiaminetetraacetic Acid

EGF - Epithelial Growth Factor

EGFR - Epithelial Growth Factor Receptors

ERK - Extracellular Signalregulated Kinase

EtOH - Ethanol

FBS - Fetal Bovine Serum

FGF - Fibroblast Growth Factor

FGFR - Fibroblast Growth Factor Receptors

FLRG - Follistatin–related gene

FSH - Follicle-stimulating hormone

Fst - Follistatin

GDF - Growth Differentiation Factor

GFP/EGFP - Green Fluorescent Protein/Enhanced Green Fluorescent Protein

HBS - Heparin Binding Site

HGF - Hepatocyte Growth Factor

HRP - Horseradish Peroxidaase

ICH - Immunohistochemistry

IRES - Internal Ribosomal Entry Site

MAPK - Mitogen Activated Protein Kinase

MeOH - Methanol

o/n - Overnight

P/S - Penecilin/Streptomycin

PAGE - Polyacrylamide Gelelectrophoresis

PBS - Phophate Buffered Saline

PFA - Paraformaldehyd

PVDF - Polyvinylidenefluoride

rh Act A – recombinant human activin A

Rb - Retinoblastoma

ROS - Reactive Oxygen Species

RPMI - Roswell Park Memorial Institute

RT - Room Temperature

SCID - Severe Combined Immunodeficiency

SDS - Sodium Dodecyl Sulfate

STAT - Signal Transducers and Activators of Transcription

STET - Sodiumchloride Tris EDTA Triton X-100 Buffer Solution

T/E - Trypsin/EDTA

TBE - Tris/Borate/EDTA

TBS - Tris Buffered Saline

TE - Tris EDTA

TEMED - N,N,N´,N´-Tetramethylethylenediamine

TGF-β - Transforming Growth Factor beta

Tris - Trishydroxymethyl Aminomethane

6. List of Figures

7. List of Table

8. References

Albano, R. M., N. Groome, et al. (1993). "Activins are expressed in preimplantation mouse embryos and in ES and EC cells and are regulated on their differentiation." Development **117**(2): 711-723.

Algazi, A. P., C. W. Soon, et al. (2010). "Treatment of cutaneous melanoma: current approaches and future prospects." Cancer management and research **2**: 197-211.

Allerstorfer, S., G. Sonvilla, et al. (2008). "FGF5 as an oncogenic factor in human glioblastoma multiforme: autocrine and paracrine activities." Oncogene **27**(30): 4180-4190.

Ball, E. M. and G. P. Risbridger (2001). "Activins as regulators of branching morphogenesis." Developmental biology **238**(1): 1-12.

Ball, E. M. and G. P. Risbridger (2001). "Activins as regulators of branching morphogenesis." Dev Biol **238**(1): 1-12.

Barden, H. and S. Levine (1983). "Histochemical observations on rodent brain melanin." Brain Res Bull **10**(6): 847-851.

Barton, D. E., T. L. Yang-Feng, et al. (1989). "Mapping of genes for inhibin subunits alpha, beta A, and beta B on human and mouse chromosomes and studies of jsd mice." Genomics **5**(1): 91-99.

Becker, D., P. L. Lee, et al. (1992). "Inhibition of the fibroblast growth factor receptor 1 (FGFR-1) gene in human melanocytes and malignant melanomas leads to inhibition of proliferation and signs indicative of differentiation." Oncogene **7**(11): 2303-2313.

Beeram, M., A. Patnaik, et al. (2005). "Raf: a strategic target for therapeutic development against cancer." Journal of clinical oncology : official journal of the American Society of Clinical Oncology **23**(27): 6771-6790.

Berking, C., R. Takemoto, et al. (2001). "Transforming growth factor-beta1 increases survival of human melanoma through stroma remodeling." Cancer Res **61**(22): 8306-8316.

Betke, H., M. Korabiowska, et al. (1998). "The role of nm23 in melanoma progression and its prognostic significance." Pol J Pathol **49**(2): 93-96.

Bissell, M. J. and D. Radisky (2001). "Putting tumours in context." Nat Rev Cancer **1**(1): 46-54.

Brissette, J. L., N. M. Kumar, et al. (1994). "Switch in gap junction protein expression is associated with selective changes in junctional permeability during keratinocyte differentiation." Proc Natl Acad Sci U S A **91**(14): 6453-6457.

Brose, M. S., P. Volpe, et al. (2002). "BRAF and RAS mutations in human lung cancer and melanoma." Cancer Res **62**(23): 6997-7000.

Burdette, J. E., J. S. Jeruss, et al. (2005). "Activin A mediates growth inhibition and cell cycle arrest through Smads in human breast cancer cells." Cancer Res **65**(17): 7968-7975.

Butler, C. M., E. J. Gold, et al. (2005). "Should activin betaC be more than a fading snapshot in the activin/TGFbeta family album?" Cytokine & growth factor reviews **16**(4-5): 377-385.

Caniggia, I., S. J. Lye, et al. (1997). "Activin is a local regulator of human cytotrophoblast cell differentiation." Endocrinology **138**(9): 3976-3986.

Carreira, S., J. Goodall, et al. (2005). "Mitf cooperates with Rb1 and activates p21Cip1 expression to regulate cell cycle progression." Nature **433**(7027): 764-769.

Chen, Y. G., H. M. Lui, et al. (2002). "Regulation of cell proliferation, apoptosis, and carcinogenesis by activin." Experimental biology and medicine **227**(2): 75-87.

Chen, Y. G., H. M. Lui, et al. (2002). "Regulation of cell proliferation, apoptosis, and carcinogenesis by activin." Exp Biol Med (Maywood) **227**(2): 75-87.

Chen, Y. G., Q. Wang, et al. (2006). "Activin signaling and its role in regulation of cell proliferation, apoptosis, and carcinogenesis." Exp Biol Med (Maywood) **231**(5): 534-544.

Cho, S. H., Z. Yao, et al. (2003). "Regulation of activin A expression in mast cells and asthma: its effect on the proliferation of human airway smooth muscle cells." J Immunol **170**(8): 4045-4052.

Danila, D. C., W. J. Inder, et al. (2000). "Activin effects on neoplastic proliferation of human pituitary tumors." J Clin Endocrinol Metab **85**(3): 1009-1015.

Davies, H., G. R. Bignell, et al. (2002). "Mutations of the BRAF gene in human cancer." Nature **417**(6892): 949-954.

de Winter, J. P., P. ten Dijke, et al. (1996). "Follistatins neutralize activin bioactivity by inhibition of activin binding to its type II receptors." Molecular and cellular endocrinology **116**(1): 105-114.

de Winter, J. P., P. ten Dijke, et al. (1996). "Follistatins neutralize activin bioactivity by inhibition of activin binding to its type II receptors." Mol Cell Endocrinol **116**(1): 105-114.

Deeds, J., F. Cronin, et al. (2000). "Patterns of melastatin mRNA expression in melanocytic tumors." Hum Pathol **31**(11): 1346-1356.

Delbaere, A., Y. Sidis, et al. (1999). "Differential response to exogenous and endogenous activin in a human ovarian teratocarcinoma-derived cell line (PA-1): regulation by cell surface follistatin." Endocrinology **140**(6): 2463-2470.

Deli, A., E. Kreidl, et al. (2008). "Activins and activin antagonists in hepatocellular carcinoma." World J Gastroenterol **14**(11): 1699-1709.

DePaolo, L. V., T. A. Bicsak, et al. (1991). "Follistatin and activin: a potential intrinsic regulatory system within diverse tissues." Proc Soc Exp Biol Med **198**(1): 500-512.

Derynck, R. and Y. E. Zhang (2003). "Smad-dependent and Smad-independent pathways in TGF-beta family signalling." Nature **425**(6958): 577-584.

Emuss, V., M. Garnett, et al. (2005). "Mutations of C-RAF are rare in human cancer because C-RAF has a low basal kinase activity compared with B-RAF." Cancer research **65**(21): 9719-9726.

Erickson, C. A. and M. V. Reedy (1998). "Neural crest development: the interplay between morphogenesis and cell differentiation." Curr Top Dev Biol **40**: 177-209.

Esch, F. S., S. Shimasaki, et al. (1987). "Complementary deoxyribonucleic acid (cDNA) cloning and DNA sequence analysis of rat ovarian inhibins." Mol Endocrinol **1**(5): 388-396.

Esch, F. S., S. Shimasaki, et al. (1987). "Structural characterization of follistatin: a novel follicle-stimulating hormone release-inhibiting polypeptide from the gonad." Mol Endocrinol **1**(11): 849-855.

Ethier, J. F. and J. K. Findlay (2001). "Roles of activin and its signal transduction mechanisms in reproductive tissues." Reproduction **121**(5): 667-675.

Fitzpatrick, T. B. and A. S. Breathnach (1963). "[the Epidermal Melanin Unit System.]." Dermatol Wochenschr **147**: 481-489.

Forage, R. G., J. M. Ring, et al. (1986). "Cloning and sequence analysis of cDNA species coding for the two subunits of inhibin from bovine follicular fluid." Proc Natl Acad Sci U S A **83**(10): 3091-3095.

Fujita, E., A. Jinbo, et al. (1999). "Akt phosphorylation site found in human caspase-9 is absent in mouse caspase-9." Biochem Biophys Res Commun **264**(2): 550-555.

Furukawa, F., K. Fujii, et al. (1997). "Roles of E- and P-cadherin in the human skin." Microsc Res Tech **38**(4): 343-352.

Gallagher, S., S. E. Winston, et al. (2008). "Immunoblotting and immunodetection." Curr Protoc Mol Biol **Chapter 10**: Unit 10 18.

Gallagher, S. R. (2007). "One-dimensional SDS gel electrophoresis of proteins." Curr Protoc Toxicol **Appendix 3**: Appendix 3F.

Gold, E., N. Jetly, et al. (2009). "Activin C antagonizes activin A in vitro and overexpression leads to pathologies in vivo." The American journal of pathology **174**(1): 184-195.

Goliger, J. A. and D. L. Paul (1995). "Wounding alters epidermal connexin expression and gap junction-mediated intercellular communication." Mol Biol Cell **6**(11): 1491-1501.

Goodall, J., C. Wellbrock, et al. (2004). "The Brn-2 transcription factor links activated BRAF to melanoma proliferation." Mol Cell Biol **24**(7): 2923-2931.

Graham, F. L., J. Smiley, et al. (1977). "Characteristics of a human cell line transformed by DNA from human adenovirus type 5." J Gen Virol **36**(1): 59-74.

Gray, A. M. and A. J. Mason (1990). "Requirement for activin A and transforming growth factor--beta 1 pro-regions in homodimer assembly." Science **247**(4948): 1328-1330.

Gray, P. C., C. A. Harrison, et al. (2003). "Cripto forms a complex with activin and type II activin receptors and can block activin signaling." Proc Natl Acad Sci U S A **100**(9): 5193-5198.

Grusch, M., C. Drucker, et al. (2006). "Deregulation of the activin/follistatin system in hepatocarcinogenesis." Journal of hepatology **45**(5): 673-680.

Grusch, M., M. Petz, et al. (2010). "The crosstalk of RAS with the TGF-beta family during carcinoma progression and its implications for targeted cancer therapy." Current cancer drug targets **10**(8): 849-857.

Gumienny, T. L. and R. W. Padgett (2002). "The other side of TGF-beta superfamily signal regulation: thinking outside the cell." Trends Endocrinol Metab **13**(7): 295-299.

Gumienny, T. L. and R. W. Padgett (2002). "The other side of TGF-beta superfamily signal regulation: thinking outside the cell." Trends in endocrinology and metabolism: TEM **13**(7): 295-299.

Gurtu, V., G. Yan, et al. (1996). "IRES bicistronic expression vectors for efficient creation of stable mammalian cell lines." Biochem Biophys Res Commun **229**(1): 295-298.

Haass, N. K. and M. Herlyn (2005). "Normal human melanocyte homeostasis as a paradigm for understanding melanoma." J Investig Dermatol Symp Proc **10**(2): 153-163.

Haass, N. K., K. S. Smalley, et al. (2004). "The role of altered cell-cell communication in melanoma progression." J Mol Histol **35**(3): 309-318.

Haass, N. K., E. Wladykowski, et al. (2006). "Differential induction of connexins 26 and 30 in skin tumors and their adjacent epidermis." J Histochem Cytochem **54**(2): 171-182.

Haluska, F. G., H. Tsao, et al. (2006). "Genetic alterations in signaling pathways in melanoma." Clinical cancer research : an official journal of the American Association for Cancer Research **12**(7 Pt 2): 2301s-2307s.

Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." Cell **100**(1): 57-70.

Harrington, A. E., S. A. Morris-Triggs, et al. (2006). "Structural basis for the inhibition of activin signalling by follistatin." EMBO J **25**(5): 1035-1045.

Harrison, C. A., E. Wiater, et al. (2004). "Modulation of activin and BMP signaling." Mol Cell Endocrinol **225**(1-2): 19-24.

Hashimoto, O., T. Nakamura, et al. (1997). "A novel role of follistatin, an activin-binding protein, in the inhibition of activin action in rat pituitary cells. Endocytotic degradation of activin and its acceleration by follistatin associated with cell-surface heparan sulfate." J Biol Chem **272**(21): 13835-13842.

Hashimoto, O., K. Tsuchida, et al. (2002). "cDNA cloning and expression of human activin betaE subunit." Molecular and cellular endocrinology **194**(1-2): 117-122.

Hayette, S., M. Gadoux, et al. (1998). "FLRG (follistatin-related gene), a new target of chromosomal rearrangement in malignant blood disorders." Oncogene **16**(22): 2949-2954.

Heldin, C. H., K. Miyazono, et al. (1997). "TGF-beta signalling from cell membrane to nucleus through SMAD proteins." Nature **390**(6659): 465-471.

Herlyn, M., C. Berking, et al. (2000). "Lessons from melanocyte development for understanding the biological events in naevus and melanoma formation." Melanoma Res **10**(4): 303-312.

Hirobe, T. (1994). "Keratinocytes are involved in regulating the developmental changes in the proliferative activity of mouse epidermal melanoblasts in serum-free culture." Dev Biol **161**(1): 59-69.

Ho, J., C. de Guise, et al. (2004). "Activin induces hepatocyte cell growth arrest through induction of the cyclin-dependent kinase inhibitor p15INK4B and Sp1." Cell Signal **16**(6): 693-701.

Hohenester, E., P. Maurer, et al. (1997). "Crystal structure of a pair of follistatin-like and EF-hand calcium-binding domains in BM-40." EMBO J **16**(13): 3778-3786.

Hsu, M., T. Andl, et al. (2000). "Cadherin repertoire determines partner-specific gap junctional communication during melanoma progression." J Cell Sci **113 (Pt 9)**: 1535-1542.

Hsu, M. Y., F. E. Meier, et al. (2000). "E-cadherin expression in melanoma cells restores keratinocyte-mediated growth control and down-regulates expression of invasion-related adhesion receptors." Am J Pathol **156**(5): 1515-1525.

Hsu, M. Y., D. T. Shih, et al. (1998). "Adenoviral gene transfer of beta3 integrin subunit induces conversion from radial to vertical growth phase in primary human melanoma." Am J Pathol **153**(5): 1435-1442.

Hsu, S. Y. and A. J. Hsueh (2000). "Tissue-specific Bcl-2 protein partners in apoptosis: An ovarian paradigm." Physiol Rev **80**(2): 593-614.

Huang, X., D. Li, et al. (2002). "[Expression of activins, follistatin mRNA in the development of hepatic fibrosis]." Zhonghua gan zang bing za zhi = Zhonghua ganzangbing zazhi = Chinese journal of hepatology **10**(2): 85-88.

Hussein, M. R. (2005). "Transforming growth factor-beta and malignant melanoma: molecular mechanisms." J Cutan Pathol **32**(6): 389-395.

Innis, C. A. and M. Hyvonen (2003). "Crystal structures of the heparan sulfate-binding domain of follistatin. Insights into ligand binding." J Biol Chem **278**(41): 39969-39977.

Jakowlew, S. B. (2006). "Transforming growth factor-beta in cancer and metastasis." Cancer Metastasis Rev **25**(3): 435-457.

Jeruss, J. S., C. D. Sturgis, et al. (2003). "Down-regulation of activin, activin receptors, and Smads in high-grade breast cancer." Cancer research **63**(13): 3783-3790.

Jhaveri, S., R. S. Erzurumlu, et al. (1998). "Defective whisker follicles and altered brainstem patterns in activin and follistatin knockout mice." Mol Cell Neurosci **12**(4-5): 206-219.

Jimbow, K., W. C. Quevedo, Jr., et al. (1976). "Some aspects of melanin biology: 1950-1975." J Invest Dermatol **67**(1): 72-89.

Kanamaru, C., H. Yasuda, et al. (2002). "Involvement of Smad proteins in TGF-beta and activin A-induced apoptosis and growth inhibition of liver cells." Hepatol Res **23**(3): 211-219.

Kelber, J. A., G. Shani, et al. (2008). "Cripto is a noncompetitive activin antagonist that forms analogous signaling complexes with activin and nodal." J Biol Chem **283**(8): 4490-4500.

Knowles, B. B., C. C. Howe, et al. (1980). "Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen." Science **209**(4455): 497-499.

Korpal, M. and Y. Kang (2010). "Targeting the transforming growth factor-beta signalling pathway in metastatic cancer." Eur J Cancer **46**(7): 1232-1240.

Krneta, J., J. Kroll, et al. (2006). "Dissociation of angiogenesis and tumorigenesis in follistatin- and activin-expressing tumors." Cancer research **66**(11): 5686-5695.

Lagna, G., A. Hata, et al. (1996). "Partnership between DPC4 and SMAD proteins in TGF-beta signalling pathways." Nature **383**(6603): 832-836.

Leto, G. (2010). "Activin A and bone metastasis." Journal of cellular physiology **225**(2): 302-309.

Li, G. and M. Herlyn (2000). "Dynamics of intercellular communication during melanoma development." Mol Med Today **6**(4): 163-169.

Lin, S. Y., R. G. Craythorn, et al. (2008). "Female infertility and disrupted angiogenesis are actions of specific follistatin isoforms." Mol Endocrinol **22**(2): 415-429.

Lopez-Bergami, P., B. Fitchman, et al. (2008). "Understanding signaling cascades in melanoma." Photochem Photobiol **84**(2): 289-306.

Luisi, S., P. Florio, et al. (2001). "Expression and secretion of activin A: possible physiological and clinical implications." European journal of endocrinology / European Federation of Endocrine Societies **145**(3): 225-236.

Markert, C. L. and W. K. Silvers (1956). "The Effects of Genotype and Cell Environment on Melanoblast Differentiation in the House Mouse." Genetics **41**(3): 429-450.

Mason, A. J. (1994). "Functional analysis of the cysteine residues of activin A." Mol Endocrinol **8**(3): 325-332.

Mason, A. J., L. M. Berkemeier, et al. (1989). "Activin B: precursor sequences, genomic structure and in vitro activities." Mol Endocrinol **3**(9): 1352-1358.

Mason, A. J., J. S. Hayflick, et al. (1985). "Complementary DNA sequences of ovarian follicular fluid inhibin show precursor structure and homology with transforming growth factor-beta." Nature **318**(6047): 659-663.

Mason, A. J., H. D. Niall, et al. (1986). "Structure of two human ovarian inhibins." Biochem Biophys Res Commun **135**(3): 957-964.

Massague, J. (2000). "How cells read TGF-beta signals." Nat Rev Mol Cell Biol **1**(3): 169-178.

Matzuk, M. M., N. Lu, et al. (1995). "Multiple defects and perinatal death in mice deficient in follistatin." Nature **374**(6520): 360-363.

Mayo, K. E. (1994). "Inhibin and activin Molecular aspects of regulation and function." Trends Endocrinol Metab **5**(10): 407-415.

McCarthy, J., F. Hopwood, et al. (2003). "Carbamylation of proteins in 2-D electrophoresis--myth or reality?" J Proteome Res **2**(3): 239-242.

McDowall, M., N. M. Edwards, et al. (2008). "The role of activins and follistatins in skin and hair follicle development and function." Cytokine Growth Factor Rev **19**(5-6): 415-426.

McPherson, S. J., S. L. Mellor, et al. (1999). "Expression of activin A and follistatin core proteins by human prostate tumor cell lines." Endocrinology **140**(11): 5303-5309.

Mellor, S. L., M. Cranfield, et al. (2000). "Localization of activin beta(A)-, beta(B)-, and beta(C)-subunits in humanprostate and evidence for formation of new activin heterodimers of beta(C)-subunit." The Journal of clinical endocrinology and metabolism **85**(12): 4851-4858.

Meyle, K. D. and P. Guldberg (2009). "Genetic risk factors for melanoma." Hum Genet **126**(4): 499-510.

Michel, U., A. Rao, et al. (1991). "Rat follistatin: ontogeny of steady-state mRNA levels in different tissues predicts organ-specific functions." Biochemical and biophysical research communications **180**(1): 223-230.

Mintz, B. (1971). "Clonal basis of mammalian differentiation." Symp Soc Exp Biol **25**: 345-370.

Munz, B., H. Smola, et al. (1999). "Overexpression of activin A in the skin of transgenic mice reveals new activities of activin in epidermal morphogenesis, dermal fibrosis and wound repair." EMBO J **18**(19): 5205-5215.

Nakamura, T., K. Takio, et al. (1990). "Activin-binding protein from rat ovary is follistatin." Science **247**(4944): 836-838.

Newmeyer, D. D., E. Bossy-Wetzel, et al. (2000). "Bcl-xL does not inhibit the function of Apaf-1." Cell Death Differ **7**(4): 402-407.

Nichols, S. E., Jr. and W. M. Reams, Jr. (1960). "The occurrence and morphogenesis of melanocytes in the connective tissues of the PET/MCV mouse strain." J Embryol Exp Morphol **8**: 24-32.

Ogino, H., S. Yano, et al. (2008). "Follistatin suppresses the production of experimental multiple-organ metastasis by small cell lung cancer cells in natural killer cell-depleted SCID mice." Clinical cancer research : an official journal of the American Association for Cancer Research **14**(3): 660-667.

Palmieri, G., M. Capone, et al. (2009). "Main roads to melanoma." J Transl Med **7**: 86.

Palmieri, G., M. Capone, et al. (2009). "Main roads to melanoma." Journal of translational medicine **7**: 86.

Patel, K. (1998). "Follistatin." Int J Biochem Cell Biol **30**(10): 1087-1093.

Piek, E. and A. B. Roberts (2001). "Suppressor and oncogenic roles of transforming growth factor-beta and its signaling pathways in tumorigenesis." Adv Cancer Res **83**: 1-54.

Pitts, J. D., M. E. Finbow, et al. (1988). "Junctional communication and cellular differentiation." Br J Cancer Suppl **9**: 52-57.

Pollock, P. M., U. L. Harper, et al. (2003). "High frequency of BRAF mutations in nevi." Nat Genet **33**(1): 19-20.

Reed, J. A., Q. Lin, et al. (2005). "SKI pathways inducing progression of human melanoma." Cancer Metastasis Rev **24**(2): 265-272.

Risbridger, G. P., J. F. Schmitt, et al. (2001). "Activins and inhibins in endocrine and other tumors." Endocr Rev **22**(6): 836-858.

Ritvos, O., T. Tuuri, et al. (1995). "Activin disrupts epithelial branching morphogenesis in developing glandular organs of the mouse." Mech Dev **50**(2-3): 229-245.

Rizos, H., S. Puig, et al. (2001). "A melanoma-associated germline mutation in exon 1beta inactivates p14ARF." Oncogene **20**(39): 5543-5547.

Robertson, D. M., R. Klein, et al. (1987). "The isolation of polypeptides with FSH suppressing activity from bovine follicular fluid which are structurally different to inhibin." Biochem Biophys Res Commun **149**(2): 744-749.

Rocco, J. W. and D. Sidransky (2001). "p16(MTS-1/CDKN2/INK4a) in cancer progression." Exp Cell Res **264**(1): 42-55.

Saito, S., Y. Sidis, et al. (2005). "Differential biosynthesis and intracellular transport of follistatin isoforms and follistatin-like-3." Endocrinology **146**(12): 5052-5062.

Satyamoorthy, K., N. H. Chehab, et al. (2000). "Aberrant regulation and function of wild-type p53 in radioresistant melanoma cells." Cell Growth Differ **11**(9): 467-474.

Satyamoorthy, K. and M. Herlyn (2002). "Cellular and molecular biology of human melanoma." Cancer Biol Ther **1**(1): 14-17.

Schmierer, B. and C. S. Hill (2007). "TGFbeta-SMAD signal transduction: molecular specificity and functional flexibility." Nat Rev Mol Cell Biol **8**(12): 970-982.

Schneyer, A., D. Tortoriello, et al. (2001). "Follistatin-related protein (FSRP): a new member of the follistatin gene family." Mol Cell Endocrinol **180**(1-2): 33-38.

Schneyer, A. L., H. A. Hall, et al. (1996). "Follistatin-activin complexes in human serum and follicular fluid differ immunologically and biochemically." Endocrinology **137**(1): 240-247.

Schwall, R. H., K. Nikolics, et al. (1988). "Recombinant expression and characterization of human activin A." Mol Endocrinol **2**(12): 1237-1242.

Seder, C. W., W. Hartojo, et al. (2009). "Upregulated INHBA expression may promote cell proliferation and is associated with poor survival in lung adenocarcinoma." Neoplasia **11**(4): 388-396.

Sherr, C. J. and J. D. Weber (2000). "The ARF/p53 pathway." Curr Opin Genet Dev **10**(1): 94-99.

Shibanuma, M., J. Mashimo, et al. (1993). "Cloning from a mouse osteoblastic cell line of a set of transforming-growth-factor-beta 1-regulated genes, one of which seems to encode a follistatin-related polypeptide." Eur J Biochem **217**(1): 13-19.

Sidis, Y., A. Mukherjee, et al. (2006). "Biological activity of follistatin isoforms and follistatin-like-3 is dependent on differential cell surface binding and specificity for activin, myostatin, and bone morphogenetic proteins." Endocrinology **147**(7): 3586-3597.

Sidis, Y., A. L. Schneyer, et al. (2001). "Follistatin: essential role for the N-terminal domain in activin binding and neutralization." J Biol Chem **276**(21): 17718-17726.

Soengas, M. S., R. M. Alarcon, et al. (1999). "Apaf-1 and caspase-9 in p53-dependent apoptosis and tumor inhibition." Science **284**(5411): 156-159.

Soengas, M. S., P. Capodieci, et al. (2001). "Inactivation of the apoptosis effector Apaf-1 in malignant melanoma." Nature **409**(6817): 207-211.

Steinberg, M. S. and P. M. McNutt (1999). "Cadherins and their connections: adhesion junctions have broader functions." Curr Opin Cell Biol **11**(5): 554-560.

Stove, C., F. Vanrobaeys, et al. (2004). "Melanoma cells secrete follistatin, an antagonist of activin-mediated growth inhibition." Oncogene **23**(31): 5330-5339.

Strizzi, L., C. Bianco, et al. (2005). "Cripto-1: a multifunctional modulator during embryogenesis and oncogenesis." Oncogene **24**(37): 5731-5741.

Sugino, H., K. Sugino, et al. (1997). "Follistatin and its role as an activin-binding protein." J Med Invest **44**(1-2): 1-14.

Takamura, K., K. Tsuchida, et al. (2005). "Activin and activin receptor expression changes in liver regeneration in rat." The Journal of surgical research **126**(1): 3-11.

Tanaka, T., S. Toujima, et al. (2004). "Activin A inhibits growth-inhibitory signals by TGF-beta1 in differentiated human endometrial adenocarcinoma cells." Oncol Rep **11**(4): 875-879.

Teti, A., A. De Giorgi, et al. (1997). "Transforming growth factor-beta enhances adhesion of melanoma cells to the endothelium in vitro." Int J Cancer **72**(6): 1013-1020.

Theriault, L. L. and L. S. Hurley (1970). "Ultrastructure of developing melanosomes in C57 black and pallid mice." Dev Biol **23**(2): 261-275.

Tortoriello, D. V., Y. Sidis, et al. (2001). "Human follistatin-related protein: a structural homologue of follistatin with nuclear localization." Endocrinology **142**(8): 3426-3434.

Tsuchida, K., K. Y. Arai, et al. (2000). "Identification and characterization of a novel follistatin-like protein as a binding protein for the TGF-beta family." The Journal of biological chemistry **275**(52): 40788-40796.

Tuuri, T., M. Eramaa, et al. (1994). "The tissue distribution of activin beta A- and beta B-subunit and follistatin messenger ribonucleic acids suggests multiple sites of action for the activin-follistatin system during human development." J Clin Endocrinol Metab **78**(6): 1521-1524.

Tuuri, T., M. Eramaa, et al. (1994). "The tissue distribution of activin beta A- and beta B-subunit and follistatin messenger ribonucleic acids suggests multiple sites of action for the activin-follistatin system during human development." The Journal of clinical endocrinology and metabolism **78**(6): 1521-1524.

Uong, A. and L. I. Zon "Melanocytes in development and cancer." J Cell Physiol **222**(1): 38-41.

Utton, M. A., B. Eickholt, et al. (2001). "Soluble N-cadherin stimulates fibroblast growth factor receptor dependent neurite outgrowth and N-cadherin and the fibroblast growth factor receptor co-cluster in cells." J Neurochem **76**(5): 1421-1430.

Valderrama-Carvajal, H., E. Cocolakis, et al. (2002). "Activin/TGF-beta induce apoptosis through Smad-dependent expression of the lipid phosphatase SHIP." Nat Cell Biol **4**(12): 963-969.

Vejda, S., N. Erlach, et al. (2003). "Expression of activins C and E induces apoptosis in human and rat hepatoma cells." Carcinogenesis **24**(11): 1801-1809.

Vivien, D., L. Attisano, et al. (1995). "Signaling activity of homologous and heterologous transforming growth factor-beta receptor kinase complexes." J Biol Chem **270**(13): 7134-7141.

Vivien, D. and J. L. Wrana (1995). "Ligand-induced recruitment and phosphorylation of reduced TGF-beta type I receptor." Exp Cell Res **221**(1): 60-65.

Vleminckx, K. and R. Kemler (1999). "Cadherins and tissue formation: integrating adhesion and signaling." Bioessays **21**(3): 211-220.

Wan, P. T., M. J. Garnett, et al. (2004). "Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF." Cell **116**(6): 855-867.

Wang, Q., H. T. Keutmann, et al. (2000). "Analysis of human follistatin structure: identification of two discontinuous N-terminal sequences coding for activin A binding and structural consequences of activin binding to native proteins." Endocrinology **141**(9): 3183-3193.

Wang, Y. and D. Becker (1997). "Antisense targeting of basic fibroblast growth factor and fibroblast growth factor receptor-1 in human melanomas blocks intratumoral angiogenesis and tumor growth." Nat Med **3**(8): 887-893.

Weiskirchen, R., S. K. Meurer, et al. (2009). "BMP-7 as antagonist of organ fibrosis." Front Biosci **14**: 4992-5012.

Woodruff, T. K., H. Meunier, et al. (1987). "Rat inhibin: molecular cloning of alpha- and beta-subunit complementary deoxyribonucleic acids and expression in the ovary." Mol Endocrinol **1**(8): 561-568.

Wrana, J. L., L. Attisano, et al. (1994). "Mechanism of activation of the TGF-beta receptor." Nature **370**(6488): 341-347.

Xia, Y. and A. L. Schneyer (2009). "The biology of activin: recent advances in structure, regulation and function." J Endocrinol **202**(1): 1-12.

Yokomuro, S., H. Tsuji, et al. (2000). "Growth control of human biliary epithelial cells by interleukin 6, hepatocyte growth factor, transforming growth factor beta1, and activin A: comparison of a cholangiocarcinoma cell line with primary cultures of non-neoplastic biliary epithelial cells." Hepatology **32**(1): 26-35.

Yoshinaga, K., H. Inoue, et al. (2004). "N-cadherin is regulated by activin A and associated with tumor aggressiveness in esophageal carcinoma." Clin Cancer Res **10**(17): 5702-5707.

Yoshinaga, K., K. Yamashita, et al. (2008). "Activin a causes cancer cell aggressiveness in esophageal squamous cell carcinoma cells." Annals of surgical oncology **15**(1): 96-103.

Zebisch, A., P. B. Staber, et al. (2006). "Two transforming C-RAF germline mutations identified in patients with therapy-related acute myeloid leukemia." Cancer research **66**(7): 3401-3408.

Zhou, H., X. M. Li, et al. (2000). "Akt regulates cell survival and apoptosis at a postmitochondrial level." J Cell Biol **151**(3): 483-494.

9. Curriculum Vitae

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EDUCATION

Since 11/2004	University of Vienna, *Genetics and Mikrobiology* Specialization: Genetics and Pathology
2003-2004	German Courses in Vienna
2001-2002	Hacettepe University in Ankara English Courses
1997-2000	High School in Ankara
1994-1997	Secondary School in Ankara
1989-1994	Primary School in Ankara

UNIVERSITY EDUCATION (Summary)

Molecular Genetic and Pathology	**Genetics**	**Cellbiology**
Stemcell Research	Introduction to Genetic	Anatomy of Animals
Molecular Genetic and Pathology Seminars	Cell- and developmental Genetics	Introduction to Molecular Cellbiology
Project planning in Genetic and Pathology	Molecular Biotechnology	Developmental Biology
Project planning in Cell Signaling	Gene Expression	Anthropology in Molecularbiology
	Molecular Genetics A+B	Structure and Function of Plants
	Project planning in Cytogenetics	
		Mikrobiology and Immunology
	Biochemistry	Introduction to Microbiology
	Biochemistry in Molecularbiology	Immunology and Cellular Microbiology A+B
	Mathematics in Molecularbiology	Applied Microbiology
	Structural Biology	Molecular Microbiology A+B
	Cellbiology and Biochemie	Molecular Biology of Cyanobacteria

WORK / RESEARCH EXPERIENCE

Since 11/2008 Medical University of Vienna,
Department of Medicine I; Institute of Cancer Research
(*Group: Michael Grusch*)
Projects: *Investigating the role of activins and follistatins in melanoma physiology and cancer*
Methods*:* Expression Analysis; MTT-Assays; Soft Agar Assays; Xenotransplantation Experiments in SCID Mice; ELISA; Wastern Blot; Transwell-Chamber Migration Assays; Angiogenesis (Tube Formation) Assays; FACS Analysis; Immunohistochemistry.

07/2007-09/2007 Max F. Perutz Laboratories,
Department of Genetics
(Group: Rudolf Schweyen and Karin Nowikovsky)
Project: *Investigation the influence of divalent cations on mitochondrial K^+ / H^+ antiporter systems in yeast. Investigation of the role of mdm38 in the cation homeostasis by yeast mitochondria.*
Methods: Site-Specific Homologue Recombination (Generation of "Knock-Out"-strains); High Efficiency Transformation; Yeast Colony PCR; Growth Assays; Mitochondrial activity assays; Photometrical swelling assay; Submitochondrial Particle Assay.

PUBLICATIONS and PRESENTATION

- Kreidl E, ***Öztürk D***, Metzner T, Berger W, Grusch M. *Activins and Follistatins:*
Emerging Roles in Liver Physiology and Cancer. World Journal of Hepatology. 2009, Oct. 15; 1(1): 17-27.

- Grusch M, Petz M, Metzner T, ***Öztürk D***, Schneller D, Mikulits W. *The Crosstalk of*
Ras with the TGF-ß Family during Carcinoma Progression and its Implication forTargeted Cancer Therapy. Curr Cancer Drug Targets. 2010 Dec 1;10(8):849-57.

- *7th World Congress on Melanoma and 5th Congress of the European Association of*
Dermato-Oncology, May 12-16, 2009; Poster-Presentation; Expression of Activins and their Antagonists in Melanoma (Topic: Signaling Cascade)

- *"Academic Medicine-Does It Pay Off?"FWF Discussion*, June 17, 2009; Poster-Presentation; Activins and activin antagonists in Melanoma.

SPECIAL SKILLS

Foreign Languages	English- fluently spoken and written German- fluently spoken and written Turkish- mother language
Computer Skills	MS Office, GraphPad Prism, Clone Manager, Adobe Photoshop, Image Quant, ImageJ

INTERESTS

Photograph, theatre, painting, cinema, music, travelling.