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„Impact of activin A on the progression of small cell lung cancer?“

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„Man braucht im Leben nichts zu fürchten, man muss es nur verstehen.
Jetzt es ist es an der Zeit, mehr zu verstehen, damit wir weniger fürchten.“
(Marie Curie)

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1. INTRODUCTION

1.1. Lung cancer

Lung cancer is classified into two major groups – small cell lung cancer (SCLC), which is sometimes also named “oat cell carcinoma” due to the flat shape of the cells and scanty cytoplasm, and non-small cell lung cancer (NSCLC), which is subdivided into the three main types, adenocarcinoma (ADC), squamous cell carcinoma (SCC) and large cell carcinoma (LCC). In the last few years lung cancer was the most frequently diagnosed cancer and among men the leading cause of cancer deaths worldwide, while in women it was the leading cause only in more developed countries (Torre et al., 2015).

1.1.1 Small cell lung cancer

Epidemiology and general characterization

Small cell lung cancer (SCLC) represents approximately 10-15% of all lung cancers and is characterized by high aggressiveness, rapid growth and early progression to local and distant metastasis. Untreated SCLC patients have a median survival of 2 to 4 months (Yamamoto et al., 2015). SCLC originates from neuroendocrine cell precursors and is defined as malignant epithelial tumor consisting of small, oval and spindle shaped cells with scant cytoplasm, finely granular nuclear chromatin, ill-defined cell borders and absent or inconspicuous nucleoli (Brambilla et al., 2001; Travis et al., 2015). Genomic sequencing analyses of SCLC cell lines have identified a variety of mutations in SCLC, including inactivating mutations in TP53, Rb1, and PTEN, as well as activating mutations in EGFR, KRAS and PiK3CA (Tatematsu et al., 2008; Yokomizo et al., 1998).

1.1.2. Risk factors

The main risk factor for SCLC is the use of tobacco. Approximately 90% of small cell lung cancer deaths among men and 60% among women are caused by smoking, even though a strong variation exists between different countries (Harmsma et al., 2013). Some other factors that have been linked to the development of SCLC and lung cancer in general are passive smoking, air pollution and exposure to environmental and occupational carcinogens such as radon, asbestos, arsenic, nickel, chromium and polycyclic aromatic hydrocarbons (Hamra et al., 2015). Previous in vivo studies in mice have shown that the conditional knockout of certain genes, such as the tumor suppressor genes TP53 and Rb1, also lead to SCLC (Meuwissen et al., 2003; Park et al., 2011; Sutherland et al., 2011).

1.1.3. Diagnosis

In general, most lung cancers do not cause any problems or symptoms until they have advanced locally or metastasized. Then a large part of the patients report symptoms including shortness of breath, fatigue, weight loss and loss of appetite, infections such as bronchitis and pneumonia and chest pain which worsens during breathing or coughing. When the cancer has already metastasized to other parts of the body (such as brain or bones) it may cause back pain, numbness or weakness of arms and legs, balance problems, dizziness or seizures (Asakura et al., 2015; Kazarian et al., 2011). SCLC represents the most frequent reason for paraneoplastic syndromes compared with endocrine syndromes, which include the syndrome of inappropriate antidiuresis (15-40%), the Cushing's syndrome (2-5%) and the Lambert-Eaton syndrome (3%) (Van Meerbeeck et al., 2011). About 2 out of 3 patients diagnosed with SCLC are 70 years or older, while less than 2% are younger than 45. At the time of diagnosis, the average age is around 70 years (Travis et al., 2015). SCLC can be diagnosed on good quality tumor material by carrying out a hematoxylin and eosin–staining. In well preserved cytologic or histological samples, derived from bronchoscopic biopsies, immunohistochemical markers can be very helpful in diagnosing pulmonary neuroendocrine tumors. Ki-67, a marker for proliferation, plays an important role in separating high-grade SCLC from carcinoid tumors, especially in small biopsies with necrotic tumor cells (Travis et al., 2015).

Neuroendorcine tumors are subdivided into carcinoid tumors, small cell lung carcinoma (SCLC) and large cell neuroendocrine carcinomas (LCNEC). Carcinoid tumors show major clinical, histologic, epidemiologic and genetic differences compared to SCLC or LCNEC. In general patients with carcinoid tumors have better prognosis, are younger and are not associated with smoking which is connected to SCLC and LCNEC (Travis et al., 2015).

1.1.4. Staging of small cell lung cancer

Cancer staging, which remains a key factor for assigning the most suitable treatment for each patient, describes the size, the location and spreading of a tumor in the body. In early staging systems, SCLC has been divided into limited stage disease (LS) and extensive stage disease (ES). This helped to determine if a patient might benefit from a chemotherapy combined with radiotherapy or whether chemotherapy alone seems to be the better option. Limited stage SCLC was defined as tumor limited to one hemithorax and able to be treated with a single radiation field, whereas extensive stage SCLC was defined as tumor that has spread throughout the lung, to lymph nodes and to the other side of the chest (Bradley et al., 2004; Simon et al., 2007). Most common staging systems are mainly divided into four basic factors which include the location of the primary tumor, tumor size, involvement of lymph nodes (whether the tumor has spread to nearby lymph nodes or not) and the presence or absence of metastasis. In 2009, when the 7th edition of TNM (tumor, node and metastasis) classification for staging of the UICC (Union for International Cancer Control) based on analysis of the IASLC (International Association for the Study of Lung Cancer) database was published, SCLC was categorized under this system and classified by stages I, II, III and IV. At the beginning of 2017 the 8th edition of the TNM classification of lung cancer, based on the data from more than 100,000 patients, including more than 5,000 people suffering from SCLC, was introduced (Nicholson et al., 2016; Vallières et al., 2009) (figure 1).

T: Primary tumor	
Tx	Primary tumor cannot be assessed or tumor proven by presence of malignant cells in sputum or bronchial washings but not visualized by imaging or bronchoscopy
T0	No evidence of primary tumor
Tis	Carcinoma in situ
T1	Tumor ≤3 cm in greatest dimension surrounded by lung or visceral pleura without bronchoscopic evidence of invasion more proximal than the lobar bronchus (i.e., not in the main bronchus)[a]
T1a(mi)	**Minimally invasive adenocarcinoma**[b]
T1a	**Tumor ≤1 cm in greatest dimension**[a]
T1b	**Tumor >1 cm but ≤2 cm in greatest dimension**[a]
T1c	**Tumor >2 cm but ≤3 cm in greatest dimension**[a]
T2	Tumor >3 cm **but ≤5 cm** or tumor with any of the following features[c]: - **Involves main bronchus regardless of distance from the carina but without involvement of the carina** - Invades visceral pleura - **Associated with atelectasis or obstructive pneumonitis that extends to the hilar region, involving part or all of the lung**
T2a	**Tumor >3 cm but ≤4 cm in greatest dimension**
T2b	**Tumor >4 cm but ≤5 cm in greatest dimension**
T3	**Tumor >5 cm but ≤7 cm in greatest dimension** or associated with separate tumor nodule(s) in the same lobe as the primary tumor or directly invades any of the following structures: chest wall (including the parietal pleura and superior sulcus tumors), phrenic nerve, parietal pericardium
T4	**Tumor >7 cm in greatest dimension** or associated with separate tumor nodule(s) in a different ipsilateral lobe than that of the primary tumor or invades any of the following structures: **diaphragm,** mediastinum, heart, great vessels, trachea, recurrent laryngeal nerve, esophagus, vertebral body, and carina
N: Regional lymph node involvement	
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in ipsilateral peribronchial and/or ipsilateral hilar lymph nodes and intrapulmonary nodes, including involvement by direct extension
N2	Metastasis in ipsilateral mediastinal and/or subcarinal lymph node(s)
N3	Metastasis in contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene, or supraclavicular lymph node(s)
M: Distant metastasis	
M0	No distant metastasis
M1	Distant metastasis present
M1a	Separate tumor nodule(s) in a contralateral lobe; tumor with pleural or pericardial nodule(s) or malignant pleural or pericardial effusion[d]
M1b	**Single extrathoracic metastasis**[e]
M1c	**Multiple extrathoracic metastases in one or more organs**

Figure 1: TNM staging system to describe the growth and spread of SCLC, based on the size of the primary tumor (T), whether the tumor has spread to regional lymph nodes (N) or to other organs of the body (M) (Nicholson et al., 2016).

1.1.5. Treatment options

The differentiation between the various lung cancer types is paramount, because treatment efficacies differ considerably between these types. However, this differentiation is not always evident, as a remarkable number of lung cancers show mixed characteristics, with various percentages of cells expressing different levels of

neuroendocrine, alveolar and bronchiolar markers (Adelstein et al., 1986). Screening for neuroendocrine markers, such as CD56, chromogranin and synaptophysin can be helpful to identify SCLC (Van Meerbeeck et al., 2011). At the time of diagnosis 60-70% of all SCLC patients show extensive-stage (ES) disease that has already metastasized beyond one hemithorax and remains incurable with present treatment options (Govindan et al., 2006; Schneider et al., 2015). Chemotherapy and radiation initially improve the symptoms in 60-80% of SCLC patients, and lead to shrinkage of the tumor within the first weeks of treatment. Even though SCLC initially seems sensitive to chemo- and radiotherapy, disease relapses within a short time and patients are rarely cured (Pedersen et al., 2003; Teicher et al., 2014). While the number of new therapeutics and treatment options has clearly increased in other malignancies, for SCLC, chemotherapy still remains the main pillar of treatment and so far no novel therapeutics for systemic treatment have entered into clinical practice in the past three decades (Kalemkerian et al., 2014). In patients with extensive stage disease, platinum compounds and etoposide remain the standard combination treatment. For limited stage disease (LS) the currently used standard treatment option is a platinum-based chemotherapy combined with etoposide and radiotherapy. Surgery is limited to a small percentage of patients that show only a small peripheral primary tumor and no lymph node involvement (Zaba et al., 2011). At the moment, there are no alternative therapeutic options for patients with platinum resistance after first-line treatment for SCLC. Second-line treatment in patients with SCLC is limited, due to the impared general clinical condition of more than 60% of patients, limiting any additional therapeutic option (Torre et al., 2015).

1.2. The transforming growth factor-β superfamily

The TGF-β superfamily of cytokines can be divided into several subfamilies, including bone morphogenetic proteins (BMPs), the activin subfamily, TGF-β isoforms 1-3 and growth and differentiation factors (GDFs). Furthermore, there are also some distant family members, such as myostatin that is also known as growth and differentiation factor 8 (GDF8), Nodal and the Anti-Mullerian Hormone (AMH) also known as the Mullerian Inhibiting Substance (MIS) (Deli et al., 2008; Herpin et al., 2004). Among these proteins, TGF-β and BMPs have been specifically well studied for their role in development, angiogenesis and epithelial to mensenchymal transition, above all for their influence in cell migration and invasion (Brown et al., 2011; Faure et al., 2000). TGF-β has been found to play an important role as a multifunctional polypeptide that regulates among other things, cell proliferation, differentiation, angiogenesis and embryonic development (Chen et al., 2000).

1.2.1. Activins

Activins, cytokines representing one subfamily of the transforming growth factor-beta (TGF-β) superfamily (Rodgarkia-Dara et al., 2006), were named after their first discovery in the 1980's as gonadal proteins, that act as activators of follicle stimulating hormone (FSH) from pituitary cells (Ling et al., 1986). Like other members of the TGF-β family, activins are secreted dimeric proteins consisting of 2 β-subunits covalently linked by a disulfide bond. In humans and also other mammals four different subunits have been identified, named βA, βB, βC and βE, which are each encoded by a single gene, named INHBA, INHBB, INHBC and INHBE. A fifth subunit, named βD, has only been identified in Xenopus laevis (Oda et al., 1995). Activins can either form homo- or hetero-dimers by dimerization of two β- subunits (a list of possible activins formed is shown in table 1). Activin A is a homodimer with 2 βA-chains covalently linked by a disulfide bond (βAβA). Activins AB and AC have been described under physiological conditions in vivo, while activins AE, BC and CE have only been described in in vitro systems, such as after ectopic expression of the appropriate cDNAs in diverse cell lines (Kreidl et al., 2009; Mellor et al., 2003).

All activin subunits are synthesized as protein proforms of 350-426 amino acids and a molecular weight between 38–50 kDa (Rodgarkia-Dara et al., 2006). Activin β-

subunits contain nine conserved cysteines in the mature peptides, where the sixth is used for dimerization and the others form intramolecular disulfide bonds which determine the three dimensional protein structure. In contrast to activins, inhibins are heterodimers consisting of one α- and one β-subunit linked by a single covalent disulfide bond. The β-subunit can either be a βA or a βB subunit, leading to the formation of inhibin A (α- and β-A) or inhibin B (α- and β-B) (Kreidl et al., 2009).

Table 1: List of possible activin subunit combinations

Activin	**Activin Subunits**
Activin A	βA and βA
Activin B	βB and βB
Activin C	βC and βC
Activin E	βE and βE
Activin AB	βA and βB
Activin AC	βA and βC
Activin AE	βA and βE
Activin BC	βB and βC
Activin BE	βB and βE
Activin CE	βA and βE

1.2.2. Activin receptors and signaling

Activins signal, like other members of the TGF-β superfamily, via two types of enzyme linked receptors that are single-pass transmembrane proteins with an intracellular serine- threonine kinase domain (Attisano et al., 1993).

Activin A first binds to and activates the type II activin receptors ActR-II (ACVR2) or ActR-IIB (ACVR2B), leading to the activation, recruitment and phosphorylation of the activin type I receptor ActR-IB (ACVR1B), also known as activin receptor-like kinase 4 (ALK4) (Gurdon et al., 1994; Hashimoto et al., 1998). Receptor-regulated Smads (R-Smads), such as Smad2 and Smad3, are recruited to the receptor complex and phosphorylated by the type I receptor, which leads to the building of complexes with the common mediator Smad4 and translocation to the nucleus where Smads are

directly involved in the regulation of gene expression. In general, Smads can be divided into receptor Smads 1, 2, 3, 5 and 8, a common mediator Smad4 and inhibitory Smads 6 and 7. Activin A receptors, as well as TGF-β receptors, activate, recruit and phosphorylate only the receptor Smads 2 and 3, whereas BMP receptors recruit and phosphorylate the receptor Smads 1, 5 and 8 (Chen et al., 2000; Deli et al., 2008) (figure2). In addition to the canonical Smad signaling pathway, recent indications attribute an important role of Smad-independent signaling of activin A to the activation of the MAP kinases ERK 1/2 and p38 as well as the Akt/PI3K pathway (Dupont et al., 2002; Murase et al., 2001). JNK and Rho were also associated with signaling of activin A (Zhang et al., 2005).

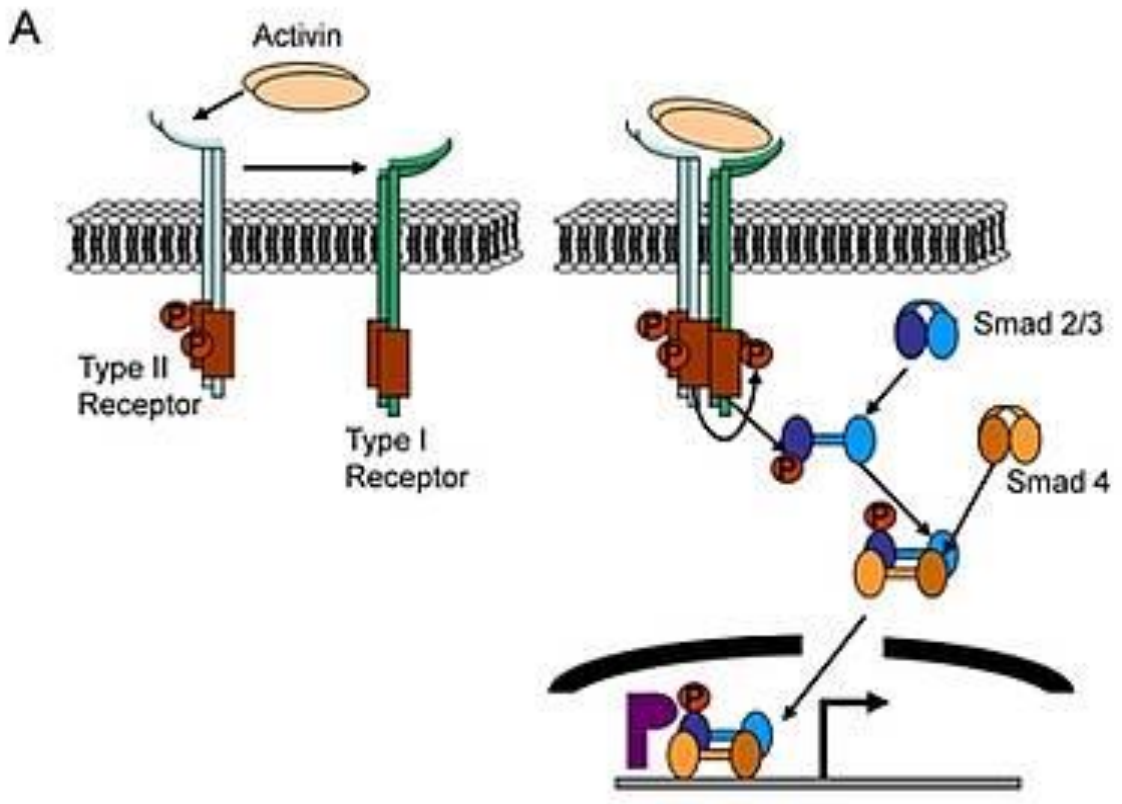

Figure 2: Activin signaling - Activins first bind a type II activin receptor, which then activates and phosphorylates a type I receptor. As a consequence of activation, receptor-regulated Smads are phosphorylated by the type I receptor, which subsequently leads to the formation of a complex with Smad 4 and translocation to the nucleus, where they regulate the transcription of target genes (Deli et al., 2008).

1.2.3. Activin A antagonists

i. Follistatin, (FST) a monomeric secreted glycoprotein and natural antagonist of activin A, can block activin signals by binding to its β-subunit and thus prevent its interaction with the type II activin receptors (ACVR2 and ACVR2B) (Hardy et al., 2015). Consequently activin A activity depends on the ratio of activin A to follistatin (Heinz et al., 2012).

ii. Follistatin-related gene (FLRG), also known as follistatin-like 3 (FSTL3) that was first characterized in leukemia as target of chromosomal rearrangement, can also bind to activin A and thus lead to the prevention of binding to the type II receptors (Hayette et al., 1998).

iii. Beside the receptor regulated Smads2 and 3 and the common mediator Smad4, there is also another group of Smad-proteins, named inhibitory Smads (I-Smads). Here the important I-Smad for activin signaling, Smad7, acts as negative regulator of the signaling pathway by binding to the activin type I receptor and subsequently preventing the co-Smad4 from binding to the type I receptors, thus blocking Smad signaling (Hayashi et al., 1997; Nakao et al., 1997). Another I-Smad protein, named Smad6, plays an important role in the inibition of the signaling pathway of BMPs (Heldin et al., 1997).

iv. In addition, the cell surface proteins cripto, a member of the epidermal growth factor-CFC family and BAMBI, also named BMP and activin membrane-bound inhibitor homolog, were found to block type I receptor activation (Gray et al., 2003).

1.2.4. Biological functions and expression of activin A

Activin A is expressed in multiple tissues and cell lines, especially in reproductive organs, and among all activins it represents the most extensively studied one (Tuuri et al., 2014). Besides activin A, activin B is also expressed in many tissues and cell types. Furthermore, it was found in human adipocytes and was suggested as a risk factor for metabolic syndrome (Palming et al., 2006). Activin C is predominantly expressed in the liver, but also in ovary, prostate and pituitary gland (Hotten et al., 1995; Hully et al., 1994; Vejda et al., 2002). Besides activin C, also another activin was found to show a high expression in the liver, activin E. Activin E is also expressed in other organs like the placenta, heart and skeletal muscle (Gold et al., 2005; Vejda et al., 2002). Activin A is involved in a variety of biological functions including cell growth, differentiation, cell death, systemic inflammation, wound healing, reproductive functions and embryonic development (de Kretser et al., 2012; Jones et al., 2004; Munz et al., 1999). Furthermore, activin A has also been implicated in stem cell biology, reproductive biology, erythroid differentiation and mesoderm induction (Beattie et al., 2005; de Kretser et al., 2002; McDowell et al., 1999). Increased levels of circulating activin A were detected in serum samples of patients suffering from angina, hepatocellular carcinoma (HCC), hepatitis, liver cirrhosis and lung adenocarcinoma (Hoda et al., 2016; Hughes et al., 2003; Kaneda et al., 2011; Norris et al., 2002). Regarding the liver, in vitro and in vivo studies showed that activin A inhibits mitogen-induced DNA synthesis and induces hepatocyte apoptosis (Hully et al., 1994; Schwall et al., 1993; Vejda et al., 2002). Activin receptors ActRI and ActRII are expressed in endothelial cells and these cells respond to activin A induction by reduced growth of cells and decreased angiogenesis (Breit et al., 2000; McCarthy et al., 1993). Furthermore, activin A was proposed as antiangiogenic factor in some tumors (Kaneda et al., 2011; Panopoulou et al., 2005; Schramm et al., 2005). Through the induction of vascular endothelial growth factor expression in corneal epithelial cells or hepatocellular carcinoma, however, activin A may also indirectly act as proangiogenic factor (Poulaki et al., 2004; Wagner et al., 2004).

Activin A plays an important role in the activation and maturation of the adaptive and innate immune system, (Hübner et al., 1996) and has also been found to stimulate

the growth of a variety of cells, including fibroblasts, keratinocytes and osteoblasts (Loomans et al., 2014).
Knock-out mice for activin A show grave craniofacial defects and die shortly after birth (Ferguson et al., 1998; Matzuk et al., 1995).

1.2.5. Activin A in cancer

Since activin A is involved in cellular differentiation and tissue architecture in multiple organs, deregulated expression of the components of activin signaling and also mutations have been found in a broad range of malignancies (Loomans et al., 2014). Like TGF-β, activin A can either promote or inhibit cell growth, depending on the tumor type. Activin A was found to inhibit cell growth in breast cancer (Burdette et al., 2005; Liu et al., 1996; Reis et al., 2004), hepatocellular carcinoma (Chen et al., 2000; Deli et al., 2008) and prostate cancer (Dowling et al., 2000; Risbridger et al., 2001). Whereas in endometrial carcinoma (Ferreira et al., 2008), oral squamous cell carcinoma (Chang et al., 2010), esophageal squamous cell carcinoma and esophageal adenocarcinoma (Pühringer et al., 2010; Yoshinaga et al., 2008), malignant pleural mesothelioma (Hoda et al., 2012), lung adenocarcinoma (Hoda et al., 2016; Seder et al., 2009) as well as in gastric cancer (Wang et al., 2015), high activin A expression led to increased tumor cell aggressiveness. Overexpression of activin A and high circulating activin A levels in the plasma and serum samples of patients were measured in various types of cancer and found associated with poor prognosis, metastasis and shorter overall survival (Chang et al., 2010; Hoda et al., 2016; Seder et al., 2009; Shimizu et al., 2007).
The tumor microenvironment could also be affected by activin A. Activin A can induce cell death of B-lymphocytes and promote T-lymphocyte immune suppression through regulatory T-cell -conversion, thereby helping the immune system in deflecting the attack of the tumor cells (Loomans et al., 2014) (figure 3).

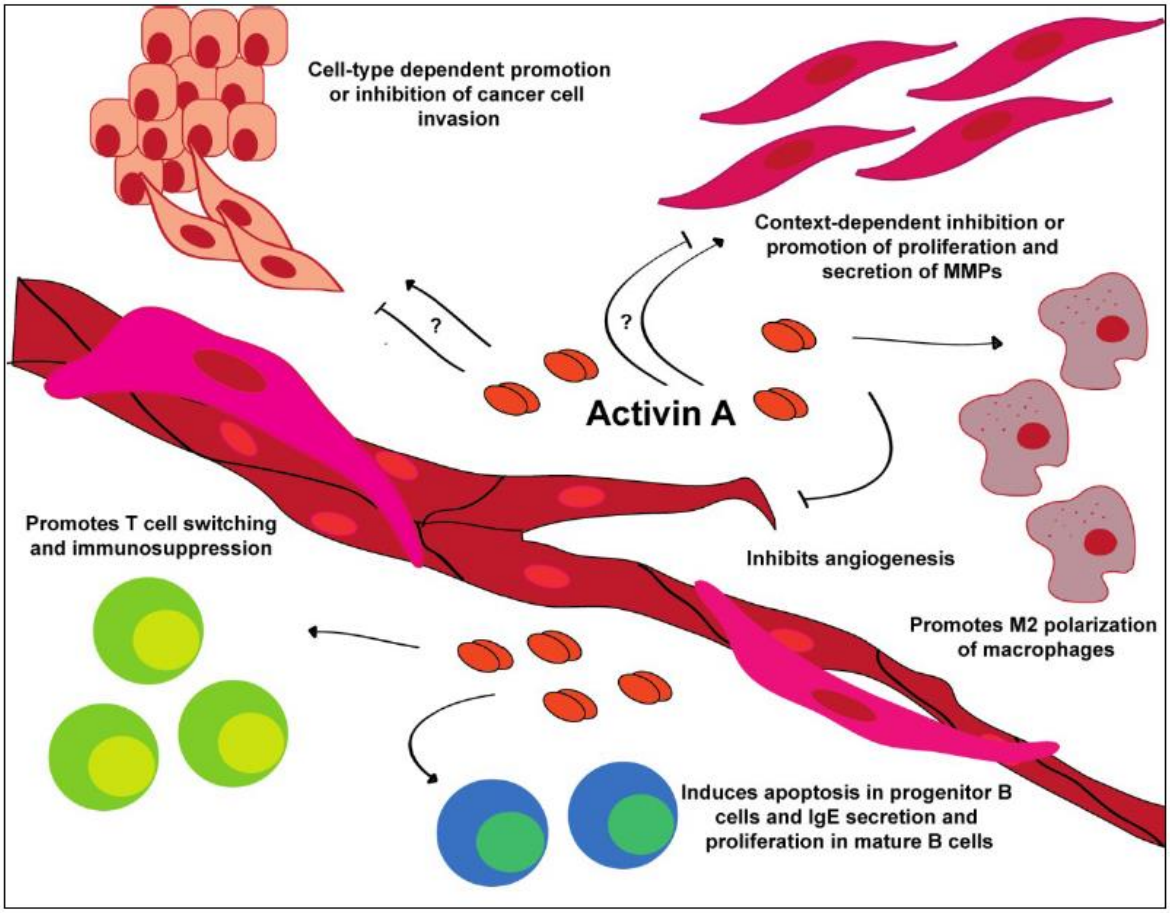

Figure 3: Effects of activin A on the tumor microenvironment (Loomans et al., 2014).

1.3. Aims of the study

Small cell lung cancer, that amounts to about 10-15% of all lung cancers, is strongly related to the usage of tobacco and characterized by its rapid growth, highly aggressive disease course, early development of local and distant metastasis and high therapeutic resistance.

Activin A, a homodimer of 2 βA-chains covalently linked by a disulfide bond and member of the TGF-β family of growth and differentiation factors has been described to impact on malignant growth in a variety of thoracic malignancies including lung adenocarcinoma, malignant pleural mesothelioma and esophageal carcinoma. So far, however, there are no published data available about the expression and function of activin A in SCLC.

Recently, an extended collection of blood samples of SCLC patients was analyzed in comparison to age-matched healthy controls by our group. Plasma levels of activin A were determined using a commercially available ELISA assay. Activin A plasma levels were found to be increased in a subgroup of SCLC patients, specifically in patients with metastatic disease (figure 4).

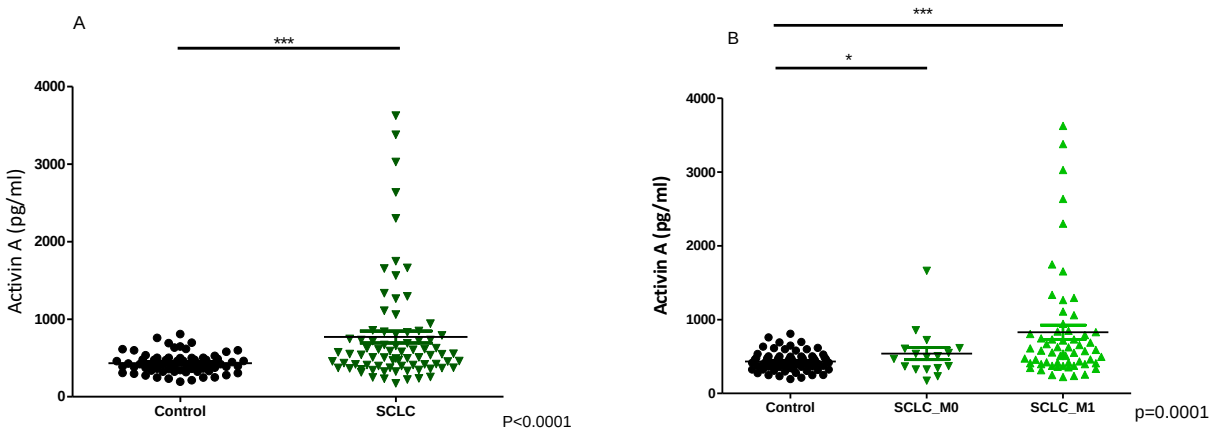

Figure 4: Plasma levels of activin A were determined in SCLC patients compared to age-matched healthy controls using a commercially available ELISA assay (R&D systems).
Activin A level is significantly elevated in the plasma samples of SCLC patients (A).
Activin A plasma levels were significantly elevated in SCLC patients with metastatic disease (B).
(data evaluated by Anita Rozsas, unpublished)

Based on these data the aims of this thesis were:

1. to determine the expression of activin A, of the activin antagonist follistatin and of activin receptors in a panel of SCLC cell lines that were obtained from international sources, including cell lines derived from metastatic disease

2. to examine the impact of activin A treatment or ectopic overexpression of activin A on cell proliferation, viability, invasion, migration, cell cycle distribution and anchorage independent growth of SCLC cell lines

3. to investigate the activation of signal trandsuction pathways by activin A and the impact on gene expression in SCLC cell lines

2. MATERIALS AND METHODS

2.1. Cell cuture

All cell lines were cultured in T-25 or T-75 tissue culture flasks in a humidified atmosphere of 5% CO_2 at 37°C in RPMI, DMEM, MEME, MNP or F12-K medium containing 10% FCS. The cells were passaged about twice a week, depending on the cell line, at a ratio of about 1:10 or 1:20. All used cell lines, their growth properties, standard growth medium and source are listed in table 2. To distinguish viable cells from dead cells, cell suspensions were mixed 1:1 with trypan blue and counted using a Neubauer chamber.

Table 2:
Cell lines used for in vitro experiments

Celllines	***Tumor type***	***Growth properties***	***Standard growth medium***	***Source***
BEC mCherry	Blood endothelial cells	adherent	EGM-2	Schoppmann S.F., Department of Pathology, Medical University of Vienna
CRL-2066 / DMS 114	carcinoma, SCLC	adherent	RPMI + 10% FCS	ATCC
CRL-2062 / DMS 53	carcinoma, SCLC	adherent	RPMI + 10% FCS	ATCC
GLC4	SCLC	semi-adherent	RPMI + 10% FCS	E.G.E. de Vries, Department of Medical Oncology, University of Groningen
GLC14	SCLC	mixed, adherent and suspension	RPMI + 10% FCS	Poulsen H.S., Institute of Pathological Anatomy, University of Copenhagen
GLC16	SCLC	suspension	RPMI + 10% FCS	Poulsen H.S., Institute of Pathological Anatomy, University of Copenhagen
GLC19	SCLC	suspension	RPMI +	Poulsen H.S.,

			10% FCS	Institute of Pathological Anatomy, University of Copenhagen
Hek293	Human embryonic kidney 293 cells	adherent	MEME + 10% FCS	ATCC
HepG2	Human hepatocellular carcinoma cell line	adherent	MNP + 10% FCS	ATCC
HLHE	Brain metastasis, SCLC	adherent	RPMI + 10% FCS	established in the group of W. Berger, Institute of Cancer Research, Medical University of Vienna
H146 / HTB-173	carcinoma, SCLC, derived from metastatic site – bone marrow	suspension, multicell aggregates	DMEM + 10% FCS	ATCC
HTB-119 / H69	carcinoma, SCLC	suspension, multicellaggregates	RPMI + 10% FCS	ATCC
HTB-171 / H446	carcinoma, SCLC, derived from metastatic site – pleural effusion	mixed, adherent and suspension	RPMI + 10% FCS	ATCC
HTB-180 / H345	carcinoma, SCLC, derived from metastatic site – bone marrow	suspension	RPMI + 10% FCS	ATCC
HTB-184 / H510A	SCLC, extrapulmonary origin – derived from metastatic site – adrenal gland	mixed, adherent and suspension	F12-K + 10% FCS	ATCC
LEC mCherry	Lymphatic endothelial cells	adherent	EGM-2	Schoppmann S.F., Department of Pathology, mCherry expression was generated in our group

2.2. Cell treatment

For different in vitro assays, the cells were treated with recombinant human activin A (R&D Systems). The cytokine was diluted as a 10 µg/ml stock solution in 4 mM HCl and stored at -80°C as recommended by the manufacturer. For further experiments, activin A was diluted in the respective growth medium. The usual standard treatment concentration was 10 ng/ml or 20 ng/ml.

2.3. Transient transfection

SCLC cells were mainly transfected using either Lipofectamine 2000 (Invitrogen) or PEI (Polyethylenimine), according to the manufacturers' instructions.
Cells (5 x 10^5 per well) were seeded into 6-well plates and left to recover over night. For a 6-well plate, 12 µl of the transfection reagent were added to 100 µl serum-free medium and incubated for 5 min. 2.5 µg of plasmid DNA (one or several plasmids) were diluted in 100 µl serum-free medium, mixed with transfection reagent and serum-free medium. The mixture was incubated for 20 min and then slowly added to the cells. After 5 h of incubation, the medium in every well was changed to the respective growth medium. Gene expression was assessed 24 h post transfection.

2.4. Establishment of stable transgenic cell lines

To study long term effects of SCLC cell lines overexpressing activin A, which cannot be mimicked well by treatment with recombinant human activin A, an isogenic derivative with stable ectopic overexpression of activin A was generated.

2.4.1. Creation of the virus

For the production of retroviral particles, Hek293 cells were seeded into a T-25 tissue culture flask and grown until they were almost confluent. Then medium was changed and transfection reagent was prepared. 625 µl serum-free medium were mixed with 25 µl PEI and incubated for 5 min at room temperature. In the meantime 625 µl serum-free medium were mixed with 3.75 µg of a plasmid containing the GagPol sequence and 1.25 µg of a plasmid containing the VSVG sequence. In addition to these two helper plasmids required for virus production, 5 µg of the plasmid containing INHBA were added. After that, both mixtures were combined and incubated for another 20 min. After the incubation period, the reagent was added to the T-25 tissue culture flask containing Hek293 cells and incubated for 5 hours. Afterwards the medium was changed to fresh MEME medium containing 10% FCS, and the cells were cultured for another 48 hours. Then the medium containing the virus was filtrated and aliquoted into 1.5 ml tubes (1 ml per tube). The virus aliquots were stored at -20°C.

2.4.2. Retroviral transduction

For retroviral transduction, 3 x 10^4 cells were seeded into 6-well plates and left to recover. The next day the growth medium was removed and one aliquot of the appropriate virus was added. The virus was left on the cells over night. After that the medium in each well was changed to the appropriate growth medium containing 10% FCS, and selection antibiotics were added (neomycin for the INHBA vector and puromycin for the GFP vector). Neomycin was added at a final concentration of 500 µg/ml and puromycin at a final concentration of 0.8 µg/ml.
Serving as control for selection efficiency, antibiotics were also added to non-transfected cells. When the colonies that formed under selection pressure were big enough, they were transferred to a new T-25 tissue culture flask and further passaged like their parental cell lines. Antibiotics were added every 3rd to 4th passage to maintain transgene expression.

2.5. Expression analysis / RNA level

2.5.1. RNA isolation

For RNA isolation, semi-adherent cells of a nearly confluent T-25 flask were pelleted, medium was discarded and 1 ml trizol was added to each tube. Next, the lysed cells were transferred into a 1.5 ml tube and incubated for 5 min at room temperature. Subsequently 200 µl chloroform per 1 ml trizol (table 3) were added and mixed by vortexing the tube. Then samples were centrifuged at 4°C for 15 min at 13.000g. The upper, aqueous phase containing the RNA was gently transferred into a new 1.5 ml tube, 500 µl isopropanol were added and mixed by vortexing. To allow the RNA to precipitate, tubes were stored at –20°C for at least 1 h. Then samples were again centrifuged at 4°C for 10 min at 13.000g, supernatant was discarded and the pellet was washed by adding 1 ml 75% ethanol. After another centrifugation step, at 4°C for 5 min at 13.000g, supernatant was discarded and the pellet air-dried. Finally RNA was dissolved by adding 15 µl nuclease-free H_2O and heating for 5 min to 60°C. RNA concentration and purity were determined by measuring the optical density at 260 nm, using a NanoDrop 1000 spectrophotometer (Peqlab), and quality of the RNA was verified using agarose gel electrophoresis.

Table 3: Trizol

0.8 M Guanidine thiocyanate
0.4 M Ammonium thiocyanate
0.1 M Sodium acetate
5% v/v Glycerol
38% Phenol

2.5.2. Agarose gel electrophoresis

Nucleic acids (RNA) were separated, according to their length (bp), by agarose gel electrophoresis. For size determination, a 1 kb DNA ladder (Thermo Scientific) was used. 1 µg of RNA was mixed with nuclease-free H_2O to a total volume of 10 µl, and mixed with 3 µl of vistra green loading dye (table 4), then loaded onto the gel,

consisting of 1.5% agarose in 1 x TBE buffer (table 5). The gel was run at 50 V for 10 min and then at 120 V for 30-40 min. To visualize the fragments, the gel was scanned by a Typhoon Trio FluorImager (GE Healthcare), which detects the fluorescence of the RNA-bound vistra green.

Table 4: Vistra green loading dye (6x)

666 µl 6x Loading dye (Thermo Scientific)
700 µl dH_2O
500 µl 80% Glycerol
133 µl 0.5M EDTA
1 µl Vistra-green 10000x (Amersham)

Table 5: Tris borate EDTA buffer – TBE

10.8 g/l Trizma base (Sigma)
5.5 g/l Boric acid (Sigma)
4ml/l 0.5M EDTA, pH=8.0
dH_2O to 1 liter

2.5.3. Synthesis of cDNA

The isolated RNA was reverse transcribed into cDNA for quantitative qPCR, using Revert Aid reverse transcriptase (ThermoScientific). Two µg RNA were diluted in 13 µl nuclease-free H_2O and heated up to 70°C for 10 min for denaturation. Then samples were put on ice for about 10 min to cool down. Afterwards 7.5 µl of cDNA master mix (table 6) were added and samples incubated at 42°C for 1 h. The reaction was stopped, by denaturating the enzyme at 70°C for 10 min. The obtained samples were finally diluted 1:1 by adding 20 µl of nuclease-free H_2O and stored at -20°C.

Table 6: 1 x cDNA master mix

4 µl 5x M-MLV RT buffer (Thermo Scientific)
1 µl dNTPs (10 mM, Thermo Scientific)
0.5 µl Random hexamer primers (0.5 µg/µl, Thermo Scientific)
0.5 µl RiboLock RNase inhibitor (40 U/µl, ThermoScientific)
1 µl Reverse transcriptase (200 U/µl, ThermoScientific)

2.5.4. Quantitative real-time Polymerase Chain Reaction (qPCR)

2.5.4.1. TaqMan qPCR

For semi-quantitative analysis of the activin A mRNA level in the transgenic cell line GLC4 or in the transiently transfected cell line CRL-2066, TaqMan qPCR was performed. 1 µl of cDNA was mixed with 11.5 µl of TaqMan quantitative real time PCR master mix (table 7) containing the appropriate TaqMan probe in a MicroAmp optical 96-well reaction plate (Applied Biosystems). All samples were analyzed in duplicates. The prepared plate was then placed in an ABI Prism 7000 SDS Thermocycler (Applied Biosystems), the PCR program was started and fluorescence was measured after every cycle. As reference for normalization, the housekeeping gene GAPDH was used.

Table 7: 1 x TaqMan qPCR master mix

6.25 µl Maxima probe qPCR master-mix (Thermo Scientific)
0.625 µl TaqMan probe (Applied Biosystems)
4.625 µl dH_2O

Table 8: Standard conditions for TaqMan qPCR

	Temperature	**Time (minutes)**	**Repetitions**
Stage 1	50°C	02:00	1x
Stage 2	95°C	10:00	1x
Stage 3	95°C	00:15	40x
	60°C	01:00	

Table 9: TaqMan probes used for expression analysis

Target	**Assay ID**
INHBA	Hs01081598 m1
FST	Hs00246256 m1
GAPDH	Hs999999 m1

2.5.4.2. SYBR Green qPCR

For semi-quantitative analysis of the activin A receptor levels in transgenic cell lines and changes in gene expression in overexpressing cell lines, SYBR Green qPCR was performed. Primers for the two different types of activin A receptors were designed using the clone manager program. The mRNA sequence of the gene of interest was taken from the NCBI database and the primer design tool in the program was used. The optimal length of the primers was set between 17 bps and 21bps, GC% range between 40 and 70 and finally acceptable PCR product length was set between 90 and 150 bps. According to the ranking of the program the best primer pair was chosen.

For the experiment, 1 µl cDNA was mixed with 12 µl master mix, containing the appropriate primers for the gene of interest. GAPDH was used as reference for normalization.

Table 10: List of primers used for SYBR Green qPCR

Target	**Sense**	**Primer Sequence**	**Annealing temperature °C**
ACVR1B	forward	GCC CTC TGA CCC TTC CAT TG	55
	reverse	CCC GCA GTG CCT CAT AAC TC	55
ACVR2A	forward	ACC CAG ATG CAG AGA CTA AC	55
	reverse	ATG GCG CAA CCA TCA TAG AC	55
ACVR2B	forward	TCA GCA CAC CTG GCA TGA AG	55
	reverse	TCA TGG AAG GCC GTG ATG AG	55
BCL2	forward	AAT TGC CAA GCA CCG CTT C	55
	reverse	TTC CAT CCG TCT GCT CTT C	55
E-cadherin	forward	CAG AGC CTC TGG ATA GAG AAC GCA	58
	reverse	GGC ATT GTA GGT GTT CAC ATC ATC GTC	58
ESM1	forward	GTG GAC TGC CCT CAA CAC	53
	reverse	GCC ATC CAT GCC TGA GAC	53
ETV4	forward	CCG GCC AGC CAT GAA TTA C	54
	reverse	CGG GAA GGC CAA AGA GAA GAG	54
GAPDH	forward	AGC TCA CTG GCA TGG CCT TC	55
	reverse	ACG CCT GCT TCA CCA CCT TC	55
MMP1	forward	TAC ATG CGC ACA AAT CCC	49
	reverse	ACA GCC CAG TAC TTA TTC CC	49
MMP3	forward	TGG GCC AGG GAT TAA TGG AG	50
	reverse	GGG AGT GGC CAA TTT CAT GAG	50
N-cadherin	forward	GCA TCA TCA TCC TGC TTA TCC	55
	reverse	TTC TCC TCC ACC TTC TTC ATC	55
PDL1	forward	CAC CAC CAA TTC CAA GAG	47
	reverse	CTG GGA TGA CCA ATT CAG	47
Smad7	forward	TGC CTT CCG CTG AAA C	54

	reverse	CGT CTT CTC CTC CCA GTA TGC	54
Vimentin	forward	GGC TCA GAT TCA GGA ACA GC	55
	reverse	CTG AAT CTC ATC CTG CAG GC	55

2.6. Protein analysis

2.6.1. Protein isolation

For total protein isolation, 5 x 10^5 -cells per well were seeded into a 6-well plate in 2 ml medium and grown over night. On the next day, the cells were washed once with PBS, then scraped off into 60-80 µl lysis buffer LB II (table 11) and transferred into a 1.5 ml tube. The suspension was first sonicated for 5 min and then centrifuged at 4°C for 10 min at 12.000 g. The supernatant was transferred into a new 1.5 ml tube and stored at -20°C. Protein concentration was determined by performing a Bradford protein assay.

Table 11: Protein Lysis Buffer II

Stock Solution	**Final Concentration**	**10ml (+bdH_20) + 1 complete tab (Roche)**
100 mM EGTA	1 mM EGTA	100 µl
5M NaCl	150 mM NaCl	300 µl
50 mM Na_3VO_4	1 mM Na_3VO_4	200 µl
100% Triton X	1% Triton X	100 µl
100 mMNaF	10 mM NaF	1 ml
1M Tris pH=8	50 mM Tris pH=8	500 µl

2.6.1.1. Bradford protein assay

To determine protein concentration, protein samples were diluted 1:10 in lysis buffer LB II, and Biorad Protein Assay Reagent was used. The assay was performed in a 96-well plate and lysis buffer LB II was used as blank (table 12). To examine total protein concentration, a standard curve that had been gained from BSA dilutions was applied. Absorption was measured at 595 nm using a Synergy HT plate reader (BioTek).

Table 12: Pipetting scheme for determination of protein concentrations according to Bradford assay

	Distilled water (µl)	**Lysis Buffer II**	**BSA (µg/µl)**	**Concentration (µg/µl)**
Blank	9	1	0	0
Standard 1	8	1	1	0.1
Standard 2	7	1	2	0.2
Standard 3	5	1	4	0.4
Standard 4	3	1	6	0.6

2.6.3. SDS PAGE

To separate the proteins according to their molecular weight (kDa), a polyacrylamide gel was used for gel electrophoresis. 25 µg protein were diluted in the appropriate amount of LBII lysis buffer to a total volume of 20 µl and mixed with 5 µl 5 x reducing Laemmli buffer (table 13). After denaturation at 100°C for 5 min, samples were spinned down and loaded, together with a page ruler plus prestained protein ladder (Thermo Scientific), serving as size marker, on a polyacrylamide gel consisting of a 10% separating and a 5% stacking gel (tables 14 and 15). The gels were placed in an electrophoresis tank filled with 1 x SDS running buffer (table 16), before being loaded with samples, and then the gel was run at 60V for 15 min and at 120V for 1.5 h.

Table 13: 5x reducing Laemmli buffer with β-mercaptoethanol

Laemmli buffer
300 nM Tris, pH=6.8
60% Glycerol
10% SDS
0.025% Bromophenol blue
7%β-mercaptoethanol (Aldrich)

Table 14: 5 ml 5% PAA stacking gel

3.6 ml ddH_2O
625 µl 0.5 M Tris –HCl, ph=6.8
100 µl 10% SDS

625 µl 30% Acrylamid/Bis; 29:1 (Bio-Rad)
50 µl APES (Merck)
7.5 µl TEMED (Amresco)

Table 15: 15 ml 10% PAA separating gel

5.8 ml ddH_2O
3.75 ml 1.6 M Tris-HCl, pH=8.8
300 µl 10% SDS
5.025 ml 30% Acrylamid/Bis; 29:1 (Bio-Rad)
37.5 µl APES (Merck)
20 µl TEMED (Amresco)

Table 16: SDS running buffer

25mM Tris base
192 mM Glycine
0.1% SDS

2.6.4. Western Blot

To transfer the separated proteins from the gel to a methanol-activated PVDF membrane, a Mini Protean 3 System (Bio-Rad) was used. The transfer was performed in transfer buffer (table 18) at 4°C for 1.5 hours. To check the quality of the protein transfer, the membrane was stained for 10 min with Ponceau S solution (table 17). Afterwards it was blocked in 5% skim milk powder in TBST (Tris-buffered saline containing 0.1% Tween 20) for 1-2 h at room temperature. Then the membrane was quickly washed once in TBST and incubated in the primary antibody (table 20) at 4°C over night. The next day the membrane was washed 3 times for 10 min with TBST and then incubated in the secondary antibody (HRP-coupled) for 1-2 h at room temperature (table 21). After this incubation step, the membrane was again washed 3 times for 10 min with TBST and once with TBS (table 19). Antibody binding was detected on an X-ray film using Immun-Star WesternC Kit (Bio-Rad), as recommended by the manufacturer. Exposure times varied between 1 second and 1 hour, depending on the antibody and the strength of the signal.

After development of the X-ray films, membranes were washed once with TBST and stored at 4°C for further usage.

Table 17: Ponceau Solution

0.5 g/l Ponceau S (Sigma)
1 ml/l Glacial acetic acid

Table 18: Towbin transfer buffer

13.39 g/l Glycine
3.03 g/l Tris base
50 ml/l Methanol

Table 19: TBS (Tris buffered saline)

8 g/l NaCl
0.2 g/l KCl
3 g/l Tris base
HCl – to adapt pH to 7.4

Table 20: List of primary antibodies used for western blotting

Antibody	**Dilution and Diluent**	**Size of target (kDa)**	**Assay ID**
Rabbit anti-Smad2	1:1000 in 3% BSA in TBST	60 kDa	Cell Signaling, # 5339
Rabbit anti-pSmad2	1:1000	60 kDa	Cell Signaling, # 3101
Mouse anti-β-actin	1:5000 in 3% BSA in TBST	42 kDa	Sigma, #A 5441

Table 21: List of secondary antibodies used for western blotting

Antibody	**Dilution and Diluent**	**Supplier**
Polyclonal Goat anti-Rabbit Immunoglobulins/HRP	1:10000	Dako
Anti-Mouse HRP	1:10000	Dako

2.7. Cell cycle analysis

The percentage of cells in each stage of the cell cycle was analyzed by determining the amount of DNA in the cell via flow cytometry of propidium iodide stained cells. For cell cycle analysis 1 x 10^6 cells were seeded into a T-25 flask in 5 ml growth medium containing 10% FCS. First, the whole cell suspension in the flask was transferred to a 15 ml tube and centrifuged for 5 min at 800 rpm for pelleting the cells. The cell suspension was then resuspended in 5 ml PBS and centrifuged a second time for 5 min at 800 rpm. Afterwards cells were resupended in 70% cold ethanol and fixed for 30 min at 4°C. After that cells were again centrifuged at 800 rpm for 10 min and supernatant was discarded. The cell pellet was then centrifuged for a last time at 800 rpm for 10 min. Supernatant was discarded and cells were finally resuspended in 0.5 ml staining solution (table 22) and transferred into 5 ml round bottom tubes (BD Falcon). After an incubation time at 4°C for 10 min, fluorescence was measured using a flow cytometer (Calibur-Becton Dickinson FACS).

Table 22: Staining solution

0.5 ml PBS
25 µg RNAse A
25 µg PI (Propidium iodide)

2.8. MTT assay

To evaluate cell viability, MTT assays were performed (Biomedica Austria, MTT kit EZ4U). This assay is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (tetrazolium dye MTT) to the orange colored formazan derivate by mitochondrial enzymes. Since the production of the derivate requires functional and active mitochondrial enzymes, the coloration reflects the number of viable cells. 3 x 10^3 cells were seeded in five replicas in 100 µl medium containing 10% FCS in a 96-well plate or in a 96-well low-adherence plate. After 72 hours, the medium of each well was aspirated and replaced by 100 µl fresh medium containing 10% EZ4U reagent. Serving as blank, three empty wells were filled with 100 µl of the mixture. Absorbance was measured at 450 nm and 620 nm, as reference, using a SynergyHT plate reader (BioTek), after an incubation time of 1 h.

2.9. Fluorescent protein-based growth assay 1

To check the growth of cells, 3 x 10^3 GFP-expressing cells were seeded in 100 µl medium into a 96-well plate. The GFP signal of the stably expressing cells, was measured every day for one week using a Typhoon Trio Fluoro Imager (GE Healthcare). (GFP signal: emission 488, PMT 600, filter 488nm, scanning height: +3 mm) Cells were seeded in five replicas. Scans were analyzed with ImageQuant software.

2.10. Fluorescent protein-based growth assay 2

In order to examine whether activin A has an influence on the growth of endothelial cells or not, 3 x 10^3 lymphatic or blood endothelial cells stably expressing the red fluorescent protein mCherry were seeded into a 96-well plate and left to recover overnight. The next day endothelial cells were either co-cultured with SCLC cells, treated with the supernatant of 5 x 10^5 SCLC cells or treated with 10 ng/ml activin A. The mCherry signal of the endothelial cells was measured every day for one week using a Typhoon Trio Fluoro Imager (for mCherry signal: emission 610, PMT 600, filter 532 nm, scanning height: +3 mm). Scans were examined using ImageQuant software.

2.11. Clonogenic assay

In order to determine the capacity of the cells to form colonies, 10 x 10^3 cells per well were seeded into a 6-well plate and left to recover over night. The next day medium was removed and replaced with 2 ml fresh growth medium containing the desired treatment and allowed to grow for 11 days. Experiments were done in duplicates.
After the incubation time of 11 days the medium was removed, cells were washed with PBS, fixed with 1ml methanol-acetic acid (3:1) for 30 min, washed again with PBS and stained with crystal violet (10% CV in ethanol, 1:1000 in PBS) for 1-3 h. Finally, the plates were washed carefully with dH_2O to remove excess crystal violet and air-dried over night. Afterwards pictures of the stained colonies were taken with a

Nikon D90 camera for image analysis and colonies were destained with 200 µl 2% SDS for some hours. The solution was then transferred to a 1.5 ml tube, vortexed, and 100 µl of the mixture were pipetted in duplicates into a 96-well microtiter plate and the absorption at 560 nm was photometrically measured using a SynergyHT plate reader (BioTek).

2.12. Adhesion assay

To evaluate the change in adhesion after coating plates with different components of the extracellular matrix, an adhesion assay was performed. Therefore 24- well plates were coated with 500 µl of the respective coating substance and incubated for 2 h at 37°C. Laminin was used at a concentration of 1:100 in dH_2O, collagen 1:12 in PBS, fibronectin 1:10 in dH_2O and poly-L-ornithin 1:10 in dH_2O, according to the manufacturers instructions (all coating substances were purchased from Sigma Aldrich). Wells without any coating substance were used as control. After the incubation time, supernatant fluid was removed and wells were washed once with PBS. 5 x 10^3 cells were seeded into 3 different wells, to allow medium change in various wells after 1, 2 or 3 days. Pictures of the same area in each well were taken before and after changing the medium.

2.13. Spheroid formation assay

In order to analyze the ability of SCLC cells to form spheroids 5 x 10^3 or 2 x 10^4 cells were seeded into a low-adherence 24-well plate in 1 ml serum-free stem cell medium (table 23). To evaluate the spheroid forming ability, pictures were taken after 1, 3, 7 and 10 days. The experiment was performed in triplicates.

Table 23: Stem cell medium

40 ml DMEM
1% P/S
10 ng/µl EGF
400 µl B27 (50 x stock)
100 ng/µl bFGF

2.14. Gap forming assay

To investigate the basic molecular mechanisms of lymph or blood vessel bulk invasion, an in vitro co-culture system was used (Kerjaschki et al., 2011). 3 x 10^5 cells were mixed with medium to a final volume of 12 ml and then 3 ml methylcellulose (table 24) were added to a final concentration of 3.2 mg/ml. 150 µl of this mixture were transferred to a 96-well round bottom plate for suspension cultures and incubated for 48 hours to allow spheroid formation. On the second day, lymphatic endothelial cells (LEC) or blood endothelial cells (BEC) were seeded at a density of 1.8 x 10^5 cells in a 24-well plate in 500 µl growth medium and incubated for 1 day. Afterwards methylcellulose was discarded, spheroids were washed once with medium, medium was replaced by 100 µl fresh endothelial growth medium and then spheroids were gently transferred onto the BEC or LEC monolayer. The co-culture was incubated for 4 h at 37°C to allow the formation of circular gaps that were named as circular chemorepellent-induced defects by Kerjaschki et al (CCID formation). Afterwards pictures were taken and analysis was carried out with ImageJ software (figure 5).

Table 24: Methylcellulose

4 g Methylcellulose
250 ml RPMI

Methylcellulose

Methylcellulose stock was produced by dissolving 4 g of autoclaved methylcellulose in 250 ml RPMI, stirring it at 4°C overnight and centrifuging the 50 ml aliquots at room temperature for 2 h at 5000 G. Afterwards approximately 40 ml were transferred to a new tube and stored at -20°C. After thawing and before use, every tube was again centrifuged at room temperature for 30 min at 5000 G.

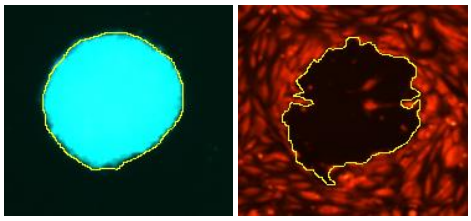

Figure 5: Representative pictures of SCLC cell spheroid induced CCIDs in lypmphatic and blood endothelial cell monolayers.

2.15. Transwell invasion assay

In order to check the invasion ability of SCLC cells through collagen and subsequent transmigration through a porous membrane, a transwell invasion assay was performed. On the first day, transwell chamber cell culture inserts (BD Falcon) with a porous membrane (8 µm diameter of pores) were placed into wells of a 24-well plate, coated with 50 µl collagen (diluted 1:12 in PBS) and then incubated overnight at 37°C. The next day, 800 µl medium with 10% FCS were placed below the insert and 200 µl medium with 4 x 10^4 cells inside the insert. One well of each cell line was immediately treated with 20 ng/ml activin A. Then cells were incubated for 72 hours at 37°C. After the incubation time, medium was discarded, inserts were washed once with PBS and cells that stayed on the upper part of the insert were removed using a cotton swab. Cells that had invaded the collagen and moved to the lower surface of the insert were fixed for half an hour with methanol/acetic acid (3:1) and then stained with crystal violet (1:1000 in PBS) for approximately one hour. Afterwards inserts were thoroughly washed with dH_2O and left to air-dry overnight. Cells were then destained using a 2% SDS solution. The absorbance was measured using a TECAN photometer at a wavelength of 562 nm.

2.16. Transendothelial migration

To investigate cell migration and invasion, a transendothelial migration assay was performed. As in the invasion assay, transwell chamber cell culture inserts were used, first coated with collagen, then incubated over night at 37°C. The next day 4 x 10^4 endothelial cells were carefully seeded on the top of the collagen layer and grown until they reached confluence. Afterwards 4 x 10^4 tumor cells were seeded on top,

immediately treated with activin A and incubated for 72 h. Cells remaining on the upper side of the membrane were gently wiped away with a cotton swab, and then the insert was placed for 1 hour in Histofix (Roti) to fix cells on the lower surface of the membrane. Membranes were afterwards cut out of the insert using a scalpel, mounted with glycerin gelatin on a microscope slide and covered with a cover glass.

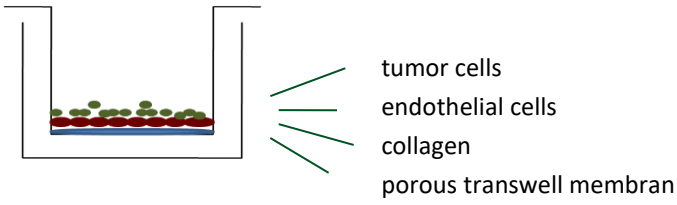

Figure 6: Setting of the transendothelial migration assay.

2.17. Soft agar assay

To analyze anchorage independent growth and ability of single cells to form cell colonies without cell-cell contact, a soft agar colony formation assay was performed. On the first day, soft agar medium as well as agar (1.2 g agar (Sigma Aldrich) dissolved in 80 ml dH_2O) were prepared and warmed up to 40°C in a water bath. Soft agar medium (table 25) and agar were mixed in a 1:1 ratio, 1.5 ml of this mixture were pipetted into each well of a 6-well plate and incubated at 37°C overnight. Medium and agar were kept at 4°C. The next day the medium was warmed to 37°C and agar from the day before was boiled again, mixed 1:1 with dH_2O, boiled up again and cooled down to 37°C in a water bath. In the meantime, cells were prepared. They were pelleted and afterwards resuspended in 750 µl soft agar medium. The cell suspension was also kept at 37°C using a heating block. Cell suspension and agar were diluted 1:1 to a total volume of 1.5 ml and gently layered on top of the prepared bottom layer from the day before. Treatment compounds were added to the tubes before transferring the solution to the 6-well plates. Plates were placed for 3 min in the refrigerator at 4°C and then in the incubator at 37°C. Plates were kept in the incubator for three weeks, until cells had formed measurable clones.

Table 25: Soft agar medium

10 ml 10 x RPMI
2 ml Sodium hydrogen carbonate
1 ml Glutamine
17 ml dH_2O
50 µl Folic acid
-> pH 8
->sterile filtering
20 ml FCS
1 ml Penicillin-Streptomycin solution (P/S)
1 ml Amphothericin B

2.18. 3-D cell culture - Alvetex

Alvetex, a porous polystyrene scaffold, was designed for 3-D cell culture that allows cells grown in the membrane, to maintain their in vivo-like 3-D architecture. The scaffold was first washed twice with 2 ml 70% ethanol and once with RPMI containing 10% FCS. 1.5 x 10^6 cells were seeded in 150 µl medium directly onto the center of the alvetex membrane and incubated for 90 min at 37°C to allow cells to attach to the scaffold. After this time each well was gently floated with 10 ml medium containing 10% FCS and 1% P/S. Plates were incubated for 7 seven days to allow cells to invade into the membrane, medium was changed every second day. After the incubation time, inserts were fixed, to preserve cells in the 3D scaffold. Medium was discarded and membranes were washed twice with PBS. Well inserts were removed and scaffolds were transferred to a 50 ml tube containing 10 ml Histofix (Roti) and incubated for one night. Afterwards scaffolds were placed into a tube with 10 ml 70% ethanol and stored at 4°C. Membranes could be stored for several weeks at 4°C until further processing.

Processing, embedding and sectioning of scaffolds

First, scaffolds were dehydrated at 65°C for 25 min in 100% ethanol using a multifunctional microwave tissue processor (KOS). Treatment for 55 min in isopropanol was used as hydrophobic clearing agent to remove the ethanol. Finally the scaffolds were placed in molten paraffin wax. Then the membranes were cut into three representative pieces, placed in the embedding cassettes and embedded with molten paraffin. Blocks were stored at room temperature until sectioning. Sections were cut at a thickness of 4 µm using a HM 355s microtome (Microm International). Two floatation baths were used (~22°C, 45°C) to let the tissue sections float on the surface of the water to enable them to flatten out. Afterwards they were carefully picked up onto microscope slides and left to air dry overnight at room temperature.

Haematoxylin and eosin staining

HE-staining, one of the principal staining methods in histology, is the most widely used staining technique in this area. Haematoxylin builds positively charged metal hematein complexes that bind to the negatively charged basophilic cell components. Eosin, an acidic dye, is negatively charged and binds to acidophilic substances, mainly proteins. Cell nuclei are stained blue with haematoxylin, whereas the cytoplasm and extracellular matrix are coloured in various shades of violet.

To perform haematoxylin and eosin staining, tissue sections were placed at 65°C for 10 min to allow melting of the paraffin. Deparaffinization was performed using two times treatment with xylol for 1 min, followed by two times 100% ethanol, 70% ethanol and dH_2O, for rehydration. Sections were stained for 1 min in haematoxylin (Merck) and washed four times in dH_2O. Haematoxylin was filtered before usage. Sections were further incubated in Scott's blueing solution (Morphisto) for 45 seconds and rinsed for 5 min in running tap water. Subsequently they were washed in dH_2O before counterstaining for 1 min in eosin solution (table 27). Then sections were washed once in dH_2O before starting with dehydration. For that, 70% ethanol was used for 6 times and then two times 96% ethanol for 1 min and two times 100% ethanol for 1 min. Finally sections were treated with butyl acetate and mounted in Entellan (Merck).

Table 26: Eosin stock solution

10 g Eosin Y disodiumsalt (Sigma Aldrich)
200 ml dH_2O
800 ml 96% Ethanol

Table 27: Eosin working solution

50 ml Eosin stock solution
150 ml 80% Ethanol
1 ml Glacial acetic acid

Haematoxylin staining – Mayer's hemalum solution

Deparaffinization was performed as in the HE staining protocol. Sections were stained for 1 min in Mayershemalum solution (table 28) and then quickly washed in dH_2O. The solution was filtered before usage. Finally tissue sections were mounted with glycerin gelatin.

Table 28: Mayer's hemalum solution

Solution 1

1 g Haematoxylin
100 ml dH_2O
0.2 g Sodium iodate
50 g Potassium aluminium sulfate

Solution 2

50 g Chloral hydrate
1 g Crystalline citric acid

Solution 2 was dissolved in Solution 1 to get Mayer's hemalum solution.

DAPI mounting

Deparaffinization was performed as in the HE staining. After the last washing step with dH_2O for 1 min, sections were mounted with Vectashield Mounting Medium with the fluorescent stain DAPI (4′,6-Diamidin-2-phenylindol), which binds strongly to AT regions of the DNA (Vector laboratories).

2.19. Luciferase assay

Transfection reagent was prepared according to the manufactures instructions as follows: the transfection reagent PEI (Polyethylenimine) was mixed with serum-free medium and incubated for 5 min, whereas the plasmid DNA (one or several plasmids) was diluted in serum-free medium to a final concentration of 100 ng/well. Then both solutions were mixed, incubated for 20 min and then pipetted into the wells before adding the cell suspension. 5 x 10^4 Hek293 cells were seeded into a white 96-well, clear bottom plate (Greiner) following reverse transfection. Plates were incubated for 5 h at 37°C, afterwards medium was changed to standard growth medium and plates were incubated for 18 h at 37°C. After this incubation time, cells were treated with different amounts of activin A (0, 0.5, 1.5, 3, 5, 7.5, 10 or 20 ng/ml) or the supernatants of SCLC cells and incubated for another 3 hours. Then the GFP signal, serving for normalization, was measured using a Typhoon Trio FluoroImager. Plates were allowed to cool down to room temperature before adding 50 µl of ONE-Glo Luciferase Assay System detection reagent (Promega). Luminescence was measured after 10 min of incubation on a TECAN photometer.

2.20. Smad phosphorylation assay

For determination of smad phosphorylation, 5 x 10^5 SCLC cells per well were seeded into a 6 well plate and incubated over night. Next day the supernatants were collected. 5 x 10^5 HepG2 cells were seeded into a 6-well plate, left to recover over night. The next day medium from HepG2 cells was removed, washed twice with PBS, and replaced with 0.5 ml SCLC supernatant. After 30 min incubation time, cells were harvested in 80 µl lysis buffer II, lysates were sonicated and centrifuged. 20 µg protein were seperated by SDS-Page, blotted onto PVDF membranes and blocked in

TBSTM (5% skim milk in TBST). Membranes were incubated over night at 4°C in the primary antibodies. Secondary antibodies were incubated for 1 h at room temperature in 5% TBSTM. Chemiluminescence was developed with western detection reagent and signals recorded on an X-ray film.

2.21. GFP analysis

The percentage of GFP positive cells, after stable GFP overexpression via retroviral transduction, was determined using flow cytometry. 1 x 10^6 cells were suspended in 5 ml cold PBS, transferred into a 15 ml tube and pelleted for 5 min at 800 rpm. Supernatant was removed, cells were resuspended in 3 ml cold 70% ethanol and fixed at 4°C for 30 min. After that, cells were pelleted for 5 min at 800 rpm, supernatant was discarded, cells were resuspended in 3 ml PBS and pelleted again for 5 min at 800 rpm. Then supernatant was removed, cells were resuspended in 2 ml PBS, transferred to 5 ml FACS tubes and measured immediately by flow cytometry (Calibur-Becton Dickinson FACS).

2.22. Statistical analysis

Graphpad Prism 5.0 software (Graphpad Software Inc.) was used for statistical analysis and calculations. Statistical significance was tested via Student's t-test or one-way ANOVA. P-values below or equal 0.05 were considered statistically significant (labelled with an asterisk, as *), from 0.001 to 0.01 very significant (**), and those below 0.001 as highly significant (***).

3. RESULTS

3.1. Expression analysis of activin A, follistatin and activin receptors

3.1.1 Quantitative RT-PCR

A panel of 9 SCLC cell lines was obtained from international sources, including cell lines derived from metastatic disease. To elucidate the activin A and follistatin levels in SCLC cells, our panel of SCLC cell lines was screened by TaqMan qRT-PCR. Expression levels of activin A type I (ACVR1B) and type II (ACVR2A, ACVR2B) receptors were determined by SYBR Green qRT-PCR. Since our previous data had shown that SCLC patients with metastatic disease showed higher levels of circulating activin A, we wanted to know, if the cell lines derived from metastatic disease also expressed a larger amount of activin A. The expression analysis of activin A and follistatin, using the corresponding TaqMan probes, revealed that most of the cell lines did not express high levels of activin A or follistatin, however, the cell line HLHE derived from brain metastasis expressed the highest amount of activin A (figure 7). The cell lines GLC4, CRL-2066 and HTB-184 expressed the lowest amount of activin A. Due do its high level of activin A, the cell line HLHE was originally considered as cell model for activin A knockout using Crispr/Cas9. However, further analysis revealed that these cells also expressed a high level of follistatin. As follistatin acts as modulator of the activity of activin A by preventing it from binding to the type II receptors and subsequently blocking activin signaling, the cell line HLHE was not suitable for knockout experiments. Activin type I and type II receptors were expressed in the whole cell line panel.

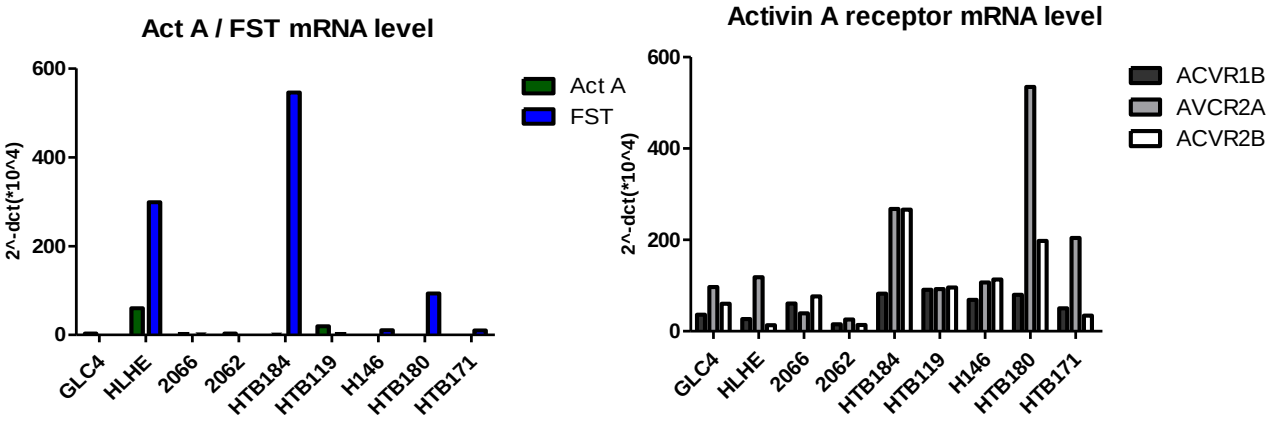

Figure 7: Relative expression of activin A, follistatin and the activin A receptors in selected SCLC cell lines, investigated by quantitative RT-PCR. Results are shown as $2^{-\Delta CT}$* 10000 values. As reference, the housekeeping gene GAPDH was used.

3.1.2. Activin A expression of three SCLC cell lines established from one patient during clinical disease progression

Activin A expression was also analyzed in three SCLC cell lines derived from one patient during clinical follow-up. Clinically, the tumor of this patient changed from sensitive (GLC14) to completely resistant to chemo- and radiotherapy (de Vries et al., 1989). The cell line GLC14 was established before therapy, GLC16 was taken 7 months after the first therapy and when the first responses from the patient to therapy were seen, and the third cell line GLC19 was derived from a biopsy after recurrence of disease (Berendsen et al., 1988). Contrary to our expectations, the expression of activin A has increased dramatically after first responses to therapy in these cell lines. The cell line GLC16 demonstrated a five fold increase of activin A, compared to the cell line GLC14 that was established before starting with chemotherapy. In the third cell line, in contrast, the expression of activin A was not detectable (figure 8).

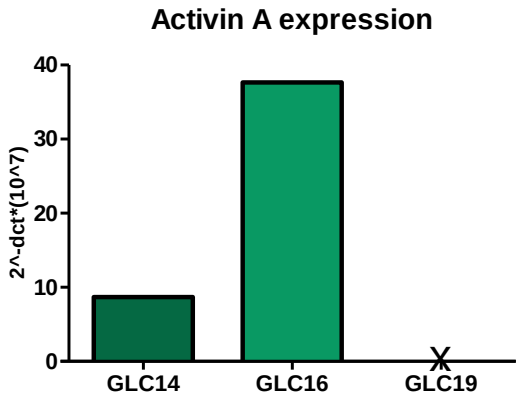

Figure 8: Activin A expression in three cell lines established from one patient during clinical follow-up.

3.2. Ectopic overexpression model

3.2.1. Transgene detection by qRT-PCR and flow cytometry

To be able to study long term effects of high activin A expression that cannot be mimicked well by treatment with recombinant activin A, an isogenic derivate with ectopic overexpression of activin A was generated via retroviral transduction. For that purpose, the semi-adherent cell line GLC4, which has only a low level of endogenous activin A, was stably transduced with GFP and activin A or only GFP serving as control. In order to check the transfection efficiency, qRT-PCR for activin A (figure 9) and flow cytometry for the GFP signal (figure 10) were performed. Results indicate nearly a 90-fold overexpression of the activin beta A subunit mRNA in the cell line GLC4 ActA compared to the control cells. The percentage of GFP positive cells was 89% in the GLC4 ActA cells and 92% in the control cells.

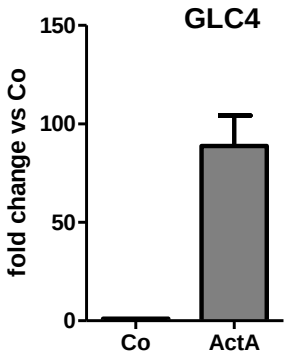

Figure 9: Expression of activin A, investigated by qRT-PCR, normalized to GLC4 control.

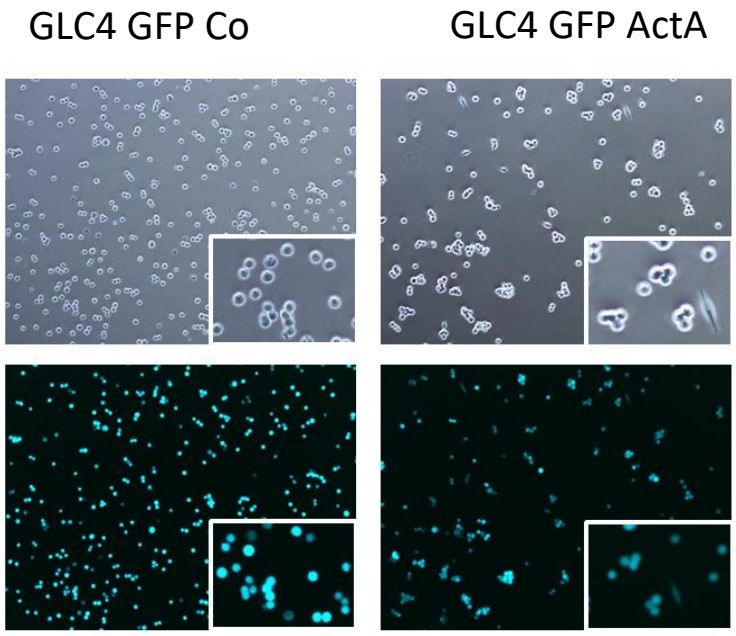

Figure 10: GFP signal after retroviral transduction of the semi-adherent cell line GLC4.

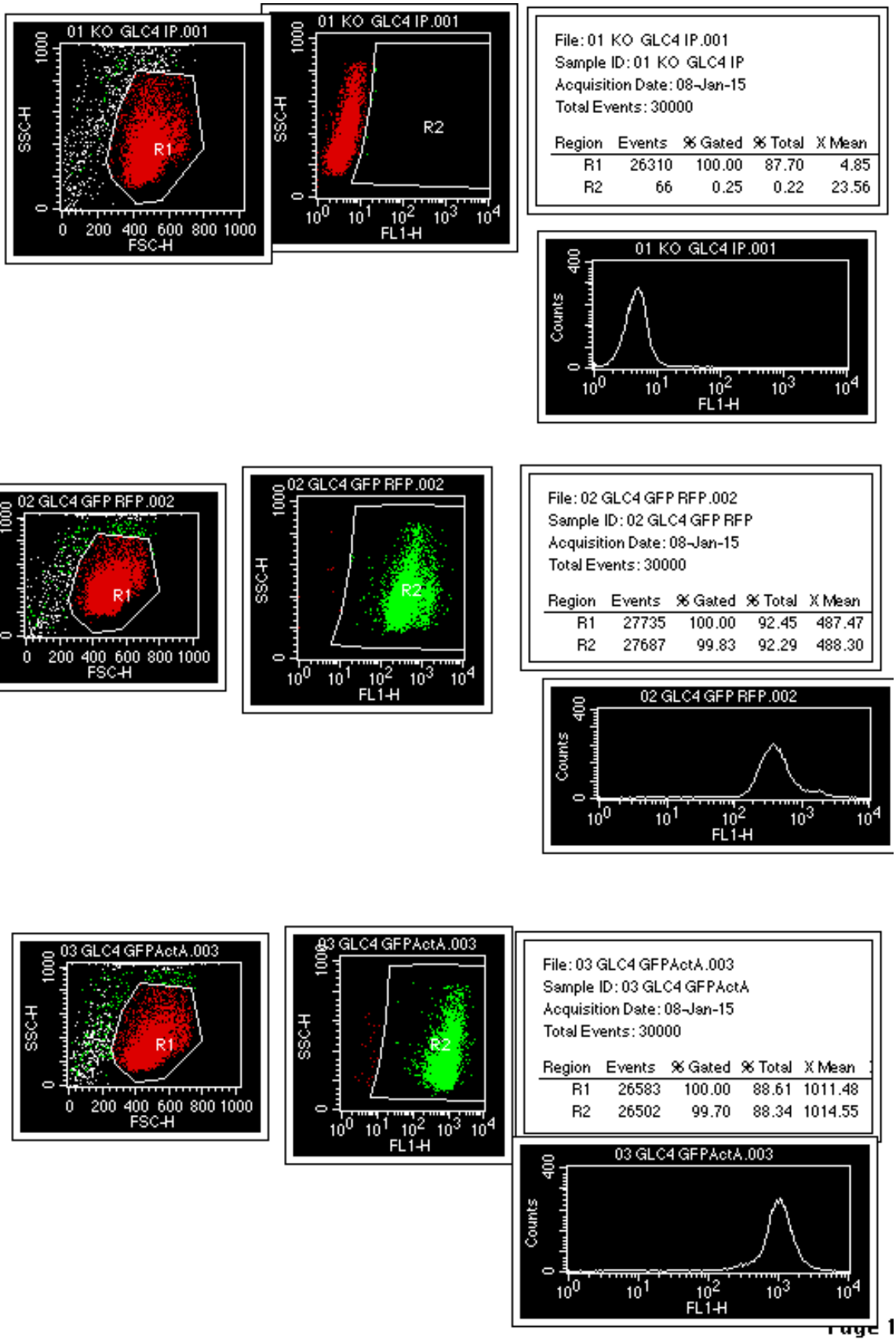

Figure 11: GFP expression in GLC4 after retroviral transduction, determined by flow cytometry. GLC4 IP served as control (empty vector).

3.2.2. Detection of bioactivity of secreted activin A

3.2.2.1 Smad phosphorylation assay 1

Since results from the retrovirally transduced cell line GLC4 showed a high expression level of the transgene INHBA by performing qRT-PCR, we wanted to verify that we got also an increase in secreted activin A. It was shown previously that treatment of HepG2 cells with exogenous activin A leads to phosphorylation of Smad2 (Razanajaona et al., 2007). Results revealed that incubation of HepG2 cells with the supernatant of GLC4 ActA cells for 30 minutes induced phosphorylation of Smad2 (figure 12). Incubation of cells for 30 minutes with 0.5 ng/ml activin A was used as control. In the GLC4 cells overexpressing activin A also an endogenous phosphorylation of Smad2 could be detected.

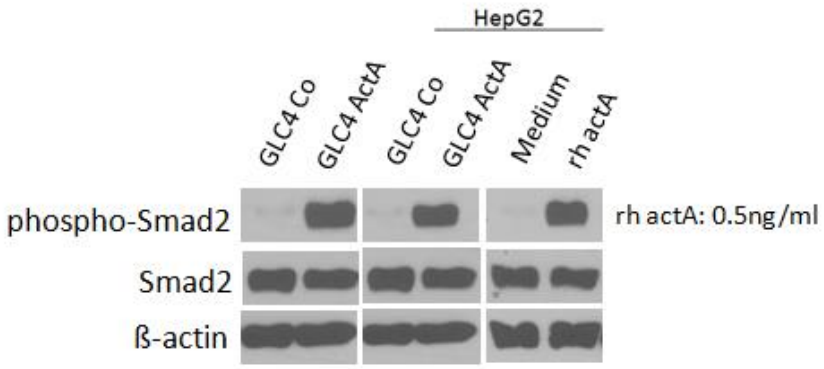

Figure 12: Phosphorylation of Smad2 in HepG2 cells treated with the supernatants of GLC4 ActA and GLC4 Co. HepG2 cells were treated with supernatants or medium with or without 0.5 ng/ml recombinant activin A. Cells were isolated and phosphorylation of Smad2 was measured by western blot analysis, using β-actin as loading control. Smad2 was phosphorylated upon treatment with the supernatant of GLC4 activin A overexpressing cells, showing that activin A produced by these cell line has biological activity.

3.2.2.2. Smad phosphorylation assay 2

Next, it was tested whether phosphorylation of Smad2 could be activated by treatment of HepG2 cells for 30 minutes with the supernatant of the other SCLC cell lines. In a second approach, endogenous levels of Smad2 phosphorylation in these

SCLC cells were tested. Treatment of HepG2 cells with medium served as negative control, whereas treatment with 0.5 ng/ml activin A was used as positive control.
Only in the overexpressing cell line, phosphorylation of Smad2 could be detected by western blot on an X-ray film when treating HepG2 cells with the supernatant of SCLC cells or looking at the endogenous levels of Smad2 phosphorylation in SCLC cells (figure 13).

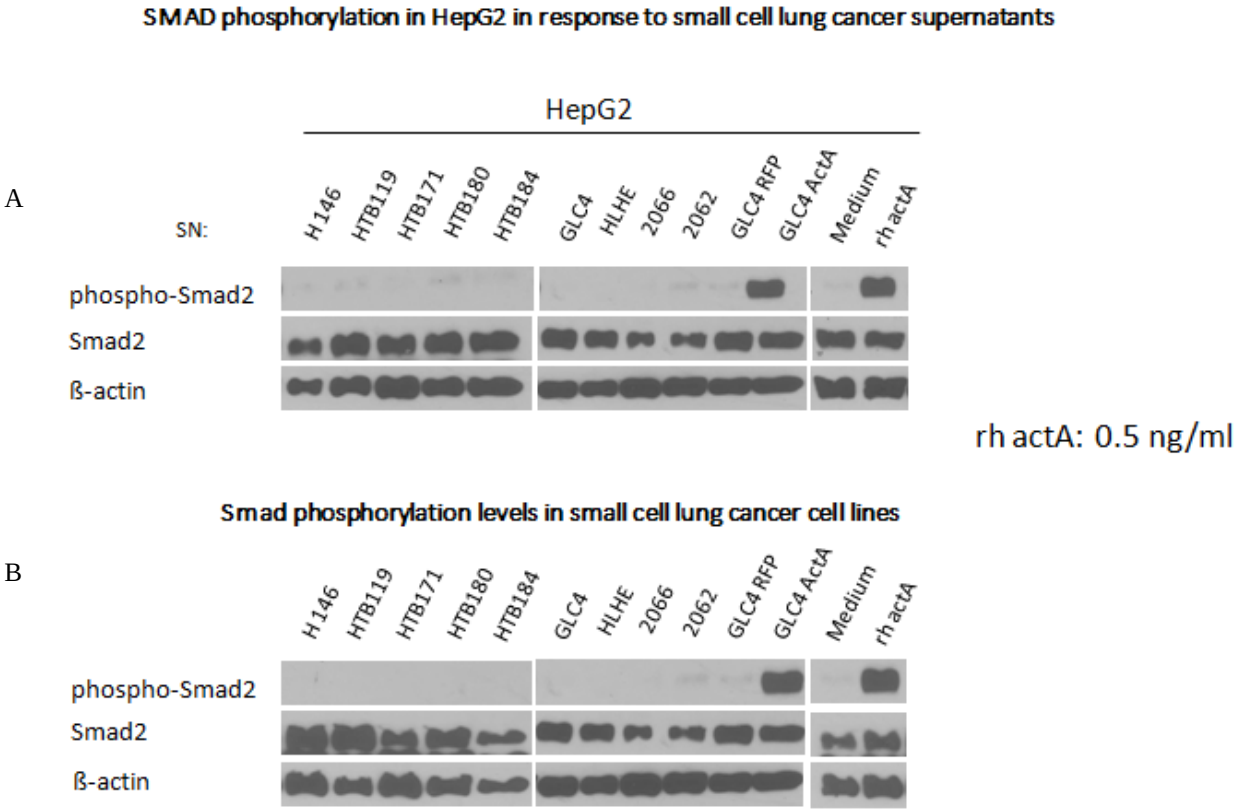

Figure 13: (A) Phosphorylation of Smad2 in HepG2 cells treated with SCLC supernatants.
(B) Endogenous levels of phosphorylation of Smad2 in SCLC cell lines.

3.2.2.3. Luciferase assay is not sensitive enough to detect different amounts of Smad signaling in SCLC cell lines

To verify Smad signaling in SCLC cells by performing a reporter gene asay, Hek293 cells were reverse transfected with a reporter gene connected to a promoter known to be activated by activin A signaling. The pGL4.48 vector used for transfecting Hek293 cells with a DNA plasmid, contains a Smad-binding element (SBE), which upon Smad2 binding leads to the transcription of the luciferase reporter gene luc2P.

The enzymatic activity of the reporter protein was measured on a photometer using ONE-Glo luciferase assay detection reagent. Results of the luciferase assay indicate that activin A treatment could only be detected from 5 ng/ml activin A upwards. The western blot seems to serve as the more sensitive assay for detecting the biological activity of SCLC cells, compared to the luciferase assay, since by performing an immunoblot already 0.5 ng/ml activin A were detectable.

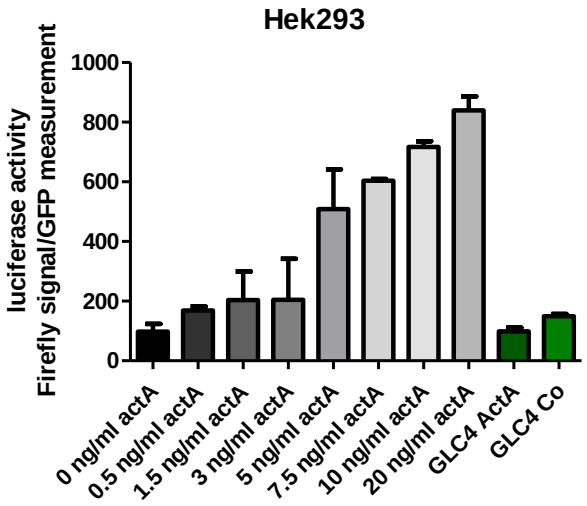

Figure 14: Luciferase assay to test for induction of Smad signaling by SCLC supernatants. Different concentrations of activin A were used to generate a standard curve for comparison with the results of SCLC cells with and without ectopic activin A expression.

3.2.2.4. Transient transfection of CRL-2066

For carrying out some assays that cannot be performed with the semi-adherent cell line GLC4, the adherent cell line CRL-2066 was transiently transfected (Lipofectamine 2000) with GFP and activin A.

GFP expression was checked under microscopic magnification (figure 15) whereas the activin A expression level was determined by TaqMan quantitative RT-PCR (figure 15). Results indicate that transfection of SCLC cells with GFP as well as activin A was achieved in the majority of cells.

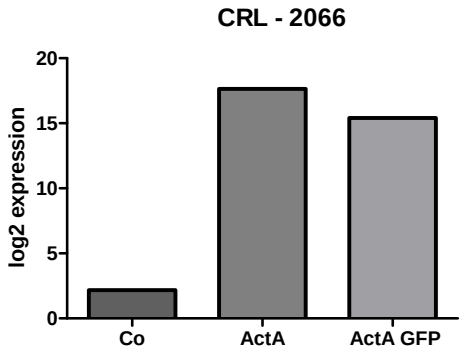

Figure 15: Expression of activin A in the transiently transfected cell line CRL-2066. The cells were either transfected with activin A, activin A and GFP or left non transfected serving as control.

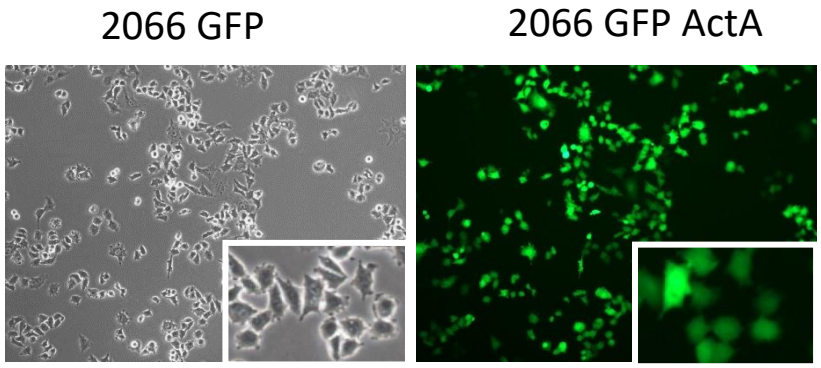

Figure 16: Microscopy of CRL-2066 cells expressing GFP.

3.3. Activin A has no effect on proliferation, cell cycle distribution and viability of SCLC cells

3.3.1. Proliferation and viability

In order to evaluate cell proliferation and viability of SCLC cells overexpressing activin A in comparison to the respective control group, growth and MTT assays were performed. The growth of SCLC cells was assessed by GFP signal measurement every day in the course of one week. To check if activin A has an effect on the viability of cells, MTT assays were performed either with the stably overexpressing cell line or with SCLC cells treated with 10 or 20 ng/ml recombinant activin A. To determine if there is a change in the behaviour of semi-adherent SCLC cells, ultra-low attachment plates featuring a covalently bound hydro gel layer that efficiently inhibits cellular attachment, were used. Results revealed no significant difference in growth (figure 17) and viability (figure 18) between the transgenic cell line and the control group. Similar results were obtained with the treatment of activin A (figure 18). The usage of low-attachment plates had no significant effect on the behaviour of semi-adherent cells (figure 18).

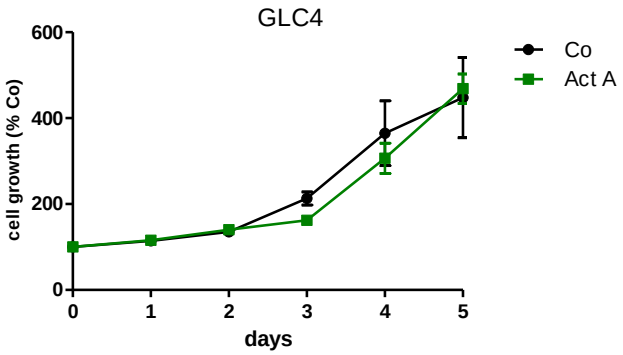

Figure 17: Growth curve of SCLC cells overexpressing activin A compared to control cells, assessed by GFP signal measurement.

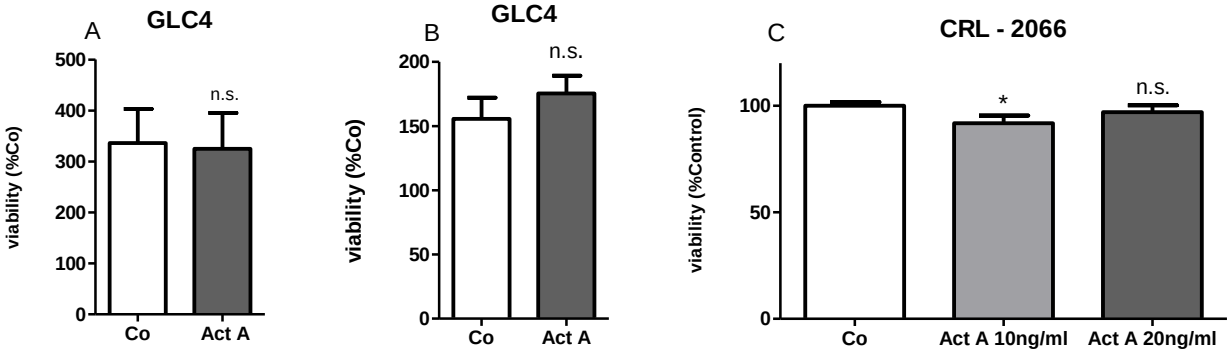

Figure 18: (A) Effect of activin A overexpression on the viability of SCLC cells was assessed by performing an MTT assay 72 hours after seeding the cells. (B) Viability of semi-adherent cells with and without activin A overexpression in a low-adherence plate 72 hours post seeding. (C) Effects of activin A on cell viability 72 hours after treatment with recombinant activin A.

The influence of activin A on the capacity of cells to form colonies, when seeded at a low density, was examined by a clonogenicity assay. For analysis, clones were fixed and afterwards stained with crystal violet. Pictures were taken and absorbance was measured at 560 nm after destaining the clones (figure 19). As this assay could not be done with the semi-adherent cell line GLC4, the cell line CRL-2066 was used. Cells were either treated with 10 ng/ml or 20 ng/ml activin A.

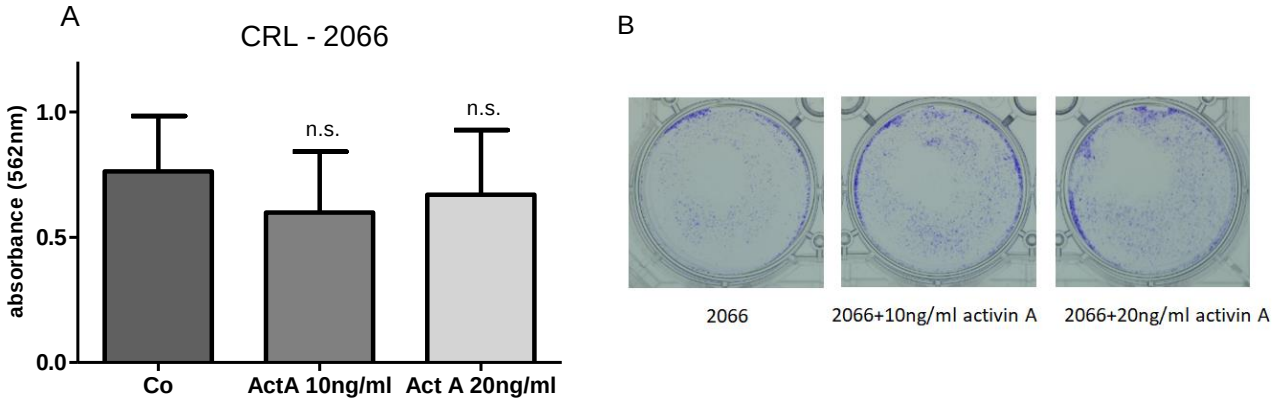

Figure 19: (A) Evaluation of clonogenic assays 11 days after treatment. (B) Representative pictures of stained cells.

Clonogenic assays revealed similar results as other proliferation assays. Treatment of SCLC cells with two different concentrations of activin A showed no significant difference in colony forming capability compared to the control cells (figure 19).

3.3.2. SCLC cells were not able to form spheroids in stem cell medium

Neither GLC4 with an increased activin A level nor the control group formed spheroids when cultured in low-attachment plates for 11 days (figure 20).

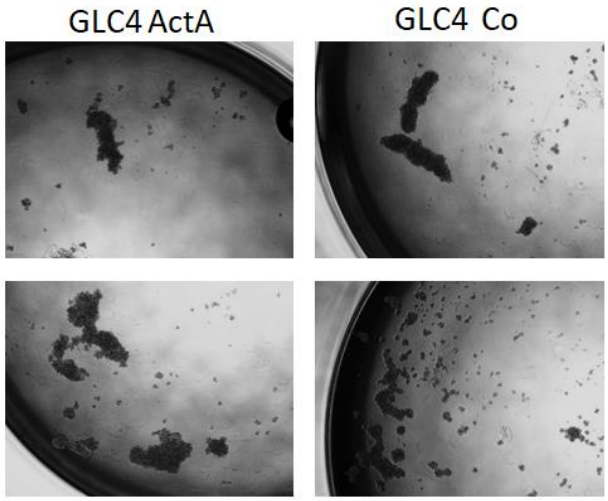

Figure 20: Pictures of cell aggregates formed in stem cell medium.

3.3.3. Cell cycle analysis

To examine whether increased activin A levels induce alterations in the distribution of the three cell cycle stages, cells were stained with propidium-iodide and analysed by flow cytometry. Results showed no obvious increase or decrease in any cell cycle stage compared to the respective control cells (figure 21).

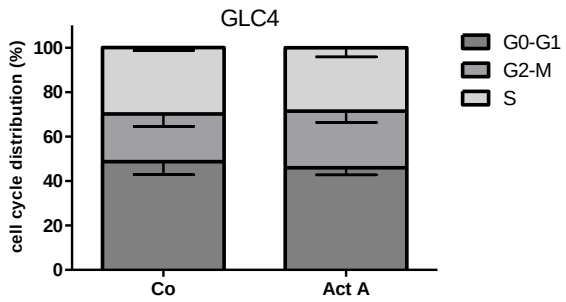

Figure 21: Cell cycle analysis. Percentage of cells in each of the three stages of the cell cycle.

3.4. Activin A has no influence on the adhesion ability of SCLC cells

To examine possible changes in adhesion of GLC4 cells stably overexpressing activin A, an adhesion assay was performed on collagen, fibronectin, laminin or poly-L-ornithin. Non-coated wells served as controls to determine the initial attachment of the cells as well as the adhesive strength after coating the plates.
Medium was changed after 24, 48 or 72 hours. For each time point, the same amount of cells was seeded into separate wells. Pictures of representative parts of the wells were taken before and after changing the medium. In summary, the adhesion assays with different coating substances, indicated that there was a huge day to day variation of adherence with no apparent link to activin A expression or type of substrate.

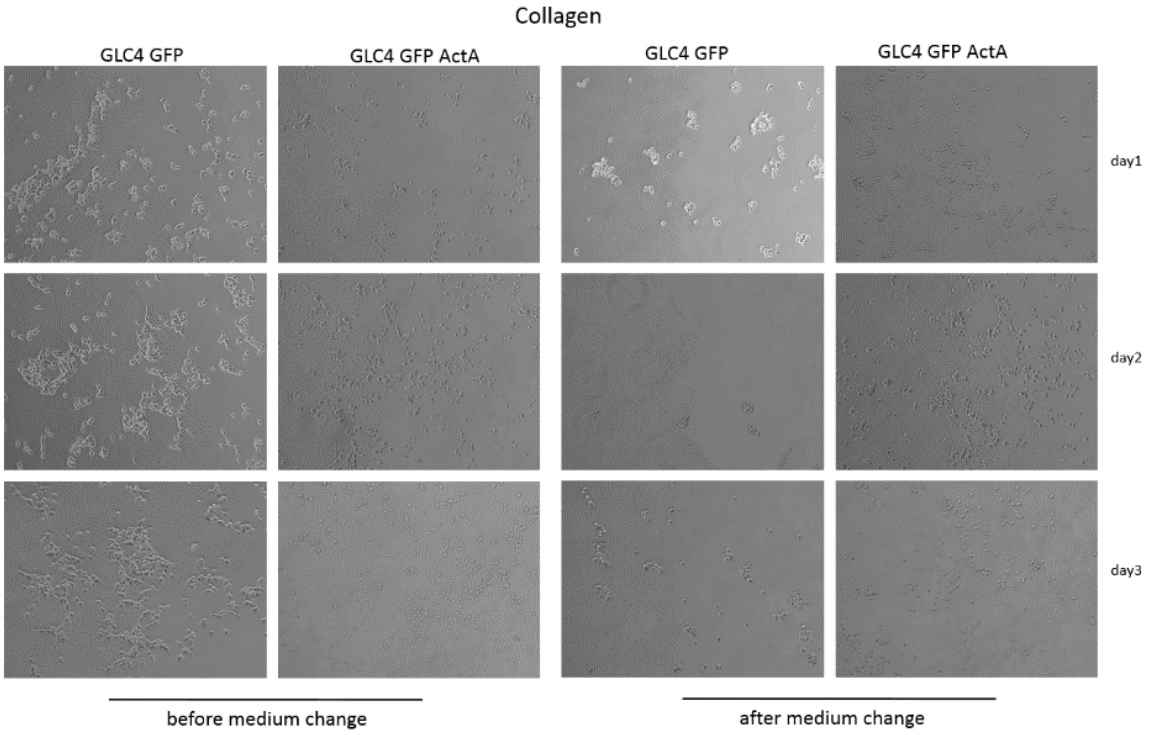

Figure 22: Adhesion assay performed in culture plates coated with collagen - that acts as fibrillar protein and gives structural help to resident cells.

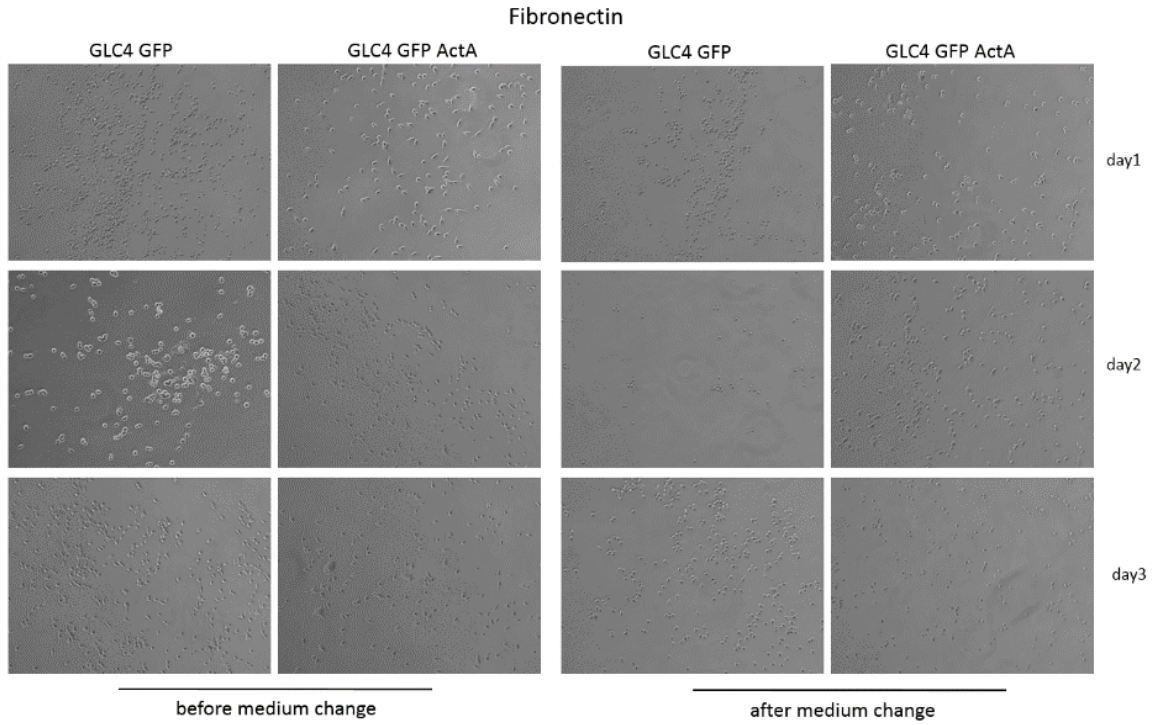

Figure 23: Adhesion assay performed in culture plates coated with fibronectin, a glycoprotein of the extracellular matrix.

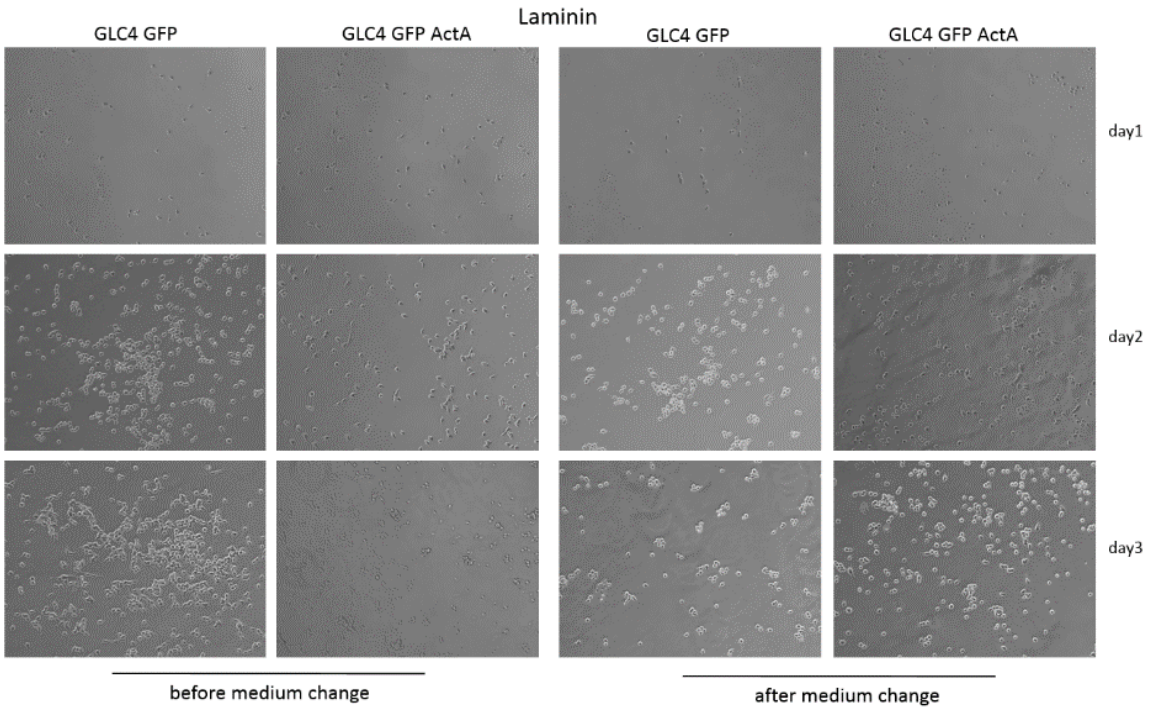

Figure 24: Adhesion assay performed in culture plates coated with laminin, a protein in the basal lamina.

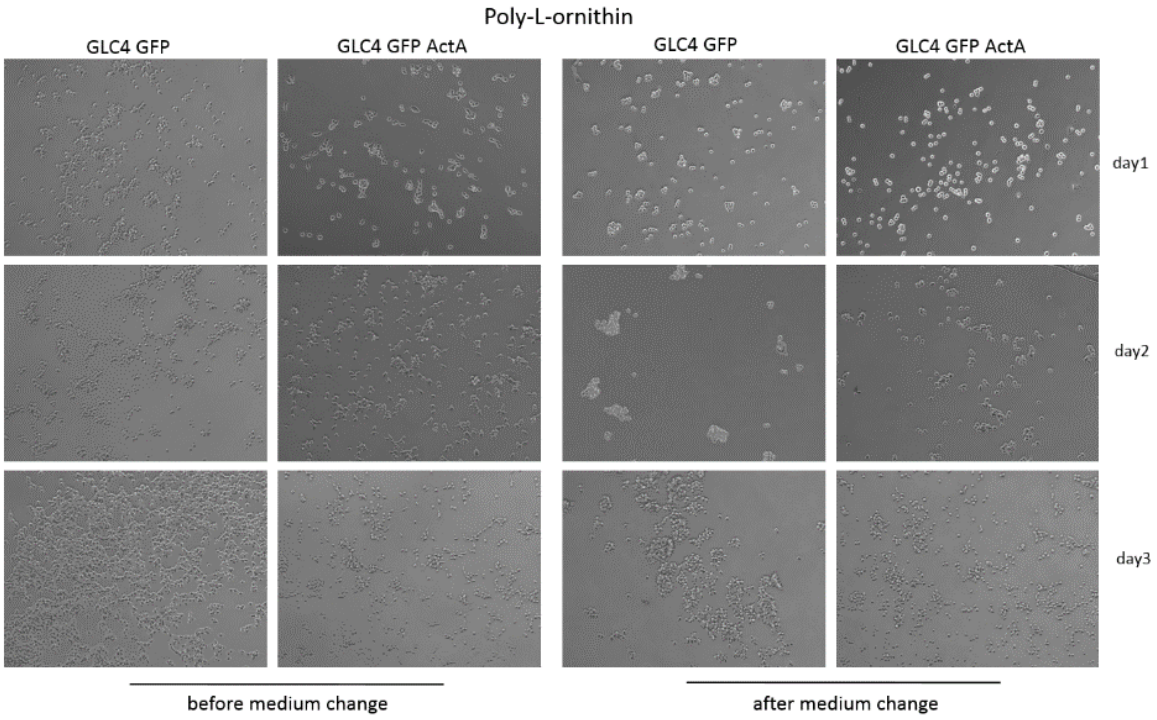

Figure 25: Adhesion assay performed in culture plates coated with poly-L-ornithin before seeding the cells.

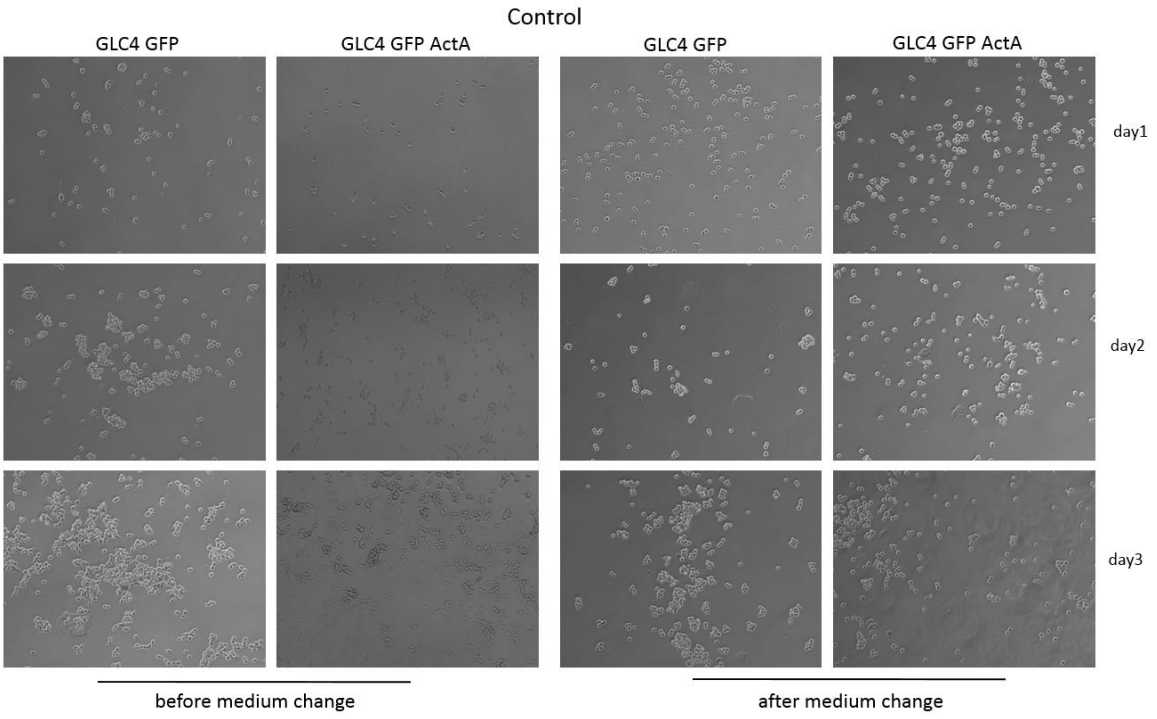

Figure 26: Non-coated 24-well plate, serving as control.

3.5. Gene expression

Selected genes, involved in cancer progression, were examined via quantitative RT-PCR. Compared to the control group, Smad7 was found to be slightly upregulated, which could be explained by the known feedback regulation of activin A via increasing the expression of the activin A signaling antagonist Smad7. MMP1, a metalloproteinase involved in extracellular matrix breakdown and metastasis was upregulated, compared to control cells. Whereas E-cadherin (CDH1), a typical epithelial protein, which is often downregulated in cancer, resulting in less cell to cell adhesion and in an increase in cellular motility, was found to be 2-fold downregulated in our study compared to control cells. No significant change in the expression of PDL1 (CD274), ESM1, N-cadherin (CDH2), BCL2 or ETV4 could be observed (figure 27).

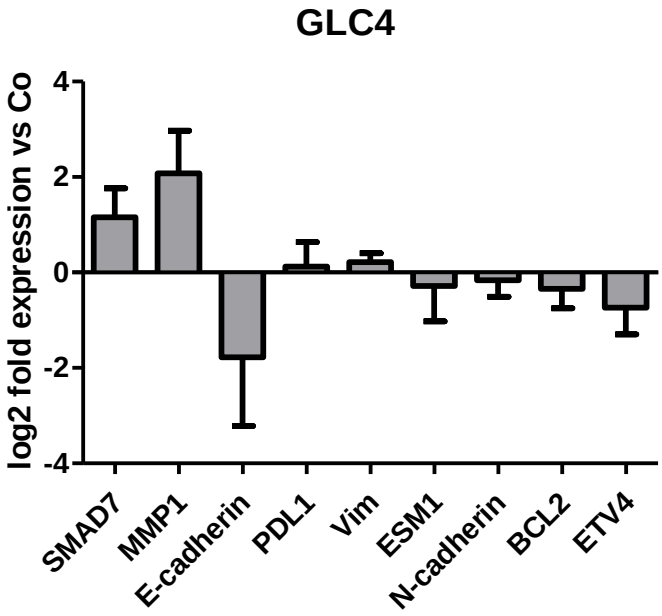

Figure 27: Gene expression in GLC4 ActA compared to respective control cells.

3.6. Activin A treatment led to decreased invasion ability of CRL-2066 cells

Invasion through a collagen-coated membrane (8 µm pores) was tested by transwell chamber invasion assays. The number of cells which were able to pass through the collagen layer and the porous filter was analysed by staining the cells with crystal violet and the subsequent photometric measurement after destaining the cells with SDS. Because of the semi-adherent character of the overexpressing cell line GLC4 this assay could only be done with the adherent cell lines CRL-2062 and CRL-2066.
Treatment with activin A resulted in reduced invasion ability of CRL-2066 cells (figure 28 A), whereas in the CRL-2062 cell line no significant difference was found (figure 28 B).

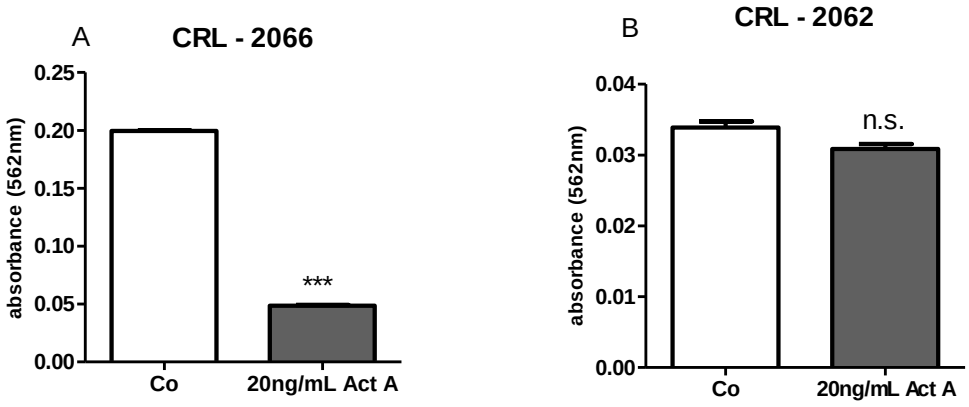

Figure 28: Analysis of the transwell invasion assay. Cells were treated with activin A immediately after seeding and allowed to invade for 72 hours.

3.7. Influence of activin A on the growth of endothelial cells

Since activin A is a secreted signaling protein, we wanted to check whether activin A has an effect on the growth of blood and lymphatic endothelial cells. Therefore, endothelial cells were seeded and, on the next day (i) treated with recombinant activin A, (ii) treated with the supernatant of SCLC cells or (iii) co-cultured with SCLC cells.

The analysis of the data showed that activin A had an inhibitory effect on the growth of endothelial cells, however, the supernatants of the tumor cells had such a strong effect also without ectopic activin A expression, that an additional influence of ectopic activin A expression could not be observed (figure 29).

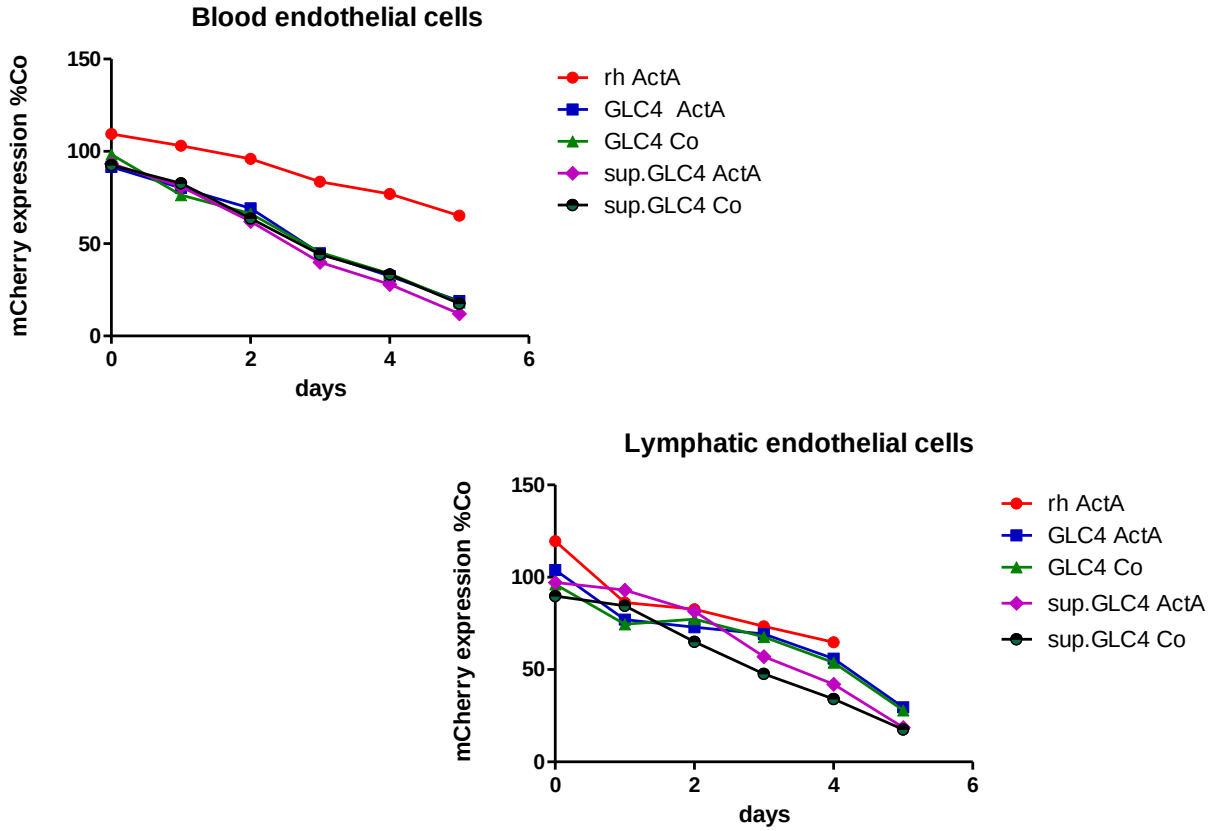

Figure 29: Incubation of blood or lymphatic endothelial cells with (i) recombinant activin A (10 ng/ml), (ii) the supernatant of GLC4 ActA and control or (iii) GLC4 ActA and GLC4 Co cells led to reduced growth of endothelial cells.

3.8. Treatment with activin A led to decreased transendothelial migration of SCLC cells through blood endothelial cell layers

To test the ability of cells to transmigrate through a layer of endothelial cells growing on a collagen matrix and subsequently pass through a membrane containing pores of 8 µm in diameter, transwell chamber inserts were used. Tumor cells were transiently transfected with GFP 24 hours before seeding, immediately treated with activin A after seeding and incubated for 72 hours. Membranes fixed on a microscopic slide

were scanned using a Midi Slide Scanner (brightfield mode, auto settings, 40x, 3D Histech, Budapest, Hungary).

Treatment with activin A led to reduced transendothelial migration of SCLC cells through blood endothelial cells. Through lymphatic endothelial cells, however, a significant increase in migration could be found in the cell line CRL-2066 (figure 30).

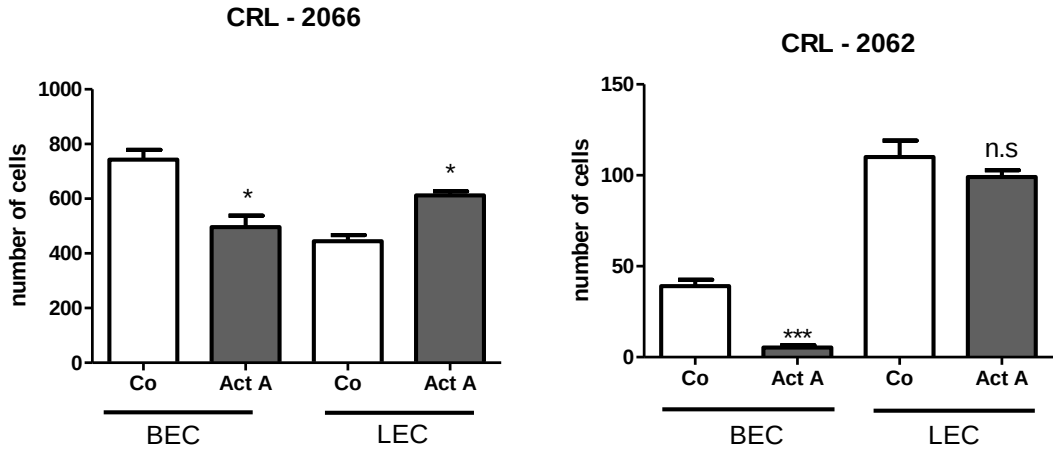

Figure 30: Number of GFP positive, invaded cells per well, on the lower surface of the membrane.

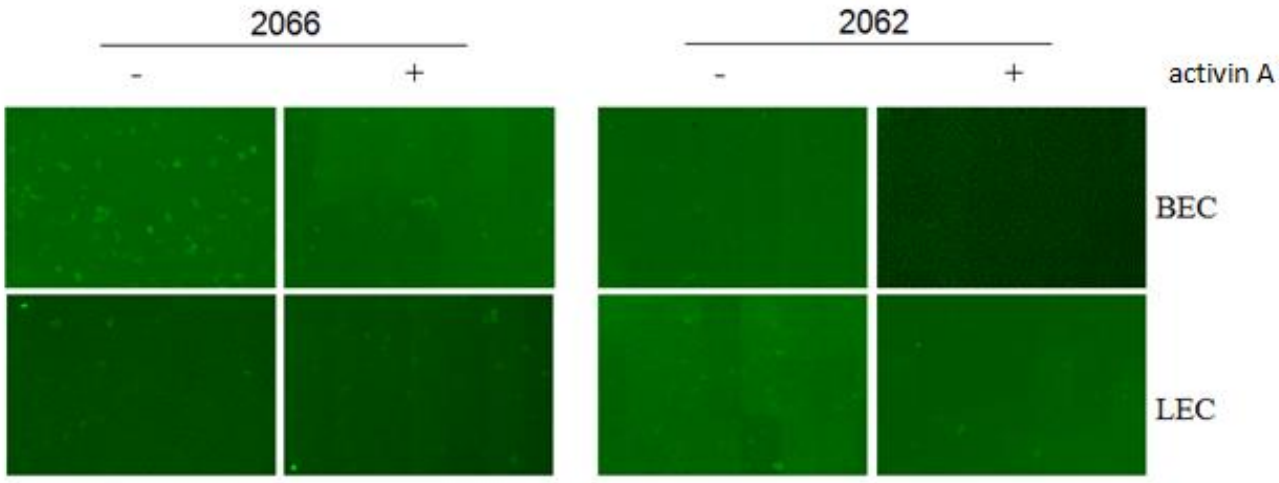

Figure 31: Representative pictures of the scanned transwell chamber membranes representing SCLC cells transmigrated through collagen and the filter containing pores of 8 µm in diameter.

3.9. SCLC cell spheroids with an increased level of activin A generated smaller CCIDs in lymphatic endothelial cell monolayers

To study the formation of circular chemorepellent-induced defects (CCID) an in-vitro co-culture system for investigating mechanisms of blood or lymphatic bulk invasion was used. Previous studies had shown that CCID formation correlates with metastatic spread in breast cancer (Kerjaschki et al., 2011). Spheroid formation of SCLC cells was performed by incubating the cells for 48 hours with medium containing methylcellulose. After this time, spheroids were placed on top of lymphatic- or blood endothelial cell monolayers and co-cultured for 4 hours to allow CCID formation. Representative pictures were taken with a 10 x objective using the FITC channel for tumor spheroids and the TRITC filter for CCIDs (figure 33). Gap area and spheroid size were determined with Image J and afterwards ratios of gap area to spheroid area were calculated. There was no significant difference in gap forming activity in blood endothelial cell monolayers (figure 32). In lymphatic endothelial cells, however, SCLC spheroids with an increased level of activin A generated significantly smaller circular defects (figure 32).

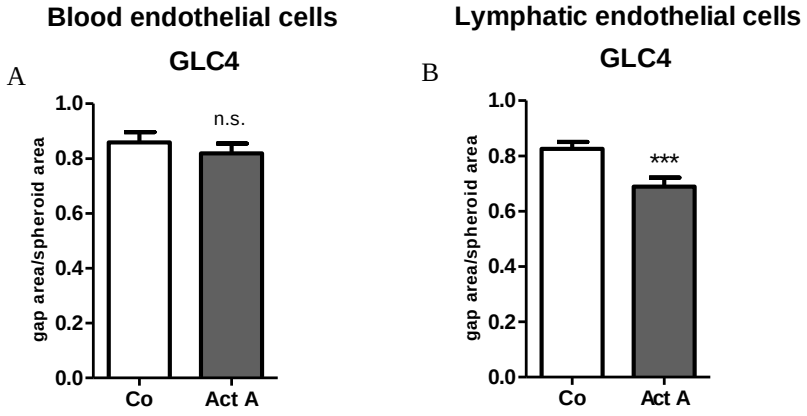

Figure 32: Quantitative analysis of gap formation on blood endothelial and lymphatic endothelial cell monolayers. CCID ratios were calculated by determining gap area and spheroid area.

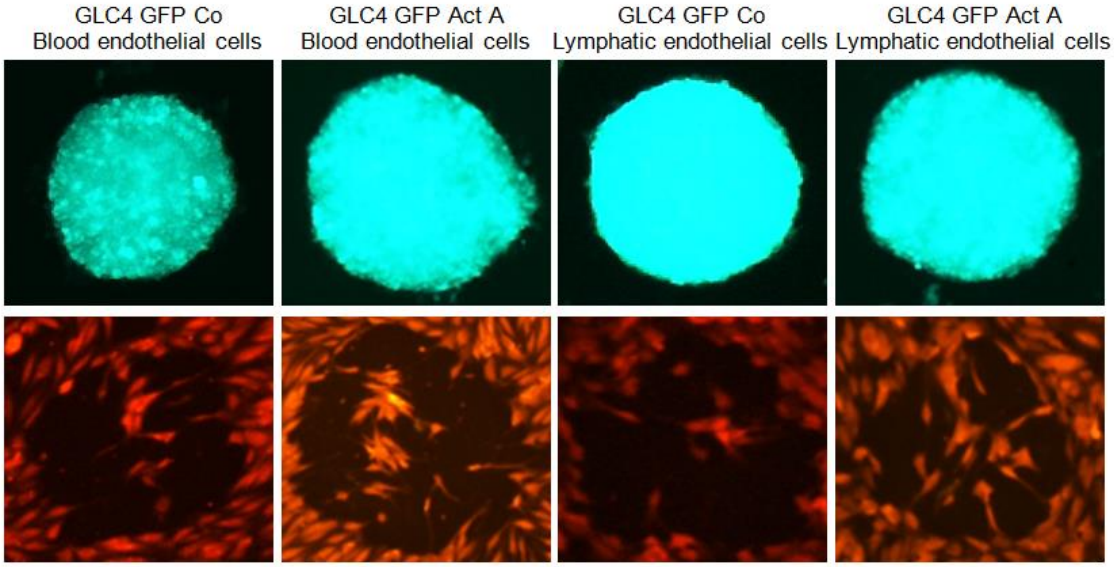

Figure 33: Spheroid and gap formation. The tumor cells are stably overexpressing GFP whereas the lymphatic and blood endothelial cells are stably transduced with mCherry.

3.10. In SCLC cells an increased level of activin A enhances colony forming ability in soft agar

By performing a soft agar assay, the ability of single cells to form colonies, independent of anchorage to the substratum, was tested. Cells were seeded into a soft agar matrix and incubated for three weeks to allow colony formation. Clones were then counted under microscopic magnification. Cells overexpressing activin A demonstrated a greater capacity to form colonies (figure 33 and 34), whereas in cells treated with recombinant activin A no significant difference could be detected (figure 34 and 35).

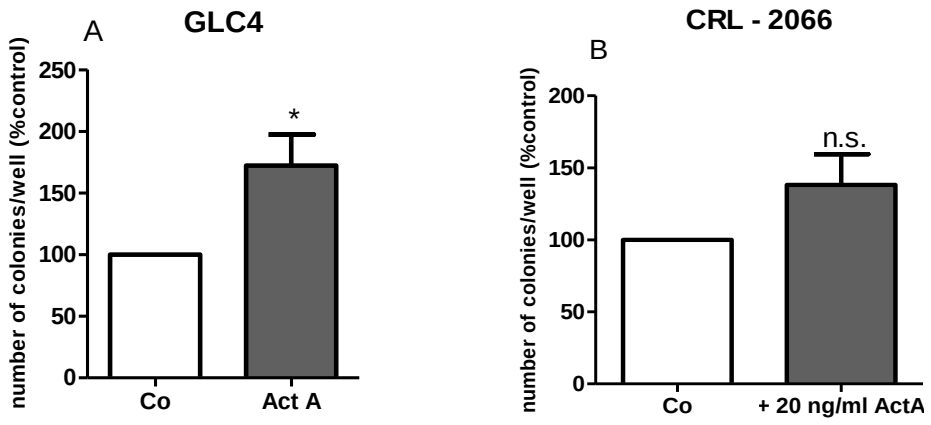

Figure 34: Number of colonies per well normalized to control cells. (A) Cells overexpressing activin A. (B) Cells treated with 20 ng/ml activin A.

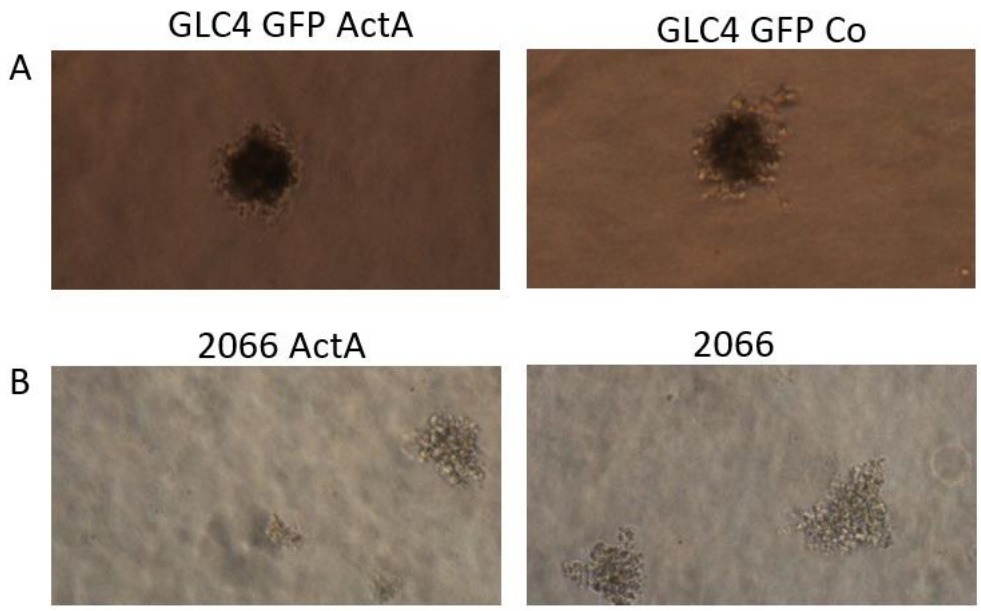

Figure 35: Representative pictures of colonies formed by performing an anchorage independent growth assay. (A) Cells overexpressing activin A. (B) Cells treated with 20 ng/ml activin A.

3.11. Growth in 3-D scaffolds could not be evaluated due to non-adherence of the scaffolds

CRL-2066 and CRL-2062 SCLC cells were cultured for seven days in a 3-D scaffold and then fixed with Histofix (Roti). Representative pieces of the membrane, embedded in paraffin were prepared for histological examinations. After HE-staining, the 3D-polystyrene scaffold was only visible in some parts of the membrane. It seems as if the scaffold was generally only able to be seen in parts where cells were attached to the membrane. Since we do not know how many parts of the membrane containing cells got detached from the slide, results could not be used for further investigations.

When Mayer's hemalum was used for staining the cells, the whole membrane got detached from the microscopic slide, during the staining process. Scaffolds were mounted with Vectashield Mounting Medium with DAPI, after deparaffining. Results showed that scaffolds got detached during the deparaffining process.

4. DISCUSSION

Lung cancer is by far the leading cause of cancer death among men and also increasing in women. About 14% of all diagnosed cancers per year are lung cancers (Torre et al., 2015). Small cell lung cancer, located in the bronchi near the center of the chest, is characterized by its early spread, poor prognosis and high therapeutic resistance. Thus the identification of non-invasive biomarkers and more efficient treatment strategies are urgently needed. In recent publications it has been shown that activin A, a cytokine of the TGF-β family that plays an important role in cell proliferation, differentiation or wound healing, could have both oncogenic and tumor suppressor roles in cancer development (Loomans et al., 2014). Tumor suppressive effects have been shown in breast, hepatocellular carcinoma or prostate cancer (Burdette et al., 2005; Deli et al., 2008; Dowling et al., 2000). While in lung, head or endometrial carcinoma it has been demonstrated that high activin A expression correlates with increased proliferation, invasion and poor patient outcome (Ferreira et al., 2008; Hoda et al., 2016; Seder et al., 2009). In contrast to other thoracic malignancies, such as lung adenocarcinoma or malignant pleural mesothelioma, nothing was previously known about the function and influence of activin A in SCLC. The only published data about members of the TGF-β family in SCLC shows, that forced overexpression of the activin A antagonist follistatin causes suppression of metastatic potential, when SCLC cells were injected into the tail veins of natural killer cell-depleted SCID mice (Ogino et al., 2008). Since the role of activin A signals in SCLC has not been characterized so far, we first analyzed the mRNA expression levels of activin A, the activin type I and type II receptors and the activin antagonizing factor follistatin (FST). With this study, we could show that activin A is moderately expressed in a panel of SCLC cell lines. Activin A receptors type I (ACVR1B) and type II (ACVR2A and ACVR2B) were determined with RT-qPCR and are expressed in all SCLC cell lines investigated. Follistatin, a monomeric glycoprotein and antagonist of activin signaling, that binds with high affinity to activin receptors, thus preventing the receptor-ligand interaction was highly expressed in three of the nine cell lines and hardly expressed in the other cell lines. Other antagonists of activin A signaling like FLRG have not been analyzed in this study.

Results of the current study reveal that forced overexpression of activin A has no effect on cell proliferation in SCLC cells. In blood endothelial cell monolayers no

significant difference in gap forming ability was found between SCLC cell spheroids overexpressing activin A compared to the control group. In lymphatic endothelial cell layers, however, SCLC cell spheroids with an ectopic expression of activin A formed smaller circular defects compared to the respective control group. Treatment with recombinant activin A led to decreased transendothelial migration capacity in the SCLC cell line 2066.

It has been shown previously that activin A has no effect on cell proliferation in melanoma cells, whereas activin A induced a significant decrease in sprouting in lymphatic endothelial cells (Heinz et al., 2012). The cumulative sprout length as well as the number of sprouts of lymphendothelial cell spheroids embedded in a collagen-methocel mixture was significantly decreased when treated with the supernatants of activin A overexpressing cells. Activin A was also shown to decrease the number of lymphatic vessels in an in vivo mouse xenotransplant model (Heinz et al., 2012).

In our experiments, co-cultivation of SCLC cells overexpressing activin A or incubation with the supernatant of the cell line, containing secreted activin A, decreases the growth of blood or lymphatic endothelial cells. Previous studies examined that treatment of endothelial cells with activin A resulted in a loss of tube formation and decreased proliferation via induction of the CDK-inhibitor 1 and inhibition of cyclin D1 (Kelner N. et al., 2015).

Incubation of HepG2 cells with the supernatant of SCLC cells for 30 min resulted in an activation of the Smad-pathway via phosphorylation of Smad2 only in the ectopic overexpression model. Previous publications show that an increase in p-Smad2 could correlate with an increase of invasiveness (Yamagata et al., 2005). In colorectal cancer it was found that JNK dependent phosphorylation of Smad2 could contribute to the development of distant metastasis (Yamagata et al., 2005). TGF-β signaling activates MMP3 and collagen type XI alpha via phosphorylation of Smad2, thus promoting invasion in ovarian cancer (Chang et al., 2013).

Selected genes, involved in cancer progression, were examined via quantitative RT-PCR. The inhibitory Smad7 was found to be 2-fold upregulated compared to the control group, which could be explained by feedback regulation of activin A, via increasing the expression of Smad7 (Massagué et al., 2005). E-cadherin (CDH1), a cell-cell adhesion glycoprotein and one of the typical EMT-markers, was found to be 2-fold downregulated in the activin A overexpression cell model compared to the respective control group. Recent studies have shown that TGF-β induces the

characteristic features of EMT in mammary and lung cells along with down regulation of E-cadherin, while activin A does not affect EMT in keratinocytes or mammary cells, but is responsible for the scattering and spindle-like morphology of lung cells (Thiery et al., 2009). When cells at the barrier to other organs contain decreased E-cadherin expression, single cell migration takes place. Matrix metalloproteinase-1 (MMP1) was found to be upregulated in SCLC cells overexpressing activin A, compared to its control group. The expression of matrix-metalloproteinases was shown to be upregulated by activin A expression in a variety of tumorigenic cell lines, indicating increased metastatic potential (Krstic et al., 2014; Yoshinaga et al., 2008). Matrix metalloproteinase 7, able to degrade components of the extracellular matrix and also to activate additional MMPs responsible for an elevated cell invasion, was examined to be upregulated in the presence of activin A (Ogawa et al., 2008; Yoshinaga et al., 2008). In our study, MMP7 was not screened by qRT-PCR in the SCLC cell line models.

It has been suggested that cells in the tumor microenvironment, such as fibroblasts, increase their production of activin A to try inhibiting the growth and extension of the tumor – the tumor cells, in contrast, try to adapt and evade these signals (Barron et al., 2012; Loomans et al., 2014). In normal tissue samples, fibroblasts express activin A at inconsiderable levels (Abe et al., 2001). It has been shown that overexpression of activin A by esophageal fibroblasts inhibits growth in an autocrine and parcrine way, though it can turn into a tumor promoter in the occurrence of malignant cells (Taylor et al., 2015). Besides this, overexpression of activin A derived from fibroblasts, decreases vascular endothelial growth factor (VEGF) expression, one of the main factors of tumor angiogenesis (Taylor et al., 2015).

Activin A, as well as TGF-β, plays an important role in wound healing. Injured vessels start to clot due to the inclusion of extracellular matrix proteins, such as collagen and fibronectin. During the process of recuperation, all types of cells require the ability to move and replace tissue. The levels of activin A are increased in wounded tissues, likely due to the early inflammatory response (Seder et al., 2009; Yndestad et al., 2004). Activin A is likely to support all features of wound granulation tissue, such as initiating wound angiogenesis and attracting fibroblasts and macrophages to the injury site (Duggal et al., 2013; Yoshinaga et al., 2008). Dvorak H. has recently described cancer as “the wound that does not heal” (Dvorak, 2015). This statement

could reflect that activin A is constantly involved during cancer progression, due to its role in wound healing.

Various studies have revealed, that activin A increases in tissues and in the serum when the patient suffers from acute or chronic inflammatory diseases, including asthma, chronic CNS imflammation, gastric ulcers, heart failure, hepatitis, meningitis, rheumatoid arthritis or traumatic brain injury (Cho et al., 2003; Ebert et al., 2007; Hübner et al., 1996; Gribi et al., 2001; Hardy et al., 2010; Jones et al., 2004; Karagiannidi et al., 2006). Furthermore, activin A is produced by many distinct cell types during the regulation of the immunesystem, inflammation and fibrosis, including endothelial cells, neuronal cells, myeloid cells, such as monocytes, macrophages and dendritic cells, hepatocytes, various epithelial cells, some T-cell subsets (such as T-helper 1, T_h1), keratinocytes, smooth muscle cells and bone marrow stromal cells (Cho et al., 2003; G. Hübner, 1996; Gribi et al., 2001; Ogawa, Funaba et al., 2008; Robson et al., 2017; Scutera et al., 2008; Tannetta et al., 2003). Moreover, activin A plays an important role in the activation and maturation of the adaptive and innate immune systems. In the innate immune response, activin A plays a key role in the defence against infections (Hübner et al., 1996; Jones et al., 2004). Whereas in the humoral immune response activin A was found to be secreted by and also to play an important role in the function of adaptive immune cells (Cho et al., 2003; Karagiannidis et al., 2006). Activin A could further be secreted by T_h2- helper cells, which also secrete high amounts of IL-4 (interleukin-4) and IL-13 (interleukin-13), which support the activation pathway of macrophages that is often associated with cancer and wound healing (Huber et al., 2009; Ogawa et al., 2006).

5. CONCLUSION AND OUTLOOK

Due to the increasing incidence of lung cancer and the poor clinical outcome, SCLC research has become more clinically relevant in recent years (Tanoue et al., 2015). Thus, further work on the identification of non-invasive biomarkers and more efficient treatment options are urgently needed.

In our study we showed that activin A is moderately expressed in SCLC cells. We also demonstrated that overexpression of activin A has no direct influence on the rapid growth of SCLC cells, nor on the invasion ability of the cells. In anchorage independent growth, however, SCLC cells with high activin A expression, demonstrated a greater capacity to form colonies.

Our data suggest only a moderate direct impact of activin A expression on SCLC growth and aggressiveness in vitro and, moreover, show that SCLC cells in in vitro culture secrete only limited amounts of activin A. This highlights the necessity to further examine the source of elevated activin A levels in patients with SCLC and the link to metastatic disease. In addition to cancer cells, also other cell types in the tumor microenvironment, such as fibroblasts and neutrophils could contribute to the activin A production of the primary tumors. In vivo experiments in mouse models are planned to examine the role of activin A in SCLC. Staining of tissue samples with activin A specific antibodies could help to define the source of activin A.

6. APPENDIX

6.1. List of abbreviations

ACVR1B	Activin receptor 1b
ACVR2A	Activin receptor 2a
ACVR2B	Activin receptor 2b
APES	Ammonium persulfate
BCL2	B-cell lymphoma 2
BSA	Bovine serum albumin
CV	Crystal violet
DAPI	4′,6-Diamidin-2-phenylindol
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylendiaminetetraacetic acid
ESM1	Endothelial cell-specific molecule 1
ETV4	ETS translocation variant 4
FACS	Fluorescence - activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldeyhyde 3-phosphate dehydrogenase
HCl	Hydrochloric acid
HE	Haematoxylin eosin
IASLC	International association for the study of lung cancer
LB II	Lysis buffer
MEME	Minimum essential eagle medium
MMP	Matrix metalloproteinase
MNP	MEME non essential amino acids pyruvate
MTT	Dimethyl thiazolyl diphenyl tetrazolium salt
NSCLC	Non small cell lung cancer
P/S	Penecilin/Streptomycin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PDL1	Programmed death-ligand 1
PEI	Polyethylenimine
PI	Propidium iodide
PVDF	Polyvinyllidene fluoride
q(RT)PCR	quantitative (real-time) polymerase chain reaction
RNA	Ribonucleic acid
RPMI	Roswell park memorial institute
SCLC	Small cell lung cancer
SDS Page	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBE	Tris borate EDTA
TBS	Tris buffered saline
TEMED	N, N, N', N - tetramethylenethylenediamin
TGF-β	Transforming growth factor beta
TRITC	Tetramethylrhodamine
UICC	Union for international cancer control

6.2. List of figures

6.3. List of tables

6.4. References

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7. ABSTRACT

Background: Small cell lung cancer (SCLC) that originates from neuroendocrine-cell precursors in the lung is characterized by rapid growth, a highly aggressive disease course, initially high response rates to chemo- and radiotherapy and early development of local and distant metastasis. Activins, cytokines of the TGF-β family of growth and differentiation factors, play an important role in growth, inflammation, cell death and wound healing. Specifically, activin A, the dimer of two βA subunits, has been described to impact on malignant growth in different types of solid tumors. So far, however, there was little information about activin A in SCLC.

Initial studies in our group had found elevated levels of activin A in an extended collection of blood samples in SCLC patients, especially in those with metastatic disease, that were determined using a commercially available ELISA assay. These data suggest that activin A is associated with - and in line with the known functions in other malignancies could contribute to - the aggressiveness of SCLC. Aim of this thesis was to clarify the impact of activin A on the malignant growth and aggressive behavior of SCLC cells.

Materials and Methods: To study effects of high activin A expression, a transgenic cell line with stable overexpression of activin A was generated from the SCLC cell line GLC4 via retroviral transduction. The cell line CRL-2066 was used for treatment with recombinant activin A. The expression levels of endogenous activin A, activin receptors and the activin A antagonist follistatin were determined in a panel of 9 SCLC cell lines by qRT-PCR.

For characterizing cell proliferation, viability and cell cycle distribution upon an elevated level of activin A, clonogenicity, growth and MTT- assays as well as flow cytometry of propidium iodide stained cells were performed. In addition, mechanisms underlying metastasis were investigated. To analyze transendothelial migration, endothelial cells stably overexpressing mCherry were used and tumor cells were transiently transfected with GFP and immediately treated with activin A after seeding. Invasion through a layer of collagen was tested by transwell invasion assays. Anchorage-independent growth was measured by performing a soft agar colony formation assay. For investigating activin A signaling, phosphorylation of Smad2 was

analyzed by immunoblotting using phospho-specific antibodies and transcriptional activity of Smad proteins was assessed with a luciferase reporter gene assay.

Results: Results revealed no significant difference in proliferation, viability or cell cycle distribution between SCLC cells overexpressing activin A or treated with recombinant activin A and the respective control groups. Treatment with recombinant activin A led to decreased invasion of 2066 SCLC cells and impaired the transendothelial migration capacity of SCLC cells through blood endothelial cell layers. SCLC cells with an increased level of activin A showed smaller circular chemorepellent-induced defects in lymphatic endothelial cell layers. However, activin A overexpressing cells demonstrated a greater capacity to form colonies in an anchorage-independent growth assay. Phosphorylation of Smad2 could only be detected in SCLC cells overexpressing activin A but not in cell lines expressing endogenous levels of activin A.

Conclusion: The results of this thesis indicate that in SCLC an increased level of activin A has no effect on cell proliferation but could contribute to properties relevant for metastasis, such as colony forming ability in soft agar. Further investigations to clarify the source of the elevated activin A level in patients with SCLC are needed.

8. ZUSAMMENFASSUNG

Hintergrund: Das kleinzellige Lungenkarzinom (SCLC), das von neuroendokrinen Zellen in der Lunge ausgeht und in etwa 10-15% aller Fälle von Lungenkrebs ausmacht, ist charakterisiert durch schnelles Zellwachstum, frühe Bildung von lokalen Metastasen sowie Fernmetastasen und zunächst gutes Ansprechen auf Chemo- oder Radiotherapie. Activine, Mitglieder der TGF-β Familie von Wachstumsfaktoren, spielen eine wichtige Rolle für Zellwachstum, Zelltod, Wundheilung und im Verlauf von Entzündungen. Insbesondere Activin A, einem aus zwei βA-Ketten bestehenden Zytokin, wurde eine wichtige Rolle für die Entstehung und Progression verschiedener Tumorerkrankungen zugeschrieben. Über den Einfluss von Activin A auf die Aggressivität des kleinzelligen Lungenkarzinoms lagen bisher jedoch keine Daten vor. In Plasmaproben von Patienten mit SCLC konnte unsere Gruppe mittels ELISA Messungen erhöhte Activin A Konzentrationen feststellen. Speziell jene Patienten, die bereits Metastasen entwickelt hatten, wiesen erhöhte Werte von Activin A auf. Ziel der vorliegenden Arbeit war die Klärung, ob Activin A, wie in anderen Tumorarten, einen Einfluss auf die malignen Eigenschaften von Zellen des kleinzelligen Lungenkarzinoms hat.

Materialien und Methoden: Mittels retroviraler Transduktion wurde von der Zelllinie GLC4 eine stabile Tochterzelllinie mit ektopischer Überexpression von Activin A generiert. Des Weiteren wurde die Zelllinie CRL-2066 für Behandlungen mit rekombinantem Activin A genutzt. Die Expression von Activin A, Activin A Rezeptoren und dem Activin A Antagonisten Follistatin wurde mittels qRT-PCR untersucht. Zellwachstum, Viabilität und Zellzyklus wurden durch Klonogenizitäts-, Wachstums und MTT-Tests sowie mittels Durchflusszytometrie ermittelt. Zusätzlich wurden weitere Tests, welche zur Metastasierung beitragen, durchgeführt. Zellmigration wurde durch Transwell Assays ermittelt. Um die Ausbreitung von Tumorzellen in Blut- bzw. Lymphgefäßen zu untersuchen, wurde ein in-vitro Ko-Kultur-Testsystem, das aus einem Zellrasen von Endothelzellen und Tumorzellspheroiden besteht, verwendet. Phosphorylierung von Smad2 wurde anhand von Immunoblots mit phosphospezifischen Antikörpern analysiert.

Ergebnisse: Die Ergebnisse zeigen, dass Activin A keinen signifikanten Einfluss auf das Zellwachstum, die Viabilität, das Migrationsverhalten oder den Zellzyklus der untersuchten Zelllinien des kleinzelligen Lungenkarzinoms hat. Activin A führte einerseits zu vermindeter Invasivität und transendothelialer Migration, andererseits zu erhöhtem Wachstum im Soft-Agar. Phospho-Smad2 konnte nur nach ektopischer Überexpression von Activin A, aber nicht in Zellen mit endogener Activin Expression nachgewiesen werden.

Schlussfolgerung: Ergebnisse der Arbeit zeigen, dass Activin A keinen Einfluss auf das Wachstum von kleinzelligen Lungenkarzinomzellen hat, jedoch Zelleigenschaften beeinflusst, die an der Bildung von Metastasen beteiligt sein könnten. Die Quelle der erhöhten Activin A Plasmawerte von Patienten mit kleinzelligem Lungenkarzinom bedarf noch weiterer Untersuchungen.