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Studies on the role of integrins in corticotroph tumorigenesis

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..... Xiao Chen.....

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Ludwig-Maximilians-Universität zu München

Erster Gutachter:	Prof. Dr. Marily Theodoropoulou
Zweiter Gutachter:	Prof. Dr. Günter Stalla
Dritter Gutachter:	PD. Dr. George Vlotides
ggf. weitere Gutachter:	_____
	_____
Mitbetreuung durch den promovierten Mitarbeiter:	_____
Dekan:	Prof. Dr. med. Thomas Gudermann

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Zusammenfassung (Deutsch)

Einleitung: Integrine sind heterodimere Transmembranproteine, bestehend aus Alpha- und Beta-Untereinheiten, die Zell-Zell- und Zell-Extrazellulärmatrix (ECM)-Interaktionen vermitteln. Mehrere Integrine werden in menschlichen Krebserkrankungen überexprimiert und ihr ECM-Erkennungsmotiv, Arginin-Glycin-Aspartat (RGD), wird für die Bildgebung und das Targeting von Tumoren genutzt.

Ziel: Untersuchung der Expression und Funktion von RGD-bindenden Integrinen in kortikotrophen Tumoren.

Methoden: Wir bestimmten die Expression von RGD-bindenden Integrinen mittels qPCR in 18 kortikotropen Tumoren und verglichen die Transkriptlevel mit gonadotropen Tumoren (n=16) und normalen Hypophysen (n=2). Um die Rolle der Integrine zu untersuchen, erstellten wir ihr Expressionsprofil in murinen kortikotropen Tumorzellen AtT-20 mittels RT-PCR und untersuchten den Effekt ihrer Hemmung mit RNA-Interferenz auf die Aktivität des humanen *POMC*-Promotors und die Zellviabilität (WST-1-Kolorimetrie-Assay). Wir verwendeten Fluoreszenzmikroskopie, um die Bindung von RGD-Peptiden in diesen Zellen zu beurteilen.

Ergebnisse: Corticotrophe Tumoren exprimieren α_v (ITGAV), β_1 (ITGB1), β_5 (ITGB5), β_8 (ITGB8) und α_8 (ITGA8). Die Integrine α_v, β_1, β_5 sind in kortikotropen Tumoren überexprimiert im Vergleich zu gonadotropen Tumoren, wo die Expression fast nicht nachweisbar war (P<0.0001) und zur normalen menschlichen Hypophyse (P<0.001). Die Expression von β_8 war nur in kortikotrophen im Vergleich zu gonadotrophen Tumoren höher (P=0.04), aber nicht zur normalen Hypophyse. Wir fanden, dass AtT-20-Zellen alle diese vier Integrine exprimieren. Das Knocking down von jeweils α_v, β_1 und β_5 verringerte die Aktivität des humanen *POMC* Promotors im Vergleich zur Scramble-Kontrolle (prozentuale Suppression 63±22, 54±23, bzw 69±28; P<0.05), während β_8 kaum einen Effekt hatte. Das Knocking down von α_v und β_1 hatte einen kleinen, aber signifikanten Effekt auf die Lebensfähigkeit von AtT-20-Zellen (% Suppression 15.92±1.6 bzw. 27.4±1.4; P<0.05). Mit Hilfe der Immunfluoreszenz beobachteten wir, dass ein RGD-Peptid, das mit dem Nahinfrarot-Fluorophor Cy5.5

konjugiert war, an AtT-20-Zellen binden und diese markieren konnte, ohne schädliche Auswirkungen auf die AtT-20-Zellviabilität (WST-1-Assay) und -funktion (bestimmt durch die *POMC*-Promotoraktivität).

Schlussfolgerungen: Diese Studie zeigt, dass kortikotrophe Tumoren die Gene exprimieren, die für die Alpha- und Beta-Untereinheiten der RGD-bindenden Integrine $\alpha_v\beta_1$, $\alpha_v\beta_5$ und $\alpha_v\beta_8$ kodieren. Wir haben vorläufige Hinweise darauf, dass diese Integrine die Aktivität des *POMC*-Promotors regulieren können. RGD-Peptidkonjugate haben das Potenzial als bildgebende Mittel für kortikotrophe Tumore.

Abstract (English)

Introduction:Integrins are heterodimeric transmembrane proteins composed of alpha and beta subunits that mediate cell-cell and cell-extracellular matrix (ECM) interactions. Several integrins are overexpressed in human cancers and their ECM recognition motif, arginine-glycine-aspartate (RGD), is being utilized for tumor imaging and targeting.

Aim:To explore the expression and function of RGD-binding integrins in corticotroph tumors.

Methods: We determined the expression of RGD-binding integrins by qPCR in 18 corticotroph tumors and compared transcript levels with gonadotroph tumors (n=16) and normal pituitaries (n=2). To study the role of integrins, we established their expression profile in murine corticotroph tumor AtT-20 cells by RT-PCR and investigated the effect of their inhibition with RNA interference on human POMC promoter activity and cell viability (WST-1 colorimetric assay). We used fluorescence microscopy to assess RGD peptide binding in these cells.

Results: Corticotroph tumours express α_v (ITGAV), β_1 (ITGB1), β_5 (ITGB5), β_8 (ITGB8), and α_8 (ITGA8). Integrins α_v, β_1, β_5 are overexpressed in corticotroph compared to gonadotroph tumors, where expression was almost undetectable (P<0.0001) and human normal pituitary (P<0.001). The expression of β_8 was higher in corticotroph only compared to gonadotroph tumors (P=0.04), but not to the normal pituitary. We found that AtT-20 cells express all these four integrins. Knocking down each α_v, β_1, and β_5, decreased human *POMC* promoter activity compared to scramble control (% suppression 63±22, 54±23, and 69±28 respectively; P<0.05), while β_8 had little effect. Knocking down α_v and β_1 had a small but significant effect on AtT-20 cell viability (% suppression 15.92±1.6 and 27.4±1.4 respectively; P<0.05). Using immunofluorescence, we observed that an RGD peptide conjugated with the near-infrared fluorophore Cy5.5 could bind to and label AtT20 cells, with no deleterious effects on AtT-20 cell viability (WST-1 assay) and function (determined by *POMC* promoter activity).

Conclusions: This study shows that corticotroph tumors express the genes encoding

the α and β subunits of the RGD-binding integrins αvβ1, αvβ5, and αvβ8. We have preliminary evidence that these integrins may regulate *POMC* promoter activity. RGD peptide conjugates potential as corticotroph tumor imaging agents.

List of abbreviations

Abbreviations	Full Name
ACTH	Adrenocorticotropin
AVP	Arginine-vasopressin
AP-1	Activator protein 1
Akt	Protein kinase B
BADX	Bilateral adrenalectomy
CAM	Cellular adhesion molecules
CHO	Chinese hamster ovary cells
CRH	Corticotrophin-releasing hormone
CXCR	Chemokine receptor
DRD2	Dopamine receptor D2
Del-1	Developmental endothelial locus-1
DMEM	Dulbecco's modified eagle's medium
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EMT	Epithelial transformation
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FSH	Follicle stimulating hormone
GH	Growth hormone
GR	Glucocorticoid receptor
HGFR	Hepatocyte growth factor receptor
HPA axis	Hypothalamic-pituitary-adrenal axis
Hsp90	Heat shock protein90
ILK	Integrin-linked kinase
ICA	Internal carotid artery
kD	Kilodalton
LAP	latency associated peptide
LH	Luteinizing hormone
LTBP	Latent TGF-β-binding proteins
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
MSH	Melanocyte stimulating hormone
MRI	Magnetic resonance imaging
NaCl	Sodium Chloride
NaOH	Sodium hydroxide
ONPG	O-Nitrophenyl-β-D-galactopyranosid
PBS	Phosphate buffered saline
PI3K	Phosphatidylinositol 3-kinase
PRL	Prolactin

PKA	Protein kinase A
RGD	Arginine-glycine-aspartate
RT-PCR	Reverse transcriptase - polymerase chain reaction
SSTR	Somatostatin receptor
Src	Steroid receptor coactivator
TGF-β	Transforming growth factor β
TMZ	Temozolomide
TSH	Thyrotropin-stimulating hormone
TSP-1	Thrombospondin-1
TZD	Thiazolidinediones
UFC	Urine free cortisol
USP8	Ubiquitin specific protease 8
VEGF	Vascular endothelial growth factor
WST-1	Water-soluble tetrazolium salt-1

1. Introduction

1.1. Pituitary

The pituitary gland, located in the pituitary fossa of the sphenoid bone, is the master gland of the endocrine system. It is divided into adenohypophysis (anterior and intermediate lobe) and neurohypophysis (posterior lobe). The adenohypophysis secretes adrenocorticotropic hormone (ACTH), thyrotropin stimulating hormone (TSH), luteinizing hormone (LH), follicle stimulating hormone (FSH), growth hormone (GH), melanocyte stimulating hormone (MSH) and prolactin (PRL)[1]. ACTH, TSH, LH, and FSH act through their respective target glands, thyroid, adrenal, and gonads respectively. GH, MSH, and PRL can directly regulate growth, mammary gland development and lactation, and melanocyte activity. The secretory function of the adenohypophysis is controlled by the central nervous system, and in particular the hypothalamus, as well as by the feedback of hormones secreted by the peripheral target glands.

1.2. ACTH regulation

1.2.1. The hypothalamic pituitary adrenal axis (HPA)

A deeper understanding of the normal and pathological mechanisms of the HPA axis is essential for the pathologic, diagnostic, and treatment of Cushing's disease[2]. The trophic regulator of ACTH is the corticotropin-releasing hormone (CRH), which is secreted from the paraventricular nucleus of the hypothalamus through the median bulge. CRH can enter the pituitary portal circulation and reaches the anterior portion of the pituitary to bind to CRH receptor 1 (CRH-R1) on pituitary corticotroph cells and stimulate ACTH synthesis. The secreted ACTH binds to melanocortin receptor 2 (MC2R) in the adrenocortical cells of the zona fasciculata and zona reticularis of the adrenal cortex, thereby stimulating the generation and secretion of glucocorticoids

(cortisol)[3]. Under the physiological conditions, circulating cortisol enables to inhibit CRH and pituitary ACTH's secretion through negative feedback loop on the hypothalamus and pituitary[4] (Figure 1.1).

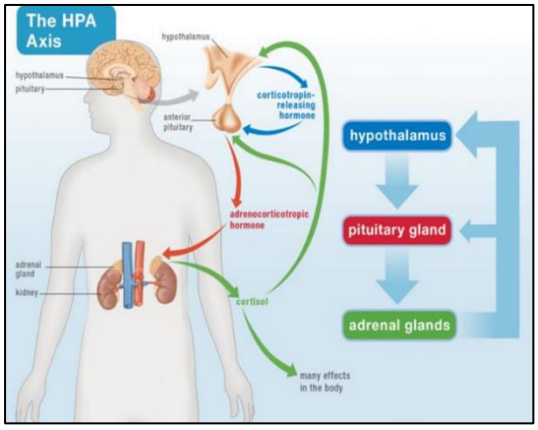

Figure 1.1: Physiological regulation of ACTH homeostasis: Neurons in the paraventricular nucleus of the hypothalamus secrete CRH to promote the release of ACTH, which in turn acts in the adrenal cortex to synthesize glucocorticoids. Glucocorticoids can feedback to the hypothalamus and pituitary gland (repress the synthesis and secretion of CRH and ACTH, respectively), forming a feedback regulation loop. Image adapted from Wikipedia.

1.2.2. POMC regulation

ACTH derives from the polypeptide precursor proopiomelanocortin (POMC). The originally translated and pre-modified form of POMC is called pre-POMC, which contains 285 amino acids. POMC is composed of 241 amino acids and obtained by removing the signal peptide from pre-POMC[5,6]. POMC is mainly expressed in the corticotroph cells in the anterior pituitary, melanotropes in the intermediate pituitary lobe that is rudimentary in humans, and the POMC hypothalamic neurons of the arcuate nucleus[7].

In corticotroph cells, POMC is enzymatically cleaved to produce ACTH, lipotropin, and a small amount of β-endorphin[1] (Figure 1.2). CRH is the main stimulator of POMC transcription and ACTH secretion[8,9]. CRH-R1 is G protein coupled receptor, which upon CRH binding activates adenylate cyclase and induces cyclic adenosine monophosphate (cAMP) to activate protein kinase A (PKA)[5,10]. The complex signaling network of PKA activates transcription factors and transcriptional coregulators that stimulate POMC transcription.

A second regulator of ACTH synthesis is arginine-vasopressin (AVP), which enhances the stimulatory action of CRH on ACTH secretion[11,12]. Similarly, *in vitro* and

in vivo findings confirmed that vasopressin has a certain inhibitory effect on the transcription level of POMC[13,14].

Glucocorticoids negatively regulate the HPA axis at hypothalamus and pituitary levels, by reducing CRH and vasopressin secretion. In addition glucocorticoids inhibit POMC transcription directly at corticotroph cell level by binding to nuclear glucocorticoid receptors[15,16].

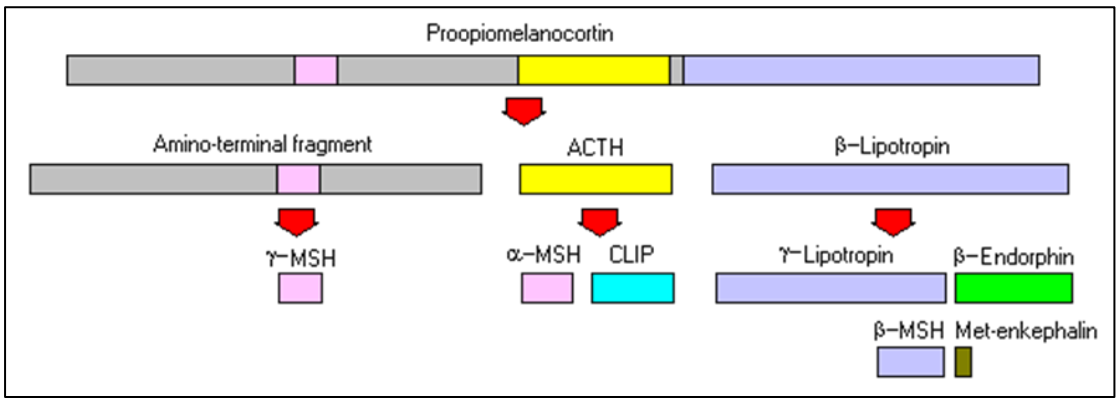

Figure 1.2: Proopiomelanocortin cleavage: The precursor protein POMC, which is synthesized in the pituitary gland, is proteolytically cleaved into multiple polypeptide fragments. MSH, melanin-stimulating hormone; CLIP, corticotropin-like intermediate lobe peptide. Image obtained from *Answers in Depth, Vol. 2(2007), pp. 98–100*.

1.3. Cushing's disease

Harvey Cushing first described the syndrome attributed to him in 1912 and proposed "*adenomas of the pituitary body*" as its cause in 1932[17]. Cushing's disease refers to the excessive secretion of ACTH by a corticotroph tumor, hypercortisolemia. Patients under chronic hypercortisolemia exhibit characteristic centripetal obesity, full moon face, buffalo back, striae, and may suffer from secondary hypertension, diabetes, fatigue, infertility and mood disorders[18]. Untreated patients often die from severe cardio-cerebrovascular diseases or severe infections[19-21]. Surgical resection of the pituitary tumors usually with transsphenoidal surgery is the main treatment[19,22].

1.3.1. Etiopathogenesis

Cushing's disease is due to corticotroph tumors, which in their majority are microadenomas (<10mm)[19,23].

1.3.1.1. Deregulated HPA axis

CRH and AVP are the main trophic factors for corticotroph cells, but there is no evidence of hypothalamic deregulation as cause of corticotroph tumorigenesis[24]. The expression of the CRH receptor is increased in corticotroph tumors. In addition corticotroph tumors express the AVP receptors (V_1 receptor, V_2 receptor and V_3 receptor)[25,26]. No mutations in the coding regions of the genes encoding for CRH and AVP receptors were found[25-27].

The glucocorticoid negative feedback is a major restraining factor of ACTH synthesis and corticotroph cell growth[28]. However, corticotroph tumor cells acquire partial resistance to the inhibitory glucocorticoid feedback[29]. Glucocorticoids bind to nuclear receptors GRα (encoded by *NR3C1*) and GRβ. GRα expression is not altered in corticotroph tumors[30]. Mutations in the *NR3C1* gene were described in two patients, but not in the majority of corticotroph tumors examined[31-34]. Glucocorticoid receptor (GR) inhibits transcription in a transcriptional project that involves brahma related-gene 1 (Brg1)[35]. Brg1 expression is frequently lost in corticotroph tumors and this could explain the resistance to glucocorticoid feedback[36]. Another factor that contributes to glucocorticoid resistance is the overexpression of the GR chaperon heat shock protein 90 (Hsp90)[37].

1.3.1.2. Genetics of Cushing's disease

Corticotroph tumors are monoclonal in origin, meaning that they are derived from a single cell transformed by a genetic event[38-41]. Until recently, the pathogenesis of corticotroph tumors was unclear[42]. Reincke et al.[20] found a mutational hotspot in the ubiquitin-specific protease 8 (USP8) gene in 4 out of ten corticotroph tumors. This mutational hotspot resides within the 14-3-3 binding region, which allows for 14-3-3 binding that protects the protein from proteolytic cleavage. 14-3-3 cannot bind to the

mutated USP8 protein, which is then cleaved to a smaller 40 kDa peptide with higher deubiquitinase activity. USP8 mutants retain epidermal growth factor receptor (EGFR) to the membrane, leading to the activation of downstream extracellular signal-regulated kinases 1/2(ERK1/2) and ultimately enhance EGFR to induce pituitary POMC transcription and ACTH secretion[20]. Somatic mutations in the USP8 gene have been reported in around 50% of corticotroph tumors[20,42-50]. USP8 mutations were also found in around 50% of progressive corticotroph tumor growth after bilateral adrenalectomy(BADX), known as Nelson syndrome[51]. USP8 mutant corticotroph tumors are mostly found in female patient with intermediate size tumors. It is hypothesized that corticotroph tumors with USP8 mutations exhibit a more "typical" corticotroph phenotype[52]. USP8 wild-type and mutant corticotroph tumors have distinct transcriptional and protein expression profiles[39,50,53].

1.3.2. Therapy

The first-line treatment of Cushing's disease is pituitary surgery, followed by a second surgery, medication, radiation therapy, and/or BADX[54,55] (Figure 1.3).

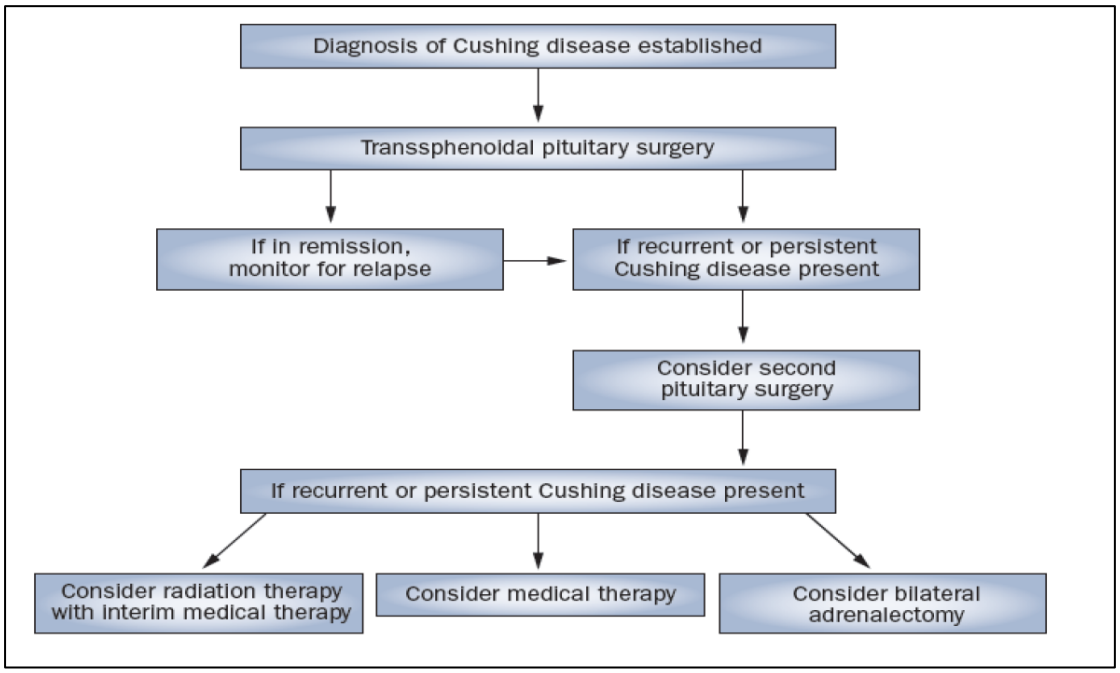

Figure 1.3. Therapeutic management of Cushing's disease. The treatment of choice for patients with Cushing's disease is transsphenoidal surgery, which provides rapid and lasting

relief from hypercortisolism. Secondary transsphenoidal surgery is considered for patients with persistent and recurrent Cushing's disease. Patients who do not achieve remission or relapse after secondary transsphenoidal surgery may be considered for individualized treatment, including medication, radiotherapy, and BADX. Image obtained from *Nature Reviews Endocrinology*,*7(2011), pp279–289.*

1.3.2.1. Surgical treatment

Selective transsphenoidal pituitary adenoma resection is the first-line therapeutic method for Cushing's disease[56]. Transsphenoidal surgery can remove more than 90% of pituitary adenoma, resulting in a 60%-90% remission rate for microadenoma and a 65% remission rate for macroadenoma[22,57-59]. The remission rates in different research reports vary greatly, which is mainly due to the size and location of adenoma, growth methods, and surgical techniques. The preoperative Magnetic resonance imaging (MRI) diagnosis of pituitary microadenoma has a remission rate of 72.3% to 100%, while the preoperative MRI negative or suspicious Cushing's disease remission rate is 64.7% to 71.4%[57]. The remission rate of non-invasively growing microadenomas is 70% to 90%, while the remission rate of invasively growing large adenomas is only 25% to 50%[60]. Surgical resection depends on the following factors: tumor size, infiltration of the cavernous sinus, preoperative drug therapy (somatostatin and bromocriptine will change the consistency of the tumor), and the surgeon's experience[58,61].

1.3.2.2. Surgery failed or secondary surgery after recurrence

Surgery failed:in several cases a second operation is recommended. Surgical methods include pituitary resection and total pituitary resection, where 38% to 67% of patients can get remission[60]. The advantage of reoperation early after surgery is that it can avoid the formation of scars and changes in anatomical structure in the pituitary body, and reduce the occurrence of complications such as cerebrospinal fluid leakage, diabetes insipidus, and hypopituitarism. The disadvantage is that some cases may cause delayed postoperative remission and contribute to unnecessary reoperation. Hameed et al. performed retranssphenoidal surgery within 10 weeks for 10 out of 16 patients who have no resuscitation after the first transsphenoidal surgery[62]. Among them, 7 patients

achieved remission and no complications; 6 patients who did not undergo reoperation were followed up for 1 year no delay remission was found[62].

Second transsphenoidal surgery: The 5-year recurrence rate of Cushing's disease patients who have been relieved after transsphenoidal surgery is 5% -10%, and the 10-year recurrence rate is 10% -20%[63]. However, recent data suggest that the 5-year recurrence rate is high to 25%[64]. Compared with pituitary microadenoma, pituitary macroadenoma is highly possible to recur after surgery with shorter recurrence time. It is worth noting that the rate of recurrence after surgery is higher in children with Cushing's disease[65]. For patients with recurrent Cushing's disease, a second transsphenoidal surgery may be considered to achieve long-term remission with an average remission rate of 64%, but this surgery also may induce the risk of cerebrospinal fluid leakage and hypopituitarism[60,66]. For patients with recurrent Cushing's disease, more neurosurgeons choose to repeat the second procedure within 2 months of surgery, when less scar tissue facilitates the surgeon to better understand the anatomical details of the patients[67].

1.3.2.3. Radiotherapy

Radiation therapy is an alternative second-line treatment for Cushing's disease[68]. Prophylactic radiotherapy is not recommended for patients with complete remission after surgery, but postoperative radiotherapy is suggested for patients with postoperative pathological atypical pituitary adenoma to reduce the chance of recurrence. Radiotherapy indications: (1) Patients with no remission or relapse after remission;(2) Patients who are not suitable or not undergoing surgery; (3)Adjuvant treatment of recurrent invasive and pituitary cancer; (4) Nelson syndrome[69]. At present, there are a variety of radiotherapy techniques available, mainly including fractional external beam irradiation and stereotactic radiosurgery[70]. Both methods have similar therapy effects, but the precise positioning of the gamma knife in stereotactic radiosurgery can limit the radiation to the tumor body, avoid damage to important tissue structures such as the saddle and the side of the saddle, and the effect is obvious. A

study on 96 patients with Cushing's disease treated with gamma knife reported a 70% remission rate, with an average remission time of 16.6 months[71]. The shortcoming of radiotherapy is that the onset of action is slow, and remission can occur only 6 months to several years (average 3 years) after radiotherapy[70]. The main complication is hypofunction of the pituitary gland, the incidence of fractional external beam irradiation exceeds 50%, and stereotactic radiotherapy is as high as 66%[72].

1.3.2.4. Bilateral adrenalectomy

BADX can rapidly regulate hypercortisolemia but contribute to permanent adrenal insufficiency, which requires lifelong steroid replacement therapy and risk the life-threatening adrenal crisis. For this reason, BADX is often considered as last option for the treatment of Cushing's disease when other resorts fail. Therefore, BADX is used strictly for the following indications. (1) Cushing's disease patients who are ineffective with chemotherapy and have persistent hypercortisolemia; (2) Intolerance to chemotherapy; (3) Radiotherapy failure and no acceptance for chemotherapy; (4) Reservation young female patients with Cushing's disease who hope for fertility[73,74]. Adrenalectomy is usually performed laparoscopically, and more than 95% of patients with refractory Cushing's disease who undergo surgery experience relief of symptoms. The median recurrence rate within 30 days of BADX is statistically 18% and the median incidence of adrenal crisis is 28%. The procedure-related mortality rate was 9%, with stroke and myocardial infarction being the most common causes of death[75,76]. In addition, there is a risk of progressive corticotroph tumor growth, also known as, Nelson's syndrome[77], which has been renamed with "Corticotroph Tumor Progression after bilateral adrenalectomy/Nelson syndrome" (CTP-BADX/NS, unclassified). The cumulative incidence of CTP-BADX/NS is approximately 40%, thus special attention should be paid close to these patients with (1) high plasma ACTH or elevated ACTH levels after BADX, (2) pre-operative adrenocorticotropic tumors visible before BADX and (3) under 35 years of age[74].

1.3.2.5. Pharmacological therapy

The medications for Cushing's disease treatment are divided into three categories: (1) corticotroph-directed agents, which inhibit ACTH synthesis; (2) steroidogenesis inhibitors that inhibit cortisol synthesis; (3) GR antagonists that block tissue response to glucocorticoids[78] (Table1). The majority of approved drugs are steroidogenesis inhibitors that inhibit cortisol syntheses like ketoconazole, mitotane, and metyrapone or GR antagonists that block glucocorticoid action in peripheral target organs like mifepristone[55,79].

Corticotroph targeted treatments include somatostatin analogs and dopamine agonists[80,55,81,82]. In corticotroph tumors, dopamine receptor 2 (DRD2) was found to be expressed by immunohistochemistry and RT-PCR in almost 80% of corticotroph tumors[83,84]. DRD2 and Somatostatin receptor 5 (SSTR5) also have a certain degree of co-expression in 60% of pituitary corticotroph tumors[85]. The DRD2 ligand cabergoline is not effective in normalizing urinary free cortisol (UFC) even when administered at high doses of 1-7mg/week[55,84,86-88]. The somatostatin analog was approved for the treatment of Cushing's disease in pasireotide, a multiagent analog with a high affinity for SSTR5[89]. SSTR5 is the main SSTR expressed in corticotroph tumors and pasireotide inhibits ACTH synthesis *in vitro* and in dogs with Cushing's disease[90-92]. Pasireotide treatment in patients with Cushing's disease normalized UFC (~25% of patients) caused tumor shrinkage and ameliorated symptoms of the disease[93-97]. Treatment with long-acting release pasireotide resulted in UFC normalization in around 40% of patients[98]. However, pasireotide also causes hyperglycemia in around half of patients, a serious side effect that requires additional treatment for diabetes[96-98]. Patients with corticotroph tumors bearing *USP8* mutations have significantly higher levels of SSTR5 expression than the wild type, suggesting that they may respond better to analogs targeting SSTR5[46,48,49].

The periphery targeting drugs are effective in normalizing cortisol but they have no effect on tumor and ACTH hypersecretion and tumor-targeted treatments are not as effective[99]. Studies have revealed potential tumor-targeted treatments (reviewed in[100]).

Several potential tumor-targeting therapies are currently in clinical trials, including gefitinib, retinoic acid, roscovitine, levoketoconazole and osilodrostat (LCI699). Gefitinib (an orally administered EGFR tyrosine kinase inhibitor) can block EGFR activity, inhibit POMC expression, suppresses corticotrophic tumor cell proliferation and induce apoptosis[101]. A clinical trial of gefitinib in Cushing's disease patients with USP8 mutations (ClinicalTrials.gov number NCT02484755) is currently underway. Retinoic acid can suppress the transcriptional activity of activator protein 1 (AP-1) and orphan receptors in corticotroph tumor cells, resulting in anti-proliferative activity and ACTH inhibition in corticotroph tumors bearing mice[102]. In two prospective proof-of-concept studies, it was shown that all-trans retinoic acid (tretinoin) treatment for 6-12 months reduced UFC by at least > 50% in patients and even normalized UFC in some patients[103,104]. Roscovitine is a competitive inhibitor of oral cytokine-dependent kinases. Which can inhibit ACTH synthesis and antiproliferative effects due to the direct regulation of POMC expression by cyclin E[105]. Roscovitine is currently in clinical trial in patients with Cushing's disease (ClinicalTrials.govno.NCT02160730). Levoketoconazole (COR-003) has higher efficacy and fewer side effects at lower doses compared to ketoconazole[106]. 11β-hydroxylase inhibitor (LCI699; osilodrostat inhibitor) is an oral non-steroidal corticosteroid synthesis inhibitor[107]. Both are currently in clinical trials (ClinicalTrials.gov numbers NCT02468193, NCT02697734 and NCT03621280).

Other potential tumor-targeting drugs currently under (pre-clinical) investigation include thiazolidinediones (TZDs) and silibinin. TZDs are ligands for peroxisome proliferator-activated receptor γ (PPARγ). Silibinin, a flavonolignan derived from Silybum marianum, can inhibit Hsp90, a chaperone protein that regulates GR[108]. Both drugs have been shown to the effective therapy effect on inhibiting ACTH synthesis and tumor growth in both *in vivo* and *in vitro* experiments[37,109].

Table 1. Summary of chemodrugs for the treatment of Cushing's disease.

	Medication	Dose	Mechanism	Effectiveness	Main side effects	Comments	REF
Corticotroph-Directed Agents	Pasireotide	600~900 μg 2 times a day (sc)	Binding to mainly SSTR5 resulting in inhibition of hormone secretion	30-80%	Hyperglycemia (40~80%), GI and biliary issues, QT prolongation, GH deficiency	Combined with cabergoline and/or steroidgehesis inhibitors Chandle	110,111
	Cabergoline	0.5–6 mg/week (oral)	Binding to Dopaminetype 2, Dopamine agonist (DA)	50-75%	GI issues, Nausea, vomiting, dizziness, mental disorders, risk of valve disease	pregnant or desire to get pregnant	86,87,112
Steroidogenesis Inhibitors	Mitotane	1.5–6g three times a day (oral)	Inhibitor of multiple enzymes in the adrenal cortex	83%	nausea,vomiting,diarrhea ,neurotoxicity dyslipidemia	avoided pregnancy within 5 years after stopping mitotane	113,114
	Ketoconazole	0.4-1.6g three times a day (oral)	Inhibiting 11β-hydroxylase and blocking 17,20-lyase	93%	GI issues, abnormal liver function tests (mild 14%, severe 3%), gynecomastia, alopecia and/or decreased libido (in men)	more suitable for female patients	115
	Metyrapone	0.75-6g/d, (oral)	Inhibits 11β-hydroxylase and 18-hydroxylase	75-80%	GI issues, dizziness, hypertension, edema and hypokalemia hirsutism and/or acne (women)	more suitable for male patients	116,117
	Etomidate	<0.1mg/kgpe r h (iv)	Inhibiting CYP11B1 with11β-hydroxylase activity	~100% (short period)	Sedation, anesthesia	For life-threatening hypercortisolemia patients, anesthesiologist monitoring	118
Glucocorticoid receptor antagonist	Mifepristone	0.3-1.2g/d, (oral)	Blockade of glucocorticoid and progesteron receptors	87%	Adrenal insufficiency, hypokalemia, Hypertension, thickened endometrium, Irregular menstruation	Pregnant women are contraindicated	119
Emerging Medical Therapy	Levo ketoconazole	Inhibition of cholesterol side-chain cleavage enzyme,11β-hydroxylase,17α-hydroxylase, and 18-hydroxylase				higher efficacy and fewer side effects than ketoconazole	120
	Osilodrostat	Inhibiting 11β-hydroxylase and aldosterone synthase				higher affinity than metyrapone	121
	ATR-101	inhibitor of acetyl-coA acyltransferase 1				Phase II study	122
	Retinoic acid	Inhibits the transcriptional activity of AP-1 and Nur77 and Nurr1				Phase II study	123
	Gefitinib	EGFR tyrosine kinase inhibitor, attenuates POMC expression				Phase II study	124
Temozolomide (TMZ)	TMZ is an oral alkylating agent that inhibits DNA repair and is used for first-line chemotherapy treatment of aggressive pituitary tumors and pituitary cancer.						125

1.4. Integrins

Integrins are a class of heterodimeric transmembrane proteins composed of α and β subunits that mediate cell-cell and cell- extracellular matrix (ECM) adhesion[126]. 18 α-subunits and 8 β-subunits combine to form at least 24 integrin subtypes[127]. Both α and β integrin subunits are type I transmembrane proteins, with 700-1100 amino acid extracellular and 30-50 amino acid intracellular domains respectively. Extracellular domains are regions of membrane proteins located outside the cell that regulate the activity of signaling pathways by defining, recognizing, specific ligands (e.g.hormones or neurotransmitters) during signal transduction. The extracellular segment of theα subunit recognizes the Arginine-glycine-aspartate (RGD) sequence of ECM, while the intracellular segment of the β subunit is connected to the cytoskeleton[128]. Integrin-containing β_1 subunit mainly mediates the adhesion between cells and ECM. Integrin-containing β_2 subunit is predominantly found on the surface of platelets, which mediates platelet aggregation and thrombosis. The β_4 subunit interacts with muscle movement protein and related protein binding. In addition to being a bridge between ECM and the cytoskeleton, integrins can also regulate a variety of intracellular signaling pathways, including actin nucleation, polymerization activation, and mitogen signaling[129]. More importantly, these signals can promote tumor cell proliferation and survival, and thus drive cancer development and progression[130].

Cell surface integrins exist in three conformations: (I) resting-state which has low affinity to ECM ligands and is called low-affinity conformation, (II) stretched state which has low affinity for ligands, and (III) activated state which has a high affinity with ECM ligands. In the resting states, the α and β subunits of the integrin tail and the helix of the transmembrane domain in the cytoplasm, forming a salt bridge to stabilize it[128].

Integrin activation is regulated by two mechanisms: (I) signal from the integrin cytoplasmic region is transmitted through the tail to the domain that binds to the extracellular ligand, which will cause conformational changes in the integrin dimer to

promote ECM ligand interaction and (II) binding of ECM ligands trigger integrin aggregation and activation[129,131].

1.4.1. Integrins and cancer

The expression of integrins varies greatly between normal and tumor tissues: $\alpha_v\beta_3$, $\alpha_4\beta_1$, $\alpha_v\beta_6$, $\alpha_6\beta_4$, and $\alpha_v\beta_5$ integrins are highly expressed in epithelial cells from a variety of solid tumors[132-135]. More importantly, $\alpha_v\beta_3$, $\alpha_v\beta_6$, and $\alpha_5\beta_1$ are typically expressed at low levels in normal epithelial cells but have higher expressions in tumor cells[136]. These highly expressed integrins regulate the migration, proliferation, and survival of tumor cells. Studies have confirmed that the expression of integrin $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_6\beta_4$, and $\alpha_v\beta_6$ in tumors correlates with tumor type and disease progression[137].

1.4.2. Integrin in cancer progression and metastasis

Integrins are involved in tumor progression and metastasis, including cell adhesion, migration, invasion, proliferation, regulation of apoptosis, and induction of angiogenesis (Figure 1.4). The high tumor expressions of $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, and $\alpha_v\beta_8$ are associated with metastasis and poor prognosis in most solid tumors[132,138-143]. Binding of $\alpha_v\beta_3$ to ECM ligands vitronectin and/or osteopontin favors tumor growth, invasion, and metastasis[144,145]. High expression of the α_5 subunit correlated with increased risk of death in patients with hepatocellular carcinoma[146]. The expression level of $\alpha_v\beta_6$ also showed the correlation with metastasis and poor prognosis, indicating that $\alpha_v\beta_6$ can be used as a marker of tumor invasiveness in solid tumors such as breast, gastric, pancreatic, and ovarian cancers[143,147-155].

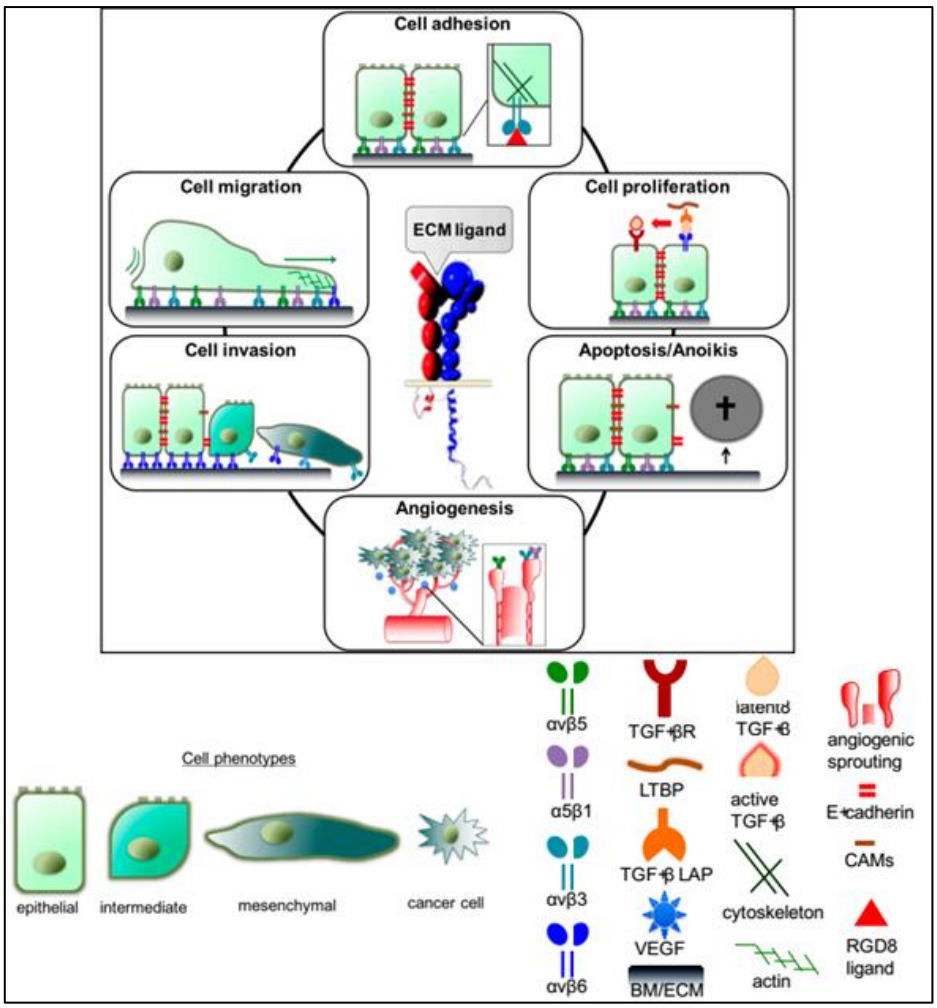

Figure 1.4: Integrin functions in tumors. Integrins highly expressed in tumor cells promote tumor progression and metastasis by increasing tumor cell migration, proliferation, adhesion, inhibiting apoptosis, and inducing angiogenesis and cell invasion. Integrins mediate cell adhesion by binding ECM components that bear the RGD recognition motif and proliferation by binding inactive TGF-β latency-associated peptide (LAP) (integrins $\alpha_v\beta_3$, $\alpha_v\beta_6$, and $\alpha_v\beta_8$). Integrin expressing cells binding to ECM in the context of mesenchymal tissue prevents apoptosis of invasive cancer cells. Integrins interact with angiogenesis factors to induce vascular sprouting. Finally, changes in integrin subtype expression during epithelial-mesenchymal transition epithelial transformation (EMT) enables tumor cells to invade. Image obtained from *Cancers 9.9 (2017), pp. 116.*

1.4.2.1. Effect of integrins on cell proliferation

Integrins maintain the integrity of normal tissues. In tumor cells, integrins not only mediate cell proliferation by binding to growth factors and their receptors, but also regulate cell proliferation by activating cytokines and oncogenes[156,157].

Integrin $\alpha_v\beta_3$ has been reported to promote the proliferation of cancerous cells[158]. $\alpha_v\beta_3$ can promote cell proliferation through integrin-linked kinase (ILK) signaling[159],

where integrins interact with growth factors and their respective receptors to promote cell proliferation[156]. Meanwhile, $\alpha_v\beta_3$ also has been reported that it can interact with EGFR, Erb-B2, and activated platelet-derived growth factor to promote cell proliferation in breast, ovarian, and pancreatic cancers as well as glioblastoma[160,161,162].

High expression of $\alpha_v\beta_6$ promotes tumor cell proliferation by binding to latency activating peptide, which associates with transforming growth factor-β (TGF-β)[157,163]. Clinical studies have shown that the expression of proliferation markers, such as p53, Ki-67, are upregulated in cervical cancer patients with high expression of $\alpha_v\beta_6$[164]. Interestingly, compared to the promoting effect of $\alpha_v\beta_6$ on pro/LAP-TGF-β, activation of TGF-β by $\alpha_v\beta_8$ inhibits the proliferation of airway epithelial cells in bronchial tissue[165,166].

1.4.2.2. Integrin mediate cell adhesion, migration, and invasion

The progression and metastasis of tumors start from the proliferation of tumor cells, where the tumor cells detach from the primary site due to the adhesion ability. Tumor cells can form local spread through epithelial transformation (EMT) and invade blood vessels and lymphatic vessels. Integrin participates in every biological process during tumor invasion and metastasis[167]. The adhesion and dissociation between tumor cells, vascular endothelial cells, and ECM play a decisive role in the progression of tumor metastasis. This adhesion and dissociation are achieved through the mediation of cellular adhesion molecules (CAMs). Change in tumor cell adhesion is an early step of tumor metastasis[168]. Integrins in tumor metastasis decrease the adhesion between tumor cells and increase the adhesion between tumor cells and host cells, thus enhancing the invasion and metastasis of tumor cells[169 170 171].

ECM is the first barrier of tumor invasion and its dissolution promotes tumor invasion and metastasis. Tumor cells must break through the ECM to metastasize. Integrin binds to matrix metalloproteinase (MMP) and positively regulates its expression. The high expression of MMP directly destroys and degrades the ECM, thus promoting the metastasis of tumor cells[172]. In addition, studies have shown that endothelial cells $\alpha_v\beta_3$ integrin can target MMP-2 and then adhere to the newly

synthesized MMP-2. Studies have found that the inhibition of $\alpha_v\beta_3$ complex by antibodies and peptides can block the invasion and metastasis of lung cancer[170]. Cathepsin D is one of the proteases secreted by tumor cells and mesenchymal cells, which promotes tumor invasion and metastasis by degrading the basement membrane. Tumor cell cathepsin D is associated with the invasion and metastasis of ovarian cancer, melanoma, and breast cancer and is related to the growth of tumor cells in distant metastases[173].

1.4.2.3. Integrin regulation of cell survival and apoptosis

Apoptosis is the process of active cell death, and the loss of the mechanism of apoptotic signal activation is the key to tumor formation. ECM removal forces the cells to enter apoptosis. As integrins specifically bind the cell surface to matrix proteins, thus they can protect from apoptosis. Monoclonal antibody against integrin β_1 disrupted the interaction of mammary epithelial cells CID-9 with ECM, resulting in apoptosis[174].

ECM not only regulates cell growth and differentiation but is also a survival factor for anchoring dependent cells[175]. When tumor cells leave the primary tumor and enter the blood lymphatic circulation, they must change from anchored to suspend. Anchored dependent mechanisms prevent tumor cells from entering the circulation, thereby inhibiting metastasis. Monoclonal antibodies against integrin $\alpha_5\beta_3$ that block the contact between human melanoma cells and collagen I, can induce apoptosis in melanoma cells[176]. Chinese hamster ovary (CHO) cells expressing $\alpha_5\beta_1$ can survive in the medium containing fibronectin without the addition of serum. $\alpha_v\beta_1$ is also a receptor for fibronectin and has the same ligand binding site as $\alpha_5\beta_1$, but CHO cells expressing $\alpha_v\beta_1$ undergo apoptosis under these conditions. Studies showed that cells adhere to fibronectin via $\alpha_5\beta_1$ and that the intracellular domain of α_5 triggers Bcl-2 and activates Bcl-2 channels, thereby inhibiting apoptosis[177]. In addition, aberrant binding of integrin to ligands can cause cell shedding and apoptosis by focal adhesion kinase (FAK), phosphatidylinositol 3-kinase/ Protein kinase B (PI3K/Akt), Mitogen-activated protein kinase (MAPK), stress-activated kinases / c-Jun N-terminal kinases (SAPK/JNK) signaling pathways. The destruction of the contact between tumor cells and stroma

could produce an activation signal. The generation of this activation signal can cause apoptosis, and the sensor of this activation signal is $\alpha_v\beta_3$[178]. Depletion of integrin α_v separates cells from ECM, thereby mediating apoptosis[179]. This integrin-regulated form of cell apoptosis is called "integrin-mediated death"[180]. Expression of integrin $\alpha_v\beta_3$ on cells grown on matrix lacking integrin ligands, such as collagen gelatin, induce caspase-8 to reappear on the cell membrane, thereby activating caspase and causing cell death[180]. Studies have shown that the mechanism by which the tumor suppressor gene pl6 can induce apoptosis in tumor cells by down-regulating $\alpha_5\beta_1$, inhibiting the activity of cyclin-dependent kinase complexes, and completing the phosphorylation of the matrix necessary for G phase[181].

1.4.2.4. The role of integrins on tumor angiogenesis

Both primary tumors and secondary metastatic tumors rely on angiogenesis in the process of growth and spread[182]. Angiogenesis not only promotes the growth of primary tumors but also increases the chance of cancer cells entering the bloodstream and promotes tumor metastasis. Multiple cytokines mediate endothelial cell proliferation and migration via integrins such as $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$ to promote tumor angiogenesis[183]. Integrins have low expressions in resting endothelial cells but have higher expressions in physiological and pathological angiogenesis, including in ischemic tissues and tumor vascularization[184]. Therefore, integrins are potential targets for blocking tumor angiogenesis in cancer therapy.

Integrins, expressed in the lumen and luminal surface of vascular endothelial cells, can mediate the migration of endothelial cells and the formation of capillary lumens[185]. Among them, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ have the most important roles. Studies have shown that the $\alpha_v\beta_3$ expression is increased in the angiogenesis of rabbit cornea, granulation tissue at human wounds, and chicken chorioallantoic allantoic. Immunohistochemical analysis also found that $\alpha_v\beta_3$ increased the expression during angiogenesis in many cancers[186]. Developmental endothelial locus-1 (Del-1) is a newly discovered ECM, which is a ligand of $\alpha_v\beta_3$ and is associated with tumor metastasis. Del-1 promotes endothelial cell connection and migration, and this effect is blocked with anti-$\alpha_v\beta_3$ antibody[187]. Del-1

overexpressing tumor xenografts show a significantly higher blood vessel density and increased tumor growth rate compared to the control group[188]. It is suggested that tumor tissue may promote the formation of tumor blood vessels by secreting Del-1 and binding to $\alpha_v\beta_3$ receptor. If the binding of $\alpha_v\beta_3$ and ECM is blocked, vascular endothelial cells undergo apoptosis, angiogenesis decreases, and tumor metastasis decreases. Humanized monoclonal antibodies against $\alpha_v\beta_3$, such as etaracizumab (MEDI-522), significantly inhibit angiogenesis[189].

The α_6 subunit is associated with angiogenesis, while monoclonal anti-α_6 antibodies can eliminate angiogenesis[190]. In metastatic prostate cancer cells, although the adhesion between tumor cells is weakened, $\alpha_5\beta_1$ integrin shows high expression and positively correlates with VEGF and VEGFR, suggesting that it may be closely related to angiogenesis and metastasis[191]. Indeed, $\alpha_5\beta_1$ and its ligand fibronectin synergistically upregulate the growth of blood vessels, leading to tumor formation while $\alpha_5\beta_1$ inhibitors block tumor angiogenesis, leading to apoptosis of human tumor cells in animal models[192].

1.4.2.5. Interaction of integrin with growth factors and cytokines

Interactions between integrin and growth factor or cytokine receptors play a crucial part in the process of tumor progression. Their interactions not only regulate tumor cell adhesion, invasive metastasis, and apoptosis but also affect host cell responses to cancer cells during angiogenesis (Figure 1.5).

Crosstalk may be mediated by integrin-growth factor receptor complex[193-196], enhancing the activation of downstream kinases such as MAPK[197] or Akt[198], thereby enhancing cell migration and survival. These crosstalks mutually regulate each other's expression[199-208], or the release of their respective ligands where these interactions are important for both tumor metastasis and drug resistance[209,210]. However, not all crosstalks are pro-tumorigenic[211].

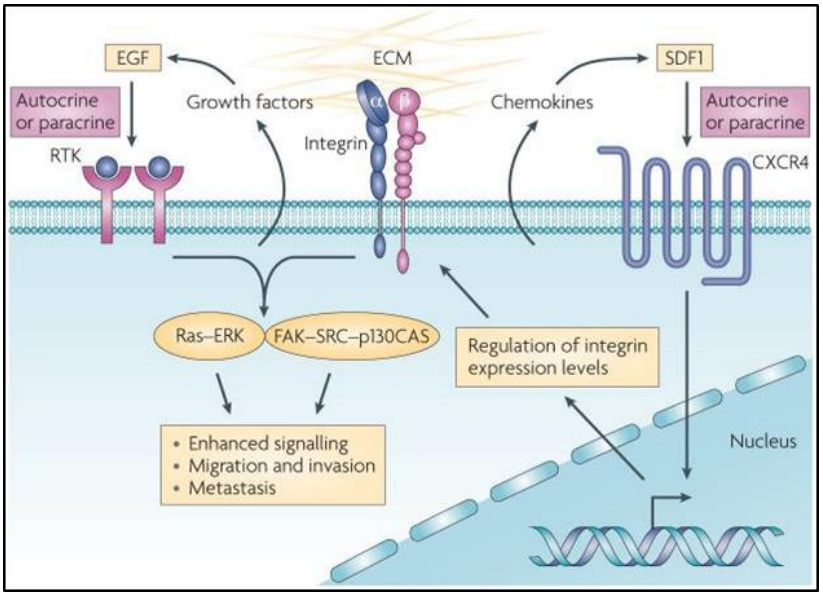

Figure 1.5: The interaction of integrin–growth factor and integrin–cytokine receptor. Growth factors and cytokines are directly affected by integrin ligation leading to an increase in their secretion, with secreted factors further inducing signaling by binding to their receptors in an autocrine or paracrine manner. Image obtained from *Nature Reviews Cancer, 10 (2010), pp 9.*

EGF and receptors

EGFR and ERBB2, members of the epidermal growth factor (EGF) receptor family, can promote tumorigenesis and metastasis in solid tumors such as breast and pancreatic cancers by cooperation with integrins. In breast cancer patients with high expression of ERBB2, $\alpha_6\beta_4$ is essential for tumorigenesis, and ERBB2 synergizes with $\alpha_6\beta_4$ to induce tumorigenesis and cell invasion. The mechanism of this action may be due to the formation of the $\alpha_6\beta_4$-ERBB2 complex that enhances the activity of signal transducer and transcription activator 3 (STAT3), thus leading to loss of cell polarity and increased proliferation. Furthermore, β_4 loss can enhance the efficacy of ERBB2-targeting therapies, antagonists of integrin and EGF receptor family members for combination therapy[212]. In pancreatic cancer, a highly aggressive disease, over-activated EGF leads to an increased ability of tumor cells to migrate and metastasize. EGF-$\alpha_v\beta_5$ stimulates migration and metastasis of pancreatic tumor cells via vitronectin[213-215]. The ability of $\alpha_v\beta_5$ to mediate cell migration requires EGF-dependent activation of steroid receptor coactivator (Src)[215]. EGF-integrin interaction happens in others cancers. For examples, EGF interacts with $\alpha_3\beta_1$ and $\alpha_6\beta_4$ to promote cell migration in colon cancer[216]; EGF

interacts with $\alpha_1\beta_1$ and $\alpha_2\beta_1$ to promote cell migration in hepatocellular carcinoma[217]. Thus, EGF signaling in tumor cells may enhance the ability of specific integrins to mediate cell migration and survival.

Integrins themselves can also modulate EGF signaling, severely affecting the sensitivity of tumor cells to therapy. Integrin can directly induce the phosphorylation of EGFR, thereby activating MAPK via the Src-p130CAS pathway[218] and promoting the proliferation and survival of tumor cells[219].

HGF and receptors

Hepatocyte growth factor receptor (HGFR) plays a central role in angiogenesis, tumorigenesis, and tissue regeneration[220]. Interaction of β_4 with HGFR enhances its oncogenic potential and promotes fibroblast transformation[221]. In breast cancer cells, HGF binding to HGFR leads to the recruitment of proteins and activation of Src and ERK by inducing phosphorylation of β_4[222]. HGFR- $\alpha_6\beta_4$ complex potentiates HGF-induced Ras and PI3K signaling[196]. Integrins of the α_v family promote HGFR signaling by regulating the expression of genes required for HGF-induced cell migration[223]. The metastasis suppressor tetraspanin CD28/KAI1 suppresses tumor cell invasion by inhibiting integrin-HGFR crosstalk[224].

TGF-β1 and receptors

TGF-β1 is highly expressed in breast cancer and other malignant cells[225]. TGF-β1 is a key regulator during the EMT of cancer metastasis and fibrosis[226]. Integrins play a role in the activation of TGF-β signaling. TGF-β is deposited in the ECM in an inactive form and binds to LAP to form TGF-β-binding proteins (LTBP)[227]. When $\alpha_v\beta_3$, $\alpha_v\beta_6$, or $\alpha_v\beta_8$ binds to the RGD motif within LAP of pro-TGF-β1, a conformational change occurs in the LAP-TGF-β-LTBP1 complex that releases TGF-β1 enabling it to bind to its receptor[228,229]. TGF-β1 activates $\alpha_v\beta_3$, $\alpha_v\beta_6$, and ECM protein ligands, which in turn trigger PI3K, Akt, and nuclear factor-κB (NF-κB) pathways[230-232]. $\alpha_v\beta_3$ activates Src-dependent phosphorylation of type 2 TGF-β receptor (TGFBR2) in concert with TGF-β to induce the EMT process in breast epithelial cells[233].

VEGF, FGF and their receptors

FGFR synergizes with $\alpha_v\beta_3$ to inhibit apoptosis through PAK-induced Raf phosphorylation[234,235,236]. In addition, VEGFR2 synergizes with $\alpha_v\beta_s$ to inhibit apoptosis which induced by inflammatory mediators such as tumor necrosis factor (TNF) through Src-dependent Raf phosphorylation[234,235]. Thus, integrin activation of inactive Raf targeting to tumor vasculature effectively inhibits tumor angiogenesis *in vivo* [237].

VEGF activates integrins like $\alpha_v\beta_3$[238]. Activation of $\alpha_v\beta_3$ increases VEGF secretion from tumor cells, leading to tumor vascularization and growth[210]. Targeted loss of the signaling portion of the β_4 cytoplasmic domain inhibits FGF-mediated angiogenesis and tumor growth[239]. These findings reveal that integrin-growth factor receptor-mediated signaling is critical for tumor angiogenesis.

1.4.3. Integrins as targets for cancer therapy

Integrin can recognize a variety of ECM proteins and receptor proteins on the cell surface, and its ability to bind to a variety of growth factor receptors makes it a target for cancer treatment and diagnosis[240]. The high expression of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ in tumor cells and angiogenic endothelial cells makes them therapeutic targets for the inhibition of angiogenesis and tumor growth[241-244]. Studies have shown that integrin antagonists can inhibit tumor growth by inhibiting the growth of tumor cells and tumor vascular endothelial cells.

The earliest integrin antagonist developed was Avastin (LM609), a monoclonal antibody (mAb) against $\alpha_v\beta_3$ that blocks angiogenesis. Etaracizumab (MEDI-522) is a humanized mAb version that not only has anti-angiogenic effects but also inhibits tumor growth by acting directly on tumor cells[245,246]. The current clinical phase I and II trials of etaracizumab have shown anti-angiogenic activity, low toxicity, and stability in patients with solid tumors, such as renal cell carcinoma and metastatic melanoma[247-249]. CNTO 95, an α_v-integrin-specific monoclonal antibody, also has anti-tumor and anti-angiogenic effects[250,251]. CNTO 95 was shown to have non-toxic and anti-tumor

activity in phase I clinical trials[252]. Antibodies against integrin β_1 inhibit the growth of breast cancer cells *in vitro* and *in vivo*[253]. Volociximab, a function-blocking monoclonal antibody against $\alpha_5\beta_1$[254,255], can inhibit angiogenesis and suppress tumor growth in phase I-II trials in patients with solid tumors[256,257]. The monoclonal antibody 6.3G9 against integrin $\alpha_v\beta_6$ blocks the growth of human pharyngeal cancer cells both *in vitro* and *in vivo* by a TGF-β regulated mechanism[258].

RGD is a cell adhesion motif expressed on many ECM and plasma proteins[259]. Among the known human integrin subtypes, only eight integrins recognize the RGD base sequence of ECM proteins, i.e., $\alpha_v\beta_1$, $\alpha_v\beta_3$,$\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_v\beta_8$, $\alpha_5\beta_1$, $\alpha_8\beta_1$, and $\alpha_{IIb}\beta_3$[260]. Many glycoproteins such as vitronectin, laminin, osteopontin and fibrinogen belong to RGD adhesion proteins which are found in the ECM[261]. RGD is a cellular recognition and attachment site for many ECM proteins as well as blood and cell surface proteins[262]. As integrins are involved in cell adhesion, cellular differentiation, migration, RGD peptides are widely used for targeted therapy of tumors. These include RGD peptides and RGD-modified targeting therapies. The inhibitory effect of cyclic RGD peptides on α_v-integrins ($\alpha_v\beta_3$, $\alpha_v\beta_5$) leads to a decrease in functional tumor vascular density, thereby inhibiting tumor growth and metastasis. RGD peptides can completely inhibit the pro-angiogenic effect of early VEGF, even at low doses[263]. Phase II/III trials showed prolonged survival with minimal side effects in patients with advanced glioblastoma treated with the RGD containing pentapeptide cilengitide that targets $\alpha_v\beta_3$ and $\alpha_v\beta_5$[264]. The non-RGD peptide ATN-161 can inhibit integrin $\alpha_5\beta_1$, which block the growth and metastasis of breast cancer *in vivo*[265]. Besides, the combination of ATN-161 and fluorouracil significantly inhibited the tumor progression and liver metastasis[266,267].

Since the discovery of RGD motifs as potent ligands for cell surface integrins, RGD-mediated chemotherapeutic drugs, lysing viruses and RGD-containing therapeutic peptides and proteins have been modified for tumor cell targeting and control of drug biodistribution *in vivo*[268] (Table 2). RGD peptides are covalently conjugated with non-viral gene vectors (Polymer, Lipid, Liposomes, Peptides) in various combinations to prepare DNA complexes, and then these compounds can be used for plasmid DNA and other short interfering RNAs (siRNAs). Integrin $\alpha_v\beta_3$

nanoparticles were successfully used for the targeted delivery to tumor blood vessels of a mutant RAF1 gene that hampers growth factor signaling, resulting in apoptosis of tumor cells and vascular endothelial cells[237]. In addition, viruses act as effective vectors for redirecting interfering genes into tumor cells or tumor neovascularization by introducing additional RGD-peptides into the adenovirus. Similarly, targeted nanoparticles containing adriamycin effectively inhibited tumor growth and metastasis when acting on $\alpha_v\beta_3$-positive tumors. Importantly, compared to systemic administration, this targeted delivery method not only significantly reduced the toxic side effects of the drug but also increased the efficiency of the drug up to 15-fold[269]. More importantly, these novel RGD-mediated drugs enhance tumor targeting by binding to integral protein receptors and controlled release the free drugs in weakly acidic lysosomes[270]. Therefore, RGD conjugated nanodrugs can be used as effective drugs or gene-delivered nanoparticles to achieve the therapeutic treatment of cancers.

Table 2.RGD-Modified Gene Carriers and Drugs for Target Therapy

	Production	Gene/drug	Experimental
RGD modified non-viral	RGD-PEG-PCL	siRNA	B16F10-luc2 lung metastatic[271]
	RGD-PEG	PEG-PEI/DNA complexes	NIH3T3 cells[272]
	RGD-PEG-PEI	Plasmid DNA	Intracranial glioblastoma[273]
	RGD-PEG-Suc	Plasmid DNA coding luciferase	Melanoma cell[274]
	Polymerized lipid nanoparticle	ATP-Raf	M21-L/CT26 colon carcinoma[237]
	RGD-SSL-DOX liposomes	RGD-Lipo-siRNA(MDR1)	Breast cancer MCF7/A cells[275]
	RGD-PEGylated liposomes	siRNA	Pigment epithelial cells[276]
	RGDGWK-lipopeptide	Anti-cancer p53 gene	B16F10 tumor[277]
	RGD-HK-branched peptides	siLacZ, siLuciferase	MDA-MB-435c, MCF7[278]
RGD modified viral	RGD4C/AAV/phage hybrid	GFP or Luc reporter genes	Human M21 Melanoma Human U87 glioblastoma[279]
	Ad/CD-PEG500-RGD	shRNA	Various cancer cells[280]
	(Adenovirus) Ad-RGD	Firefly luciferase gene	Mouse TS cells[281]
RGD modified drugs	PTX-RGD/Tf-NPs (nanoparticles)	Paclitaxel	HeLa cells[282]
	RGD-PEG-PAMAM-DOX	Doxorubicin	C6 glioma cells[283]
	RGD-CA-4 and Dox liposomes	Combretastatin A-4 (CA-4) and doxorubicin	B16 and B16F10 melanoma cells[284]
	RGD-PEG-LP-DC	Docetaxel	BT-20 and MDA-MB-231 cells[285]
	RGD-fibulin-5	Fibulin-5	A549, H1299 and H460 cells[286]
	(RGD)3/tTF	tTf	H460 lung cancer cells[287]
	RGD-Mediated angiogenic factor	The angiogenic factor Del1	Human umbilical vein endothelial cells[288]
	RGD-containing Osteopontin	Osteopontin	Avian osteoclast-like cells[289]

1.4.4. Integrin as targets for imaging

Integrins can be specifically targeted to tumor cells when combined with diagnostic agents. Integrin antagonists in combination with paramagnetic contrast agent[290] or radionuclides[291] are effective in detecting tumors and their vascularization in tumor models[292]. It has been shown that detection of xenograft tumors such as breast cancer,

brain tumors, and lung cancer can likewise be targeted to tumor cells using RGD peptides labeled with ^{64}Cu, ^{18}F, and ultra-small superparamagnetic iron oxide particles[293-295]. Recently, some integrin-targeted peptide contrast agents have been evaluated in cancer patients. Among them, scintigraphy using radiolabeled $\alpha_v\beta_3$-targeted peptide (^{99m}Tc-NC100692) in breast cancer patients detected a high percentage of malignant tumors[296,297]. In addition, $\alpha_v\beta_3$ expression in human tumors could be quantitatively assessed non-invasive positron emission tomography (PET) using ^{18}F-galacto-RGD[298]. In summary, labeled integrin antagonists can be used as an important tool for tumor diagnosis and can enable early diagnosis of tumors.

1.5. Integrins pituitary tumors

Integrins trigger different cellular responses by participating in the ECM signaling process[299]. During ECM signaling, integrins activate multiple downstream effectors in parallel through a complex signaling integration system[300]. Integrin signaling involves changes in the GTPase and kinase pathways[301]. For example, fibronectin regulate Rac small GTPases and determine fibroblast morphology through an integrin-mediated signaling system. The expression and distribution of laminin isoforms and fibronectin isoforms differ from normal pituitary and pituitary adenomas. In pituitary adenomas, fibronectin is involved in adenoma angiogenesis and is specifically associated with endothelial cells and vascular smooth muscle cells[302]. Laminin is involved in the early stages of pituitary development and is expressed in both the basement membrane and follicular stellate cells[303,304]. Besides, laminin regulates the release of prolactin and gonadotropins in the normal pituitary gland[305]. During the development of early prolactinomas, Laminin inhibits prolactin secretion and cell proliferation[306]. Collagen IV has a high expression in the pituitary gland but the expression value is reduced by MMP-9 specifically, allowing the migration and invasion of pituitary tumor cells. Therefore, collagen IV is considered as an important factor in the progression and invasion of pituitary adenomas[307]. Collagen IV has also been shown to regulate the release of prolactin[308]. High expression of MMPs have been found in human pituitary

adenomas[309]. MMPs are associated with tumor invasion and metastasis[310]. The active form of MMP-2 is found only in some pituitary tumors, whereas it is expressed as the inactive form of the enzyme (pro-MMPs) in the normal pituitary gland[311]. MMPs activity in pituitary cells anchors factors on the extracellular matrix, which subsequently controls pituitary cells. The high expression of MMPs in pituitary adenomas in relation to cell proliferation and hormone secretion, makes them as pharmacological inhibitors[312].

In pituitary tumors, α_v and β_3 subunits are expressed in tumor cells, but also highly stained in tumor cell-matrix, while α_1, α_5, and β_1 were found in tumor endothelial cells[313]. During pituitary adenoma development, the transformation of pituitary adenoma cells is associated with altered β_1-integrin expression[313]. Except for α_3 co-expression with β_1, α-integrins do not appear to be involved in pituitary tumorigenesis and therefore may deliver the signals by β_1-activation[313].

Laminin, collagen I, and collagen IV inhibit ACTH biosynthesis at the level of *POMC* gene transcription in the corticotroph tumor cells line AtT-20, but in dispersed rat anterior pituitary cells[314]. This difference in the regulation of ACTH secretion may be related to the different integrin expression in normal and pituitary adenoma cells. Interestingly, the anti-β_1 integrin activating antibody also stimulated AtT-20 cell proliferation, which is consistent with the stimulation produced by fibronectin[314]. Overexpression of the integrin $\alpha_v\beta_5$ ligand thrombospondin-1 (TSP-1) inhibits *POMC* transcription and ACTH secretion in AtT20 corticotroph tumor cells. Overexpression of TSP-1 in pituitary tumor cells inhibits cell proliferation, migration, and invasion[315].

Laminin and collagen I inhibit, fibronectin and collagen IV stimulat lactotroph cell proliferation[306]. Because fibronectin already produces maximal stimulation via the β_1-integrin-activated Rho pathway, cell proliferation cannot be further stimulated in the presence of fibronectin. In follicle-like cell proliferation in the anterior pituitary, laminin is involved in the Caveolin 3-activated integrin β_1 signaling pathway and subsequently the MAPK pathway[316]. Again, this confirms that β_1-integrins play a role in pituitary adenomas in regulating cell proliferation. Furthermore, the paracrine and autocrine factor TGF-β1 of the pituitary gland can regulate the expression of individual

integrin subunits in other cell types[317] and may be involved in pituitary tumorigenesis[318], which may be related to changes in integrin expression and its regulation of proliferation in pituitary adenomas. Finally, an RGD with high affinity and specificity for $\alpha_v\beta_3$ conjugated with the pro-apoptotic Fas ligand to induce apoptosis in pituitary tumor cells, highlighting the therapeutic potential of integrins also for pituitary neoplasms[319].

1.6. Aim of the study

There is an evidence for the role of ECM in the pathophysiology of corticotroph tumors. Metalloproteinase inhibitors suppressed ACTH secretion and cell proliferation, indicating a regulatory role of factors anchored to ECM on corticotroph function[309].

ECM components bind to integrins, but there is little information on the expression of integrins in corticotroph tumors. The present study aims to explore the expression and function of RGD-bound integrins in human corticotroph tumors compared to hormone inactive pituitary tumors, and also to test the efficacy of RGD peptides in an *in vitro* corticotroph tumor cell model.

2. Materials and Methods

2.1. Laboratory equipment

Item	Model	Manufacturer
Automated Cell Counter	TC20	Bio-Rad
Autoclave	VX-75	Systec
Benchtop centrifuge	5424R	Eppendorf
Benchtop centrifuge	5804	Eppendorf
Biological safety cabinet	Safe 2020 Class II	Thermo
CO2 incubator	BBD 6220	Thermo
Centrifuge/Vortex	FVL-2400	PeqLab
Electrophoresis chamber	Mini-Sub Cell GT Cell	Bio-Rad
Electrophoresis Apparatus	200/2.0 power supply	Bio-Rad
Fluorescence microscopy	DM 2500	Leica
Ice machine	QM20AC	Manitowoc
MiniPlateSpinner Centrifuge	230EU	Axygen
Multimode Plate Reader	VICTOR X4	PerkinElmer
Microplate Reader	iMark	Bio-Rad
Microscope	TMS	Nikon
Microwaves	iQ100	Siemens
Precision balance	440-45	KERN
Pipettes	Research® plus	Eppendorf
Rocker-Shaker	MR-12	Biosan
Real-time PCR System	Mx3000P	StrataGene
Spectrophotometer NanoDrop	ND-1000	PeqLab
Thermal cycler	T100	Bio-Rad
Thermomixer	F1.5	Eppendorf
UV-transilluminator	GEL iX20	INTAS
Vortex mixer	Vortex-Genie 2	Scientific Industries
Water bath	TSGP20	Thermo

2.2. Chemicals and reagents

Chemicals and Reagents	Manufacturer
Beetle-Juice Luciferase Assay Firefly	P.J.K. GmbH
Chloroform	Merck KGaA
Cell Proliferation Reagent WST-1 (Roche)	Sigma
C(RGDyK)(RK-5)	GL Biochem Ltd
Cyanin 5.5 NHS Ester, 5mg	Lumiprobe GmbH
Dulbecco's modified Eagle medium	Invitrogen,Gibco™
EDTA	Sigma
Ethanol 100%	Sigma
Ethanol 75%	Sigma
FBS	Invitrogen
GoTaq G2 MasterMix Polymerase HotStart	Promega
GelRed Nucleic Acid Stalt	Biotium
Isopropanol	Sigma-Aldrich
ONPG	Sigma
Opti-MEM, 500ml	Invitrogen
O'GeneRuler Express DNA ladder	Thermo
Passive Lysis 5X Buffer	Promega
PeqGOLD Universal Agarose 500g	PeqLab
Paraformaldehyde 4% (PFA)	Microcos GmbH
QuantiTect RT, 200x	Qiagen
QuantiTect Reverse Transcription Kit	Qiagen
Sso Fast Eva Green, 500 x 20µl	Bio-Rad
SuperFect Transf. 4x1,2ml	Qiagen
Trizol Reagent 100ml 2x	Thermofisher
Trypsin-EDTA, 0,05%	MWG
Tris-Borate-EDTA buffer(10X)	Sigma
Trypan Blue, 0,4%, 1ml	Invitrogen

2.3. Oligonucleotides (primers)

Primers for Real-Time PCR (mouse integrin targets)		
Target	**Sequence (5'-3')**	**Product size (bp)**
Itgav (α_v)	F:CCGTGGACTTCTTCGAGCC	162
	R:CTGTTGAATCAAACTCAATGGGC	
Itga5(α_5)	F:CTTCTCCGTGGAGTTTTACCG	163
	R:GCTGTCAAATTGAATGGTGGTG	
Itga8(α_8)	F:CGAAGCCGAACTCTTTGTTATCA	78
	R:GGCCTCAGTCCCTTGTTGT	
ItgaIIb	F:TTCTTGGGTCCTAGTGCTGTT	133
(α_{IIb})	R:CGCTTCCATGTTTGTCCTTATGA	
Itgβ1(β_1)	F:ATGCCAAATCTTGCGGAGAAT	209
	R:TTTGCTGCGATTGGTGACATT	
Itgβ3(β_3)	F:CCACACGAGGCGTGAACTC	107
	R:CTTCAGGTTACATCGGGGTGA	
Itgβ5(β_5)	F:GAAGTGCCACCTCGTGTGAA	86
	R:GGACCGTGGATTGCCAAAGT	
Itgβ6(β_6)	F:CAACTATCGGCCAACTCATTGA	186
	R:GCAGTTCTTCATAAGCGGAGAT	
Itgβ8(β_8)	F:AGTGAACACAATAGATGTGGCTC	115
	R:TTCCTGATCCACCTGAAACAAAA	
Gapdh	F:AGGTCGGTGTGAACGGATTTG	123
	R:TGTAGACCATGTAGTTGAGGTCA	

Primers for Real-Time PCR (human integrin targets)		
Target	**Sequence (5'-3')**	**Product size (bp)**
ITGAV (α_v)	F:GCTGTCGGAGATTTCAATGGT	136
	R:TCTGCTCGCCAGTAAAATTGT	
ITGA5(α_5)	F:GGCTTCAACTTAGACGCGGAG	140
	R:TGGCTGGTATTAGCCTTGGGT	
ITGA8(α_8)	F:GCAGATACCGTTTGACACCAC	237
	R:GGAGAGAACTCGGCATAGGC	
ITGAIIB	F:ACAAGCGTTACTGTGAAGCG	102
(α_{IIb})	R:GGGCCAGGAGACCTAAGAAATAA	
ITGB1(β_1)	F:CAAGAGAGCTGAAGACTATCCCA	137
	R:TGAAGTCCGAAGTAATCCTCCT	
ITGB3 (β_3)	F:AGTAACCTGCGGATTGGCTTC	164
	R:GTCACCTGGTCAGTTAGCGT	
ITGB5 (β_5)	F:GGAAGTTCGGAAACAGAGGGT	106
	R:CTTTCGCCAGCCAATCTTCTC	
ITGB6 (β_6)	F:CTCAACACAATAAAGGAGCTGGG	110
	R:AAAGGGGATACAGGTTTTTCCAC	
ITGB8 (β_8)	F:ACCAGGAGAAGTGTCTATCCAG	204
	R:CCAAGACGAAAGTCACGGGA	
GAPDH	F:GGAGCGAGATCCCTCCAAAAT	197
	R:GGCTGTTGTCATACTTCTCATGG	

2.4. Plasmid constructs

Expression Vectors and Reporter Constructs	
Vector	**Source**
Human *POMC* promoter luciferase reporter vector (humam *POMC*-luc)	Panomics
pSV-β-Galactosidase Control Vector	Promega

2.5. siRNA for integrins.

Name	Type	Product Number	Company
Integrin α_v	siRNA	sc-35694	Santa Cruz Biotech
Integrin β_1	siRNA	sc-35675	Santa Cruz Biotech
Integrin β_5	siRNA	sc-35681	Santa Cruz Biotech
Integrin β_8	siRNA	sc-43138	Santa Cruz Biotech

2.6. Human pituitary samples

This part of the study was approved by the ethics committee of the LMU Klinikum (no. 152-10).

The expression of genes encoding for RGD-binding integrin subunits was investigated in 18 corticotrophs (Cushing's disease) and 16 gonadotrophs (hormone inactive) tumors. Diagnosis for Cushing's disease was done according to the current guidelines[320]. In brief, diagnosis includes lack of response to the 0.75-1.5mg overnight dexamethasone suppression test, elevated 24 hours UFC, midnight salivary cortisol, baseline ACTH >20 pg/mL. In addition, final diagnosis was based on characteristic responses to 2 days high-dose (2mg/6 hours, 8times) overnight dexamethasone suppression testing and CRH stimulation test (1μg/kg or 100μg h-/o- i.v.) or inferior petrosal sinus sampling. The presence of corticotroph tumor was confirmed by neuropathology.

The patients' surgical approach were transsphenoidal microsurgy. Clinical manifestations, imaging, endocrinology, surgical and biochemical findings were used to make a comprehensive diagnosis of the tumor. The most commonly used is the Knosp five-level classification of pituitary adenomas: three lines（medial tangent，intercarotid line，lateral tangent）are drawn between supraclinoid internal carotid artery（ICA） and intracavernous ICA on MRI in the coronal position of the cavernous sinus to determine the relationship of the pituitary adenoma to the cavernous sinus. According to Knosp classification, pituitary tumors are divided in 4 grades: grade 0（Normal）= tumor within the medial tangent; grade 1 = tumor extends beyond the medial tangent

but not beyond the intercarotid line; grade 2 = tumor extends beyond intercarotid line,but not beyond the lateral tangent; grade 3 = tumor extends beyond the lateral tangent(3A: over ICA,3B:under ICA); grade 4 = total encasement of theintracavernous ICA[321]. After surgical resection tumors were put in DMEM supplemented with 10%FBS, 500 μg/L partricin and 10^5 U/L penicillin/streptomycin and were thus transported to the laboratory for processing. After washing with HDB+ buffer, fibers and debris were removed and the tumor was snap-frozen with dry ice and stored in -80°C for RNA extraction. Two normal human pituitary glands were obtained at autopsy after sudden death from subjects with no evidence of endocrine diseases, no more than 12h after demise.

Table 3. Clinical information of tumor specimens

Case Nr.	Clinical diagnosis	Age	Gender	Tumor grade (Knosp)	Invasive
001	Cushing's disease	76	F	4	no
005	Cushing's disease	61	F	4	yes
013	Cushing's disease	55	M	0	yes
040	Cushing's disease	31	F	0	no
042	Cushing's disease	80	M	4	yes
043	Cushing's disease	49	F	3	no
046	Cushing's disease	29	F	0	no
047	Hormone inactive	70	F	0	yes
054	Cushing's disease	55	F	1	no
055	Cushing's disease	43	M	0	no
060	Hormone inactive	70	F	3	yes
067	Hormone inactive	46	M	2	no
077	Cushing's disease	29	M	1	no
078	Cushing's disease	22	F	0	no
085	Cushing's disease	56	F	1	no
089	Cushing's disease	28	F	0	no
091	Cushing's disease	40	F	0	no
093	Hormone inactive	74	M	2	no
110	Hormone inactive	58	F	2	no
117	Cushing's disease	53	F	1	no
118	Cushing's disease	65	F	1	no
119	Hormone inactive	60	F	3	yes
122	Hormone inactive	76	M	2	no
130	Hormone inactive	73	M	3	yes
135	Hormone inactive	69	F	2	no
138	Hormone inactive	51	M	0	no
140	Hormone inactive	69	M	1	no
147	Hormone inactive	44	M	3	yes
156	Hormone inactive	83	M	2	yes
160	Hormone inactive	72	F	1	yes
163	Hormone inactive	44	F	0	yes
174	Cushing's disease	56	M	3B	yes
181	Hormone inactive	35	M	0	yes
191	Cushing's disease	21	F	0	no

2.7. Methodology

2.7.1. Cell culture

Murine pituitary corticotroph tumor AtT-20 cell line (American Type Culture Collection (ATCC®) CRL-1795™) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum(FBS) and 1% penicillin/streptomycin. Cell culture conditions were 95% air, 5% CO2, Temperature: 37°C, incubator humidity of 70%-80%; Cell transfer: cell density of 70%-80%.

2.7.2. Reverse-transcriptase polymerase chain reaction (RT-PCR)

RNA extraction

RNA was extracted using Trizol®, which contains others guanidine isothiocyanate and phenol that rapidly break down cells while blocking the release of nucleases. For RNA extraction from pituitary tissues, 1ml of Trizol was added per 50-100mg of tissue and the tissue was homogenized using Ultra Turrax. For RNA extraction from cells, the cell medium was discarded and 1ml Trizol was added (per $3x10^5$-10^7 cells).

After adding Trizol, the samples were kept at room temperature for 5 minutes. Then the samples were collected into RNase-free tubes and 0.2 ml of chloroform was added for every 1 ml of Trizol used. Samples were vortexed for 15 seconds and left at room temperature for 3 minutes before being centrifuged at 4 °C at 12,000 rpm for 15 minutes. After centrifugation, each sample is divided into three layers: the red organic phase (contains proteins), middle zone (contains DNA), and upper layer of the colorless aqueous phase, which contains the RNA and is carefully pipetted out and transferred to the new Eppendorf tube. This process was repeated once. After adding 500µl isopropanol, the sample was mixed upside down and incubated at room temperature for 10min, before being centrifuged at 12,000 rpm at 4 °C for 10 minutes. The supernatant was discarded and the RNA precipitate was washed with 1 ml 75% ethanol, was shaked for 15 seconds on a vortex shaker and centrifuged at 7500 rpm at 4 °C for 5 minutes. The supernatant was carefully discarded with a pipette taking care not to aspirate the precipitate. This step was repeated once. Samples were left to air dry for 10 minutes at

room temperature, taking care to not over dry, as the RNA is difficult to dissolve after it is completely dry. RNA pellets were dissolved in 20-50µl of RNase-free water. The purity and concentration of RNA were determined with the NanoDrop spectrophotometer.

Reverse-transcription DNA (cDNA)

Extracted mRNA was reverse transcribed using the Qiagen QuantiTect Reverse Transcriptase Kit: add the following reactants together to a sterilized RNase-free 0.2ml tube, (1) template RNA: total RNA 0.5-1µg; (2) gDNA wipeout: 2µl; (3) RNase-free ddH2O: fix to 14µl. Samples were gently mixed and centrifuged for 30 seconds before being incubated in a thermal cycler at 42° for 2 minutes. A mastermix containing 4µl of reverse transcriptase buffer, 1µl of primer mixture, and 1µl of reverse transcriptase was then added to each sample. Samples were mixed in a reaction volume of 20µl and centrifuged for 30 seconds before being incubated in a thermal cycler at 42° for 15 minutes and at 95° for 3 minutes. The reverse-transcribed cDNA samples were stored at -20°C.

Polymerase chain reaction (PCR)

PCR was operated with 1µl cDNA sample (diluted 1:10) and a mix of 10µl GoTaq G2 Master Mix, 1µl forward primer (10pmol/µl),1µl reverse primer (10 pmol/µl) and 7µl RNase-free water. PCR was carried out using a thermal cycler. In standard reactions, the three-temperature point method is used, with the PCR reaction system: double-stranded DNA is denatured at 95°C for 2 minutes, annealed at 95°C for 15 seconds, rapidly cooled to 60°C for 15 seconds, and then extended at 72°C for 3 minutes. The entire reaction system has 34 cycles.

PCR products are then analyzed by agarose gel electrophoresis. Prepare 1% agarose gels for electrophoresis according to the molecular weight of the nucleic acid. Prepare 100 ml of TBE buffer (1X) electrophoresis buffer in a triangular flask, weigh out a certain amount of agarose powder and melt it in the post-microwave oven. When cooled to 60°C, add GelRed (GelRed is a new type of fluorescent nucleic acid gel staining reagent that can replace Ethylene Bromide (EB), with high sensitivity, low toxicity, high thermal stability, and green fluorescence under UV light transmission), pour into

the electrophoresis bath and allow to solidify. Pour TBE buffer (1X) into the electrophoresis bath in an amount that is no more than 2 mm above the gel surface and remove the comb carefully making sure there are no bubbles. Add the PCR product and a marker to the sample wells. Usually, 30 minutes of electrophoresis are enough to run 200-400 bp of PCR product at 100V. Bands were visualized after GelRed staining with UV light.

Quantitative real-time PCR (qPCR)

For quantitative real-time PCR, 2µl of cDNA was mixed with the Sso Fast Eva Green master mix that contained 6µl of Eva Green Buffer, 3µl of ddH2O, and 1µl of amplification primer-F and 1µl of amplification primer-R. Real-time PCR reactions were carried out in Mxpro-Mx3000P Quantitative PCR instrument. Reaction system: 95°C activations for 3 minutes, 95°C denaturations for 10 seconds, and combined annealing/extension at 60°C for 10 seconds, for a total of 40 cycles. After the cycles are completed, the baseline and threshold values were set manually.

Data analysis: The data are represented by the following conversion expression=10^5 × [(1+E1)^Ct1(target gene)/(1+E2)^Ct2(GAPDH)]. Among them, E1: target gene primer amplification efficiency; E2: housekeeping gene primer amplification efficiency; Ct1: target gene Ct average value of the experimental group; Ct2: housekeeping gene GAPDH Ct average value.

2.7.3. WST-1 cell viability assay

The WST-1 assay was used to determine cell viability following the manufacturer's protocol. AtT-20 cells were seeded in a 96-well plate (2×10^4 cells/well) and kept culturing overnight before being treated. After adding 10µl of WST-1 reagent in each well the plate was incubated in the cell culture incubator in the dark for 0.5-1 hour 37°C. WST-1 is reduced to orange formazan by a number of dehydrogenases in the mitochondria in the presence of electronically coupled reagents. The more and faster the cells proliferate, the more formazan is reduced and the darker the color. The formation of formazan dye was quantified using a scanning multi-well spectrophotometer. The absorbance was measured at 450 nm and a dual-wavelength

measurement was carried out using 655 nm as the reference wavelength.

2.7.4. Human *POMC* promoter luciferase assay

AtT-20 cells were transfected with the human *POMC* reporter vector that has the proximal human *POMC* promoter upstream to the luciferase gene using the SuperFect reagent according to the manufacturer's instructions. In brief, cells were seeded in a 48-well plate (5×10^4 cells/well) and left overnight to attach. After incubating the cells for 3 hours in the transfection complex, the cell culture medium was changed to low serum (2%FBS) DMEM and the cells were allowed to recover overnight before being treated for 6 hours. For knockdown experiments, cells were cotransfected with human *POMC* reporter vector and the indicated siRNA and were left for 48 hours in low serum (2%FBS) DMEM.

For lysis, cells were washed with pre-cooled PBS and lysed with cell lysis buffer (Promega)(75µl/well) and stored at -80°C for up to 7 days. Cells were scraped and were collected in Eppendorf tubes that were centrifuged at 15000 rpm for 10 minutes at 4°C. After centrifugation, the supernatant was collected.

For the luciferase assay, 20µl of sample was added to the luminometer plate and assayed using the Victor x4 multilabel plate reader in the presence of 100µl luciferin.β-Galactosidase (β-gal) was used to measure the efficiency of cell transfection. For the β-Galactosidase assay, 20µl of lysate was transferred to a transparent 96-well plate and incubated with 30µl of ddH2O + 50µl of ONPG in a cell culture incubator for 30-90 minutes in dark. Galactosidase activity was measured at 450 nm and 655 nm in an ELISA plate reader. Data were expressed as the ratio of luciferase to β-galactosidase activity.

2.7.5. siRNA Transfection

Cell transfection methods for the purposes of RNA-interference, Knockdown integrin gene (e.g. αv, β1, β5, β8). Cells were attached overnight in a 48-well plate (5×10^4 cells/well) and 6-well plate (3.5×10^5 cells/well). The next day, cells are transiently transfected with siRNA (10µM) using SuperFect transfection reagents

(Follow manufacturer's protocol). After incubating the cells for 3 hours in the transfection complexes, change cell culture medium (2%FBS+1%P/S) to recover overnight. Cells were harvested for analysis 48 hours post-transfection.

2.7.6. Cy5.5-RGD conjugates synthesis

RGD peptide (1μmol) was dissolved in 1ml of PBS buffer (pH = 8.3) and then mixed with Cy5.5 (1.2μmol) in H_2O (1ml). The reaction mixture was vibrated overnight and then purified by a reversed-phase HPLC. Cy5.5-RGD conjugates were later lyophilized, and kept in the dark at -20 °C.

2.7.7. Fluorescence microscopic studies

AtT-20 cells were seeded in 8-well slides (3×10^3 cells/well) overnight. Then the cells were incubated with the Cy5.5-RGD conjugate (10nM) for 30 minutes. After the incubation, the cells were washed with ice cold PBS, gently removing unbound conjugates. Add DAPI to incubates 1 minutes at room temperature. Cells were fixed with 4% paraformaldehyde (pH=7.4) at room temperature for 10 minutes. Cy5.5-RGD uptake was monitored with fluorescence microscopy.

2.7.8. Statistical analysis

SPSS 20.0 software (IBM) was used to analyze the data in this paper, and the measures are expressed as mean ± standard deviation (SD) when they are normal distribution. One-way ANOVA was used for comparisons between groups, and the Mann-Whitney U test was used when the data did not conform to a normal distribution. All experiments were repeated three times independently, with $P<0.05$ being statistically significant.

3. Results

3.1. Expression of RGD-binding integrins in corticotroph tumors

RT-PCR analysis on 18 cases of corticotroph tumors showed expression of 5 out of 9 RGD-binding integrin subunits: α_v, α_8, β_1, β_5, and β_8 (Figure 3.1A). No expression for α_5, α_{IIb}, β_3, and β_6 was detected.

The immortalized murine corticotroph tumor AtT-20 cells expressed α_v, α_{IIb}, β_1, β_5, and β_8, but not α_5, α_8, β_3, and β_6 subunits (Figure 3.1 B).

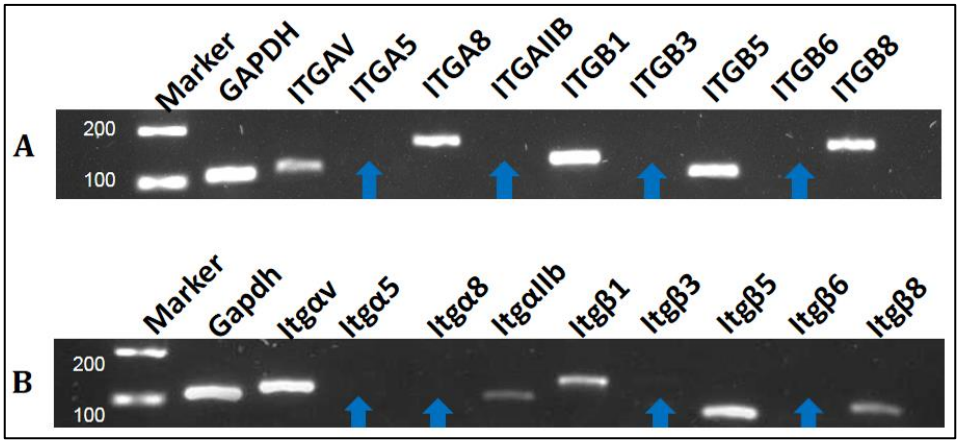

Figure 3.1: RT-PCR for integrin subunits in corticotroph tumor and AtT-20 cell. (A) Expression profiles of integrin amplification products in a corticotroph tumor (representative of 18 cases). (B) Expression profile of integrin amplification products in AtT-20 cells. Molecular markers are used to determine the size of the PCR product. The blue arrows show unexpressed integrin subunits.

All PCR products were of the predicted size. To exclude possible false negative results, primers for human α_5, β_3, and β_6 and mouse α_5, α_8, β_3, and β_6 were validated by RT-PCR on human and mouse kidney samples and primers for human α_{IIb} integrin subunit in human blood samples (Figure 3.2). Our primers for all the integrin subunits were designed to detect the presence of these targets at the mRNA level.

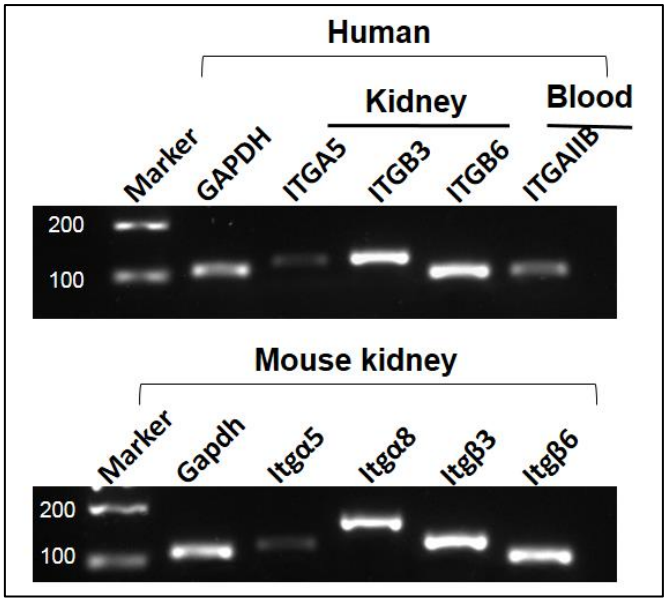

Figure 3.2: Validation RT-PCR.RNA from human and mouse kidney and human blood was reversed transcribed. Primers for human α_5 (*ITGA5*), β_3 (*ITGB3*), and β_6 (*ITGB6*) were validated on human kidney and for human α_{IIb} (*IGAIIB*) on human blood. Primers for mouse α_5 (*Itga5*), α_8 (*Itga8*), β_3 (*Itgb3*), and β_6 (*Itgb6*) were tested on mouse kidney. Molecular markers are used to determine the size of the PCR product.

3.2. Expression of RGD-binding integrins in corticotroph tumors

The expression of α_v (*ITGAV*), α_8 (*ITGA8*), β_1 (*ITGB1*), β_5 (*ITGB5*), and β_8 (*ITGB8*) in corticotroph tumors from patients with Cushing's disease (n=18), gonadotroph tumors (n=16), and normal human pituitary (n=2) was analyzed by quantitative qPCR. The heatmap in Figure 3.3 shows the variability in expression of these genes among the different samples (red: high expression, blue low expression). Corticotroph tumors express in their majority genes encoding for integrin subunits α_v (*ITGAV*; 18/18,100%), β_1 (*ITGB1*; 18/18,100%), β_5 (*ITGB5*; 13/18,72%), β_8 (*ITGB5*; 15/18, 83%), and α_8 (*ITGA8*; 11/18, 61%). In contrast, gonadotroph tumors, except for α_8 that was expressed in 12/16 of cases (75%), show very low expression of genes for α_v, β_1, β_5, and β_8 subunits.

Cluster analysis showed clustered relationships between the expression levels of multiple samples or multiple genes. Figure 3.3 shows that the two clinical pituitary tumor types, Cushing's disease and hormone inactive, cluster according to the expression patterns of *TGAV, ITGB1, ITGB5,* and *ITGB8.*

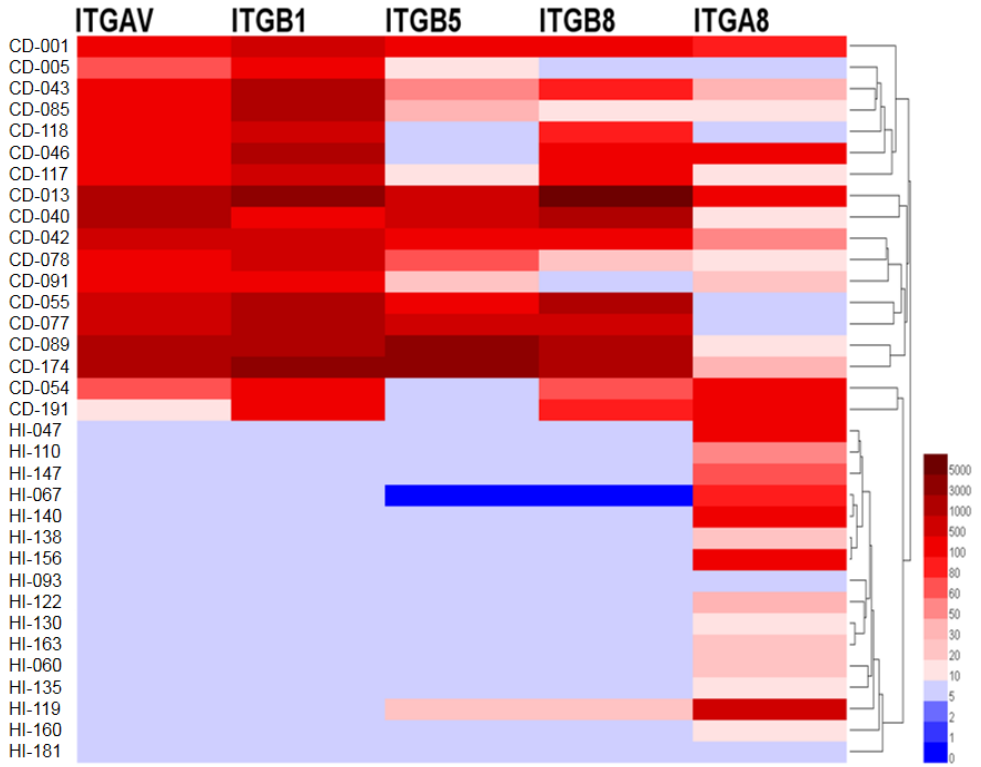

Figure 3.3: Heatmap showing RGD-binding integrin expression in corticotroph versus gonadotroph tumors. Clustering the expression of 5 genes (rows for genes, right-hand character for gene name) and 34 samples from human corticotroph (CD;n=18) and gonadotroph (HI; n=16) tumors. The sample ID is denoted according to clinical diagnosis (CD: Cushing's disease, HI: hormone inactive). Cluster analysis (K-Means Cluster, tree structure above and to the left of the graph boundary).

Human corticotroph tumors have significantly higher expression of genes encoding for α_v, β_1, β_5, and β_8 than gonadotroph (hormone inactive) tumors ($P<0.05$; Figure 3.4). The gene encoding for α_8 is highly expressed in some corticotrophs but also in gonadotroph tumors and the results showed no significant difference between these two ($P=0.0583$).

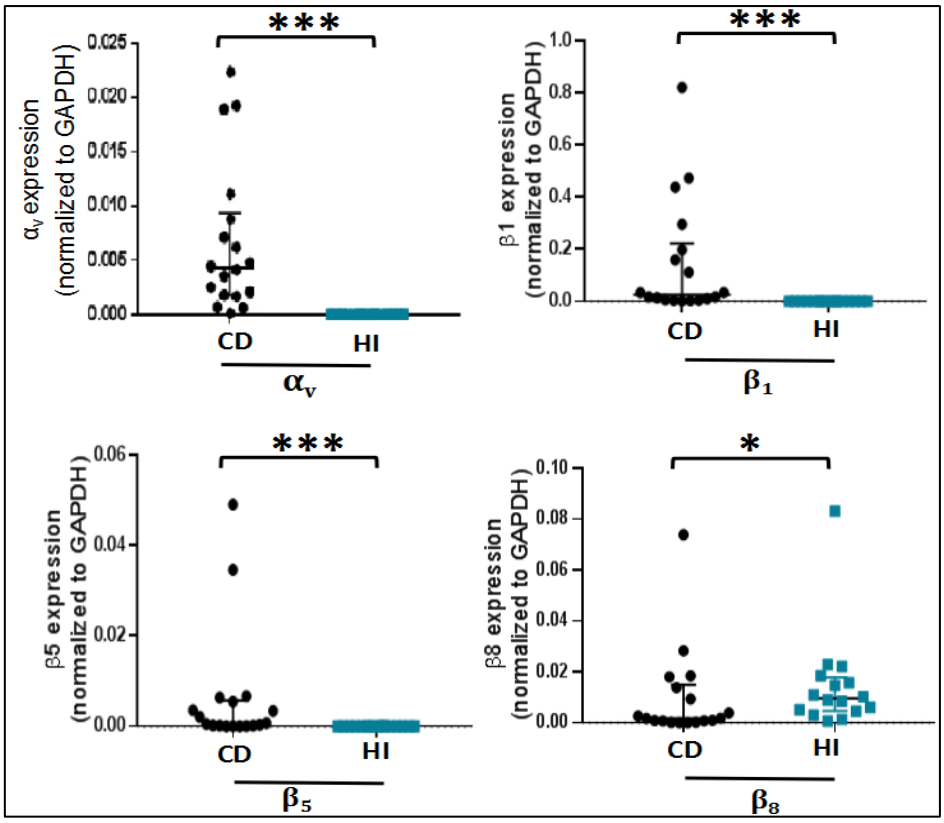

Figure 3.4: Expression of integrin in corticotroph tumors and gonadotroph tumors. CD, Cushing's disease (corticotroph tumors), HI, hormone inactive (gonadotroph) tumors. Mann-Whitney U-Test. *$P < 0.05$, ***$P < 0.001$.

The genes encoding for α_v, β_1, and β_5 are also highly expressed in corticotroph tumors compared with the human normal anterior pituitary ($P<0.05$; Figure 3.5). Although α_8 and β_8 are also highly expressed in some corticotroph tumors, the results showed no significant difference ($P=0.0583$ and $P=0.5167$ respectively), possibly due to the small number of human normal pituitary glands (n=2).

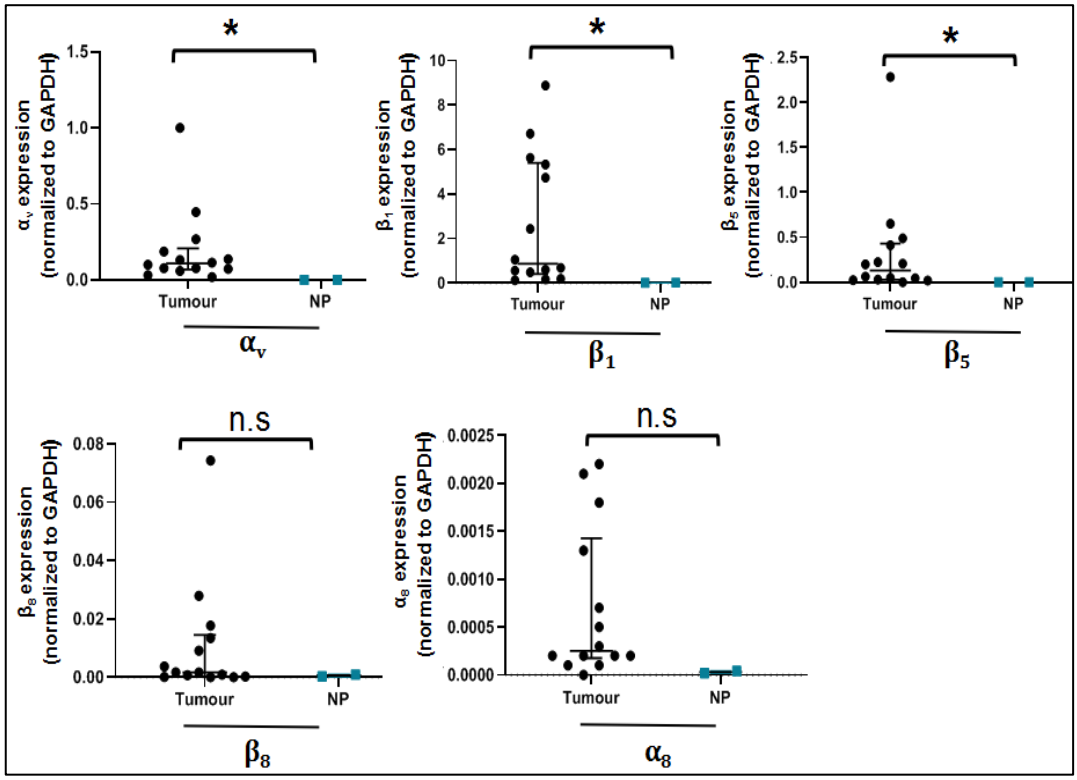

Figure 3.5: Expression of GRD-binding integrins in corticotroph tumors and human normal anterior pituitary. NP, human normal pituitary. The Mann-Whitney U-Test was used. *$P < 0.05$; n.s: no significant.

3.3. Effect of integrin knockdown on human *POMC* promoter activity

Corticotroph tumors showed high expression of genes encoding for the integrin subunits α_v, β_1, β_5, and β_8 compared to another pituitary tumor type (gonadotroph). The next step was to explore their role in corticotroph tumor function. To this end, siRNA was used to knockdown each integrin subunit in the murine corticotroph tumor cell line AtT-20 and human *POMC* promoter activity was assessed as surrogate marker of corticotroph function.

Figure 3.6 shows the effective silencing of the genes expressing for the integrin subunits α_v, β_1, β_5, and β_8 (56%, 63%, 66%, and 65.5% to scrambled RNA control respectively).

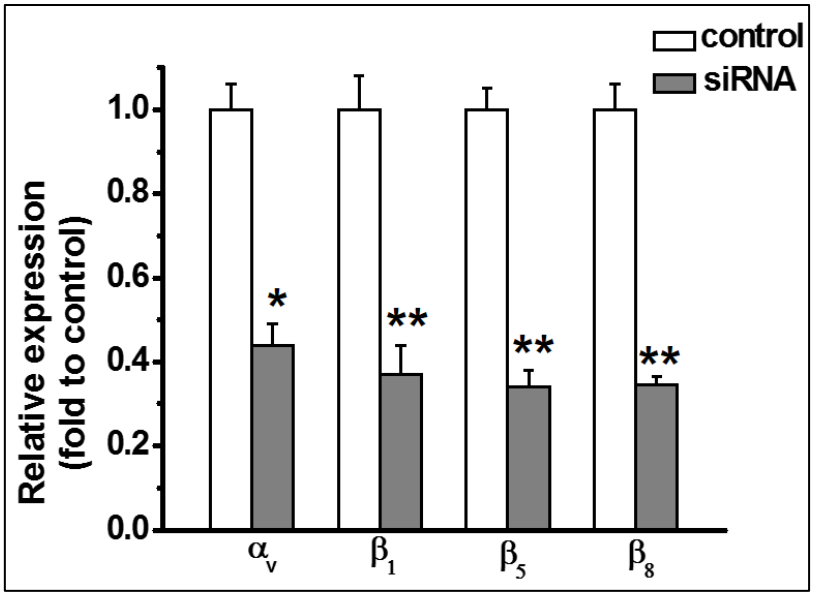

Figure 3.6: Gene silencing efficiency of siRNA targeting integrin mRNA. Each gene was normalized with the *Gapdh* housekeeping gene. Control: scrambled siRNA-transfected. Data are fold change of scrambled control, presented as mean ± SD calculated from three independent experiments. **P* < 0.05, ***P* < 0.01.

Knocking down each gene encoding for α_v, β_1, and β_5 significantly decreased the activity of human *POMC* promoter compared to scramble control (% suppression 63±22, 54±23, and 69±28 respectively; *P*<0.05), while knocking down β_8 had no effect (Figure 3.7).

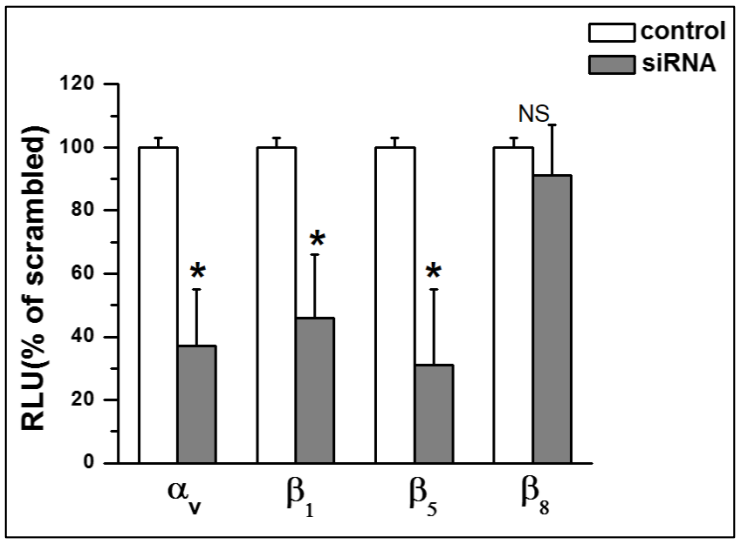

Figure 3.7: Effect of integrin knockdown on human *POMC* promoter activity. A pool of commercially available siRNAs was used to knockdown the gene encoding for α_v, β_1, β_5, or β_8 in AtT-20 cells. Control: scrambled siRNA-transfected. Luciferase was determined 48 hours

after transfection. Data are luciferase/β-galactosidase presented as percentage of scrambled control. Mean±SD of three independent experiments. *P<0.05; NS, not significant. RLU, relative luciferase activity.

3.4. Effect of integrin knockdown on corticotroph tumor cell viability

Knocking down the genes encoding for integrins α_v and β_1 had a small but significant effect on AtT-20 cell viability (% suppression 15.92±1.6 and 27.4±1.4 respectively; P<0.05). Knocking down the genes encoding for integrins β_5 and β_8 did not affect cell viability (Figure 3.8).

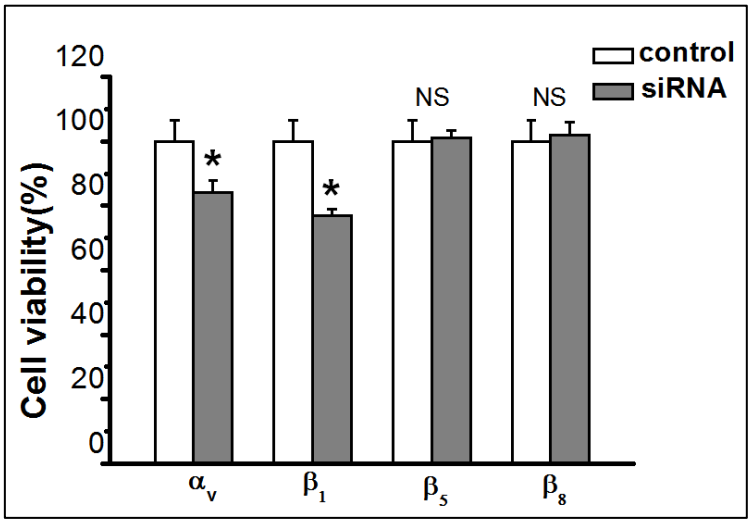

Figure 3.8: Effect of knockdown of integrins on cell viability. A pool of siRNAs was used to knockdown the gene encoding for α_v, β_1, β_5, or β_8 in AtT-20 cells. Control: scrambled siRNA-transfected. Cell viability was assessed with the WST-1 colorimetric assay. Data are mean of three independent experiments presented as percentage of scrambled control. *P < 0.05; NS, not significant.

3.5. RGD peptide conjugated with the fluorophore Cy5.5 targets AtT-20 cells

The experimental results presented above show that human and murine (AtT-20) corticotroph tumors highly express the genes encoding for α_v, β_1, β_5, and β_8 genes are highly expressed in. These four integrin subunits form 3 integrins ($\alpha_v\beta_1$, $\alpha_v\beta_5$, and $\alpha_v\beta_8$) that could bind RGD peptides. Indeed, AtT-20 cells showed active uptake of RGD-

conjugated Cy5.5 (Cy5.5-RGD) as observed by fluorescence microscopy (Figure 3.9).

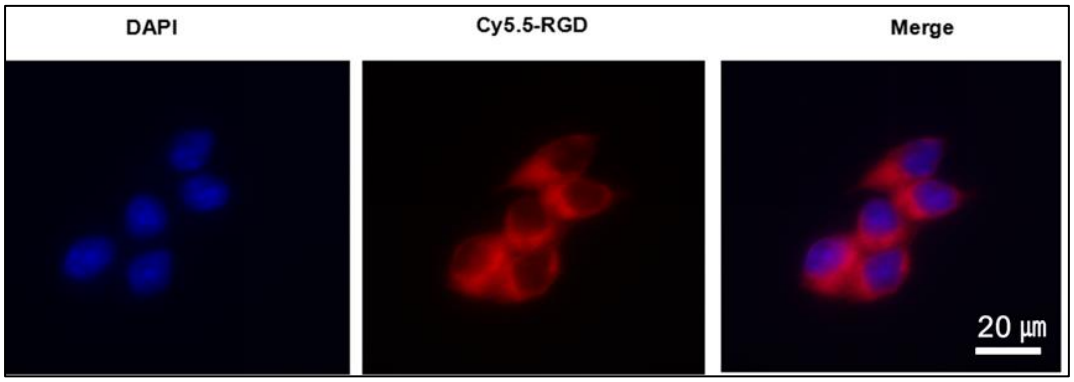

Figure 3.9: RGD peptide conjugated Cy5.5 can accurately target AtT-20 cells. Bar: 20μm

3.6. RGD effects on the cell viability

The above results show fluorophore RGD conjugate uptake by AtT-20 cells, suggesting its potential for tumor-targeted diagnostics. The next step was to determine any deleterious effects on cell viability. To this end, AtT-20 cells were treated with different RGD concentrations (0.05, 0.1, 0.5, 1, 10, 100 μM) for 24 hours. The results showed that it does not affect the viability of AtT-20 cells, even at the high μM doses of 100μM (Figure 3.10).

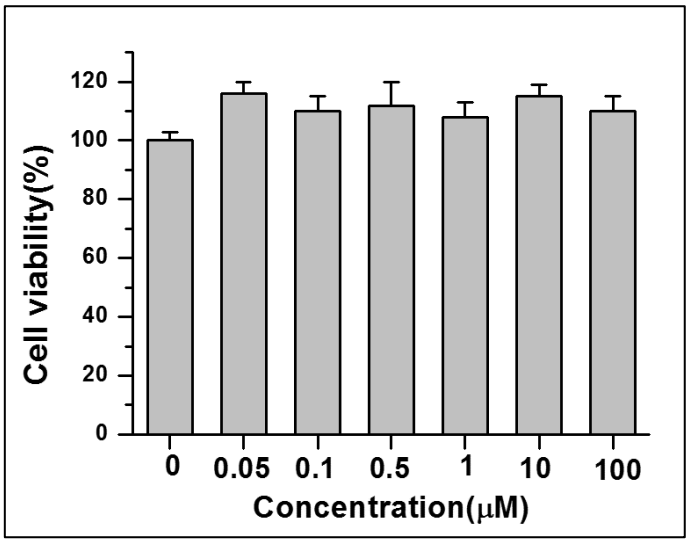

Figure 3.10: Effects of RGD on the cell viability. AtT-20 cells were treated for increasing concentrations of RGD peptide in 10%FBS DMEM for 24 hours. Cell viability was determined with the WST-1 colorimetric assay. Data are presented as percentages of untreated cells.

3.7. RGD effects on human *POMC* promoter activity

The effect of RGD conjugate on corticotroph cell function was assessed by determining human *POMC* promoter activity. Treatment with different peptide concentrations (0.05, 0.1, 0.5, 1, 10, 100 μM) did not affect human *POMC* promoter activity (Figure 3.11).

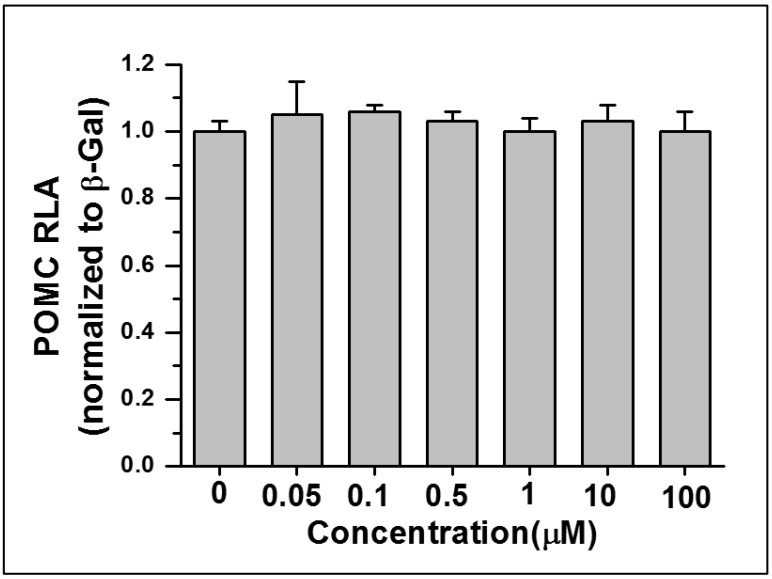

Figure 3.11: Effect of RGD conjugate on human *POMC* promoter activity. AtT-20 cells were transfected with the human *POMC* promoter luciferase reporter plasmid and were left to recover overnight before being treated with the indicated doses of RGD peptide for 6 hours. Data are luciferase/β-galactosidase expressed as fold change to untreated control and presented as mean±SD. RLA, relative luciferase activity.

4. Discussion

Cushing's disease is a serious condition of cortisol overload caused by the secretion of ACTH by corticotroph tumors. The mechanisms supporting corticotroph tumor and ACTH hypersecretion remain obscure. ECM components, such as fibronectin, laminin and collagen I, were shown to affect *POMC* gene transcription and ACTH secretion therefore playing a role in corticotroph pathophysiology[314]. Individual ECM components bind to different integrins[322]. Among the known human integrin subtypes, eight integrins recognize the RGD motif in ECM proteins. The fact that integrins recognize ECM proteins through the RGD motif is the most important reason why RGD-binding integrins have the potential as diagnostic and therapeutic targets for cancer. Integrin targeting RGD peptides can deliver theranostics agents to tumor cells and the tumor vascular system[323]. The present study investigated the expression and potential role of RGD binding integrins in corticotroph tumors.

4.1. Differential expression and potential significance of integrins in corticotroph tumors

My data show significantly high expression of the RGD binding integrins α_v, β_1, β_5, and β_8 in corticotroph tumors compared to the normal pituitary gland and other pituitary tumor types (hormone inactive tumors). This is similar to the situation in other human tumors, where the expression of integrins is highly variable between normal and tumor tissues, with integrins being highly expressed in tumor epithelial cells such as gastric cancer, glioblastoma, small cell lung cancer, breast cancer, rectal cancer, and ovarian cancer[240].

Integrin signaling is involved in the regulation of cytoskeletal, GTPase, and MAPK kinase pathways[324]. In addition to serving as a bridge connecting the ECM to the cytoskeleton, integrins can also regulate multiple intracellular signaling pathways involved in tumor progression and metastasis, including cell adhesion, migration, invasion and proliferation, regulation of apoptosis, and induction of angiogenesis. Members of the α_v sub-family of integrins, including $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, and $\alpha_v\beta_8$,

can specifically bind to RGD peptides, which are expressed in most of ECM proteins, including vitronectin, fibronectin, osteopontin, and collagen IV as well as potentially TGF-β1 and potentially TGF-β3[325, 326].

The integrin subunit-β_1 is essential for cellular interactions with the extracellular mesenchyme and can interact with many α-subunits. β_1 plays an important role in the binding and assembly of exogenous fibronectin, possibly by participating in the organization of the assembly site, regeneration, or cycling, rather than by interacting with fibronectin. $\alpha_5\beta_1$ and $\alpha_2\beta_1$ effectively convert mechanical stimuli into intracellular signals. $\alpha_4\beta_1$ interacts with vascular cell adhesion molecule 1 (VCAM-1), a member of the immunoglobulin superfamily, and fibronectin[327]. β_1-fibronectin receptor is the major integrin responsible for proliferation response in endothelial cells[328]. β_1 controls the expression of GFR and EGFR protein levels, resulting in the decrease of suspension-cultured human epithelial cells[329]. Although β_1 can promote proliferation, it also can inhibit it. For example, $\alpha_1\beta_1$ inhibits EGFR signaling in kidney cells by activating T-Cell Protein Tyrosine Phosphatase (TCPTP), whereas $\alpha_2\beta_1$ activates PP2A, which leads to Akt dephosphorylation. TCPTP phosphorylates integrin and scaffolding protein for GFR (Caveolin-1), leading to reduced EGFR activation[330,331]. Emilin-1 binds to $\alpha_4\beta_1$ and $\alpha_9\beta_1$. It antagonizes the proliferation of skin and epidermal cells to maintain a steady state in the skin, and emilin-1 deficiency leads to excessive proliferation and accelerates wound closure. Emilin-1 inhibition may occur through $\alpha_9\beta_1$-mediated PTEN activation, which blocks Akt signaling[332].

The integrin $\alpha_v\beta_5$ promotes tumor angiogenesis and mediates EGF's effect on tumor cell proliferation and metastasis. Increased expression of $\alpha_v\beta_5$ *in vitro* and *in vivo* bind to ECM proteins generated by endothelial cells and deposited in the tumor microenvironment to mediate endothelial cell proliferation and migration to promote tumor angiogenesis[183]. These integrins can directly modulate EGF signaling and affect tumor cell proliferation and metastasis. EGF-$\alpha_v\beta_5$ stimulates pancreatic tumor cell migration and metastasis *in vivo* via vitronectin. $\alpha_v\beta_5$ can induce EGFR phosphorylation, thereby activating MAPK and subsequent tumor cell proliferation and survival[218,219].

The subunit β_8 binds only to α_v subunit. $\alpha_v\beta_8$ is a receptor for a potential TGF-β

protein in the ECM-bound form and activates the TGF-β signaling pathway. $\alpha_v\beta_8$ can regulate the neovascularization in the developing retina and astrocytes[333] as well as promote the migration of astrocytes to vitronectin[334]. $\alpha_v\beta_8$-TGF signaling in astrocytes could act as a central regulator of cerebrovascular homeostasis[335]. Interestingly, TGF-β activation is a pathway used by tumor cells to suppress the immune system and evade immune checkpoints[336]. Thus, the effects of immune checkpoint inhibitors is enhanced by $\alpha_v\beta_8$ blocking TGF-β signaling or potentially TGF-β activation. $\alpha_v\beta_8$ and TGF-β proteins have recently been shown to be effective in suppressing T cells in a variety of tumor types[337,338].

4.2. Integrins regulate *POMC* transcription in corticotroph tumor cells

The high levels of integrin α_v, β_1, β_5, and β_8 in corticotroph tumors compared to the hormone inactive tumor suggest that they may play a role in corticotroph function. Integrins recognize a variety of extracellular matrix proteins as well as receptor proteins expressed on the cell surface[339,340]. Previous studies have reported that transcription of the proto-oncogene c-fos in pituitary adenomas is dependent on the integrity of the ECM[341]. Besides, MMP can regulate the proliferation and hormone secretion of pituitary tumor cells[309].

The small size of human corticotroph tumors and the low transfection efficacy of primary cell cultures did not allow for detailed *in vitro* experiments, therefore I studied the effect of integrins on corticotroph function in the immortalized mouse corticotroph AtT-20 tumor cells using the human *POMC* promoter reporter vector as readout. Human and mouse corticotroph tumors cells showed similar RGD-binding integrin expression profiles with genes encoding for integrin subunits α_v, β_1, β_5, and β_8 being highly expressed in both. The similar profile indicates that *in vitro* data obtained from the murine immortalized corticotroph tumor cells could extrapolate on human Cushing's disease tumors.

My data show that knocking down the overexpressed in corticotroph tumors

integrin subunits α_v, β_1, β_5, and β_8 significantly decreases human *POMC* promoter activity in the AtT20 cell line, indicating that they may modulate human *POMC* promoter activity and thus affect ACTH synthesis. Integrins employ multiple downstream signaling pathways, including FAK, PI3K, and ERK/ MAPK, coordinate through receptor tyrosine kinase signaling[342,343]. FAK is a key tyrosine kinase in the integrin signaling pathway. Integrins, FAK, and cytoskeletal proteins copolymerize on focal adhesions, allowing the FAK post-activation. Activated FAK binds to Src family kinases and the resulting FAK/Src complexes phosphorylate Paxillin and Cas and activate MAPK, downstream to Ras, Crk and Grb2[344]. Previous studies demonstrated that EGFR-mediated pathways regulate *POMC* and ACTH synthesis via a MAPK dependent pathway[101]. USP8, which is mutated in ~50% of corticotroph tumors, triggers *POMC* transcription in part by rescuing EGFR and enabling its downstream MAPK signaling pathway, which in turn affects ACTH synthesis in corticotroph tumor cells[20].

4.3. The role of $\alpha_v\beta_1$ in cell proliferation and survival

My data revealed that knocking down integrins α_v and β_1 (but not β_5 or β_8) reduce cell viability in AtT-20 corticotroph tumor cells. Integrin subunit β_1 plays a central role in the regulation of cell proliferation and survival[345,346]. In fibroblasts, β_1-collagen-conducting signals inactivate the transcription factor FoxO3a to promote cell proliferation via the Akt pathway[347]. In osteoblasts, the kindlin-2 signals through β_1 to Rac1, which initiates AP-1-dependent transcription for proliferation via Akt signaling[348]. Integrin β_1 has a unique role in driving cell proliferation, since in mammary epithelial cells in the absence of β_1 unable to drive proliferation[349]. Loss of β_1 in the developing pancreas *in vivo* results in dramatic reduction in β-cells[350].

The interaction between β_1 and the collagen matrix regulates cell proliferation and survival by binding to the FAK and initiating ERK signaling[351]. Any disruption of the linking integrins to the actin cytoskeleton and signaling molecules has deleterious effects because loss of adhesion to the matrix leads to apoptosis[352]. FAK activates the

downstream MAPK pathway in anchorage-dependent cells by directly binding to the cytoplasmic domain of β_1 at the site of adhesion[353-355]. Activation of MAPK by β_1-FAK signaling enhanced proliferation and survival of adult rat matrix-cultured β-cells[356]. Collagen I interacts with $\alpha_2\beta_1$ to inhibit cell death induced by T-cell activation via the MAPK pathway[357,358]. Finally, β_1 induces DNA synthesis in a laminin-dependent manner and nerve growth factor promotes proliferation upon binding to $\alpha_9\beta_1$[359].

4.4. Corticotroph tumor cell show uptake of fluorescent RGD peptides

I showed that RGD conjugated with the fluorescent dye Cy5.5 could be targeted to AtT-20 cells. Integrins that can bind specifically to RGD are among the most targeted molecules for studying the precise cancer theranostics. Several studies have shown that RGDs linked to fluorescent imaging agents or radionuclides have tumor-targeted imaging capabilities. Among them, ^{18}F-galacto-RGD has been studied in clinical trials in patients with melanoma, sarcoma, and breast cancer[360,361]. CR780RGD-NPs can effectively cross the blood-brain barrier and target brain tumors in mouse xenografts for diagnosis and image-guided resection of brain tumors[362]. Notably, Near-infrared fluorescence imaging is a non-invasive and highly sensitive imaging modality using fluorescent probes that do not require the use of ionizing radiation or radioactive materials[363]. c(RGDyK) combined with the near-infrared fluorescent dye Cy5.5 specifically targets integrin receptors in both U87MG glioblastoma cell culture and subcutaneous xenograft models[364,365]. In the human Kaposi's sarcoma (KS1767) mouse model for dynamic fluorescence imaging, the intravenous Cy5.5-c(RGDfK) conjugate had specific uptake in tumor tissue[366].

MRI imaging can detect 50-80% of corticotroph tumors. MRI-invisible corticotroph tumors are in the range of 17-63%[367]. The new imaging modality needed to improve the diagnostic rate of tumors as well as to identify tumor margins intraoperatively improves endocrine recovery rates and reduces postoperative complications. In actual surgery, pituitary adenomas may be very close to or even invade critical vascular,

skeletal, and endocrine structures, resulting in a postoperative pituitary hypoplasia incidence of between 5-25%[368]. Importantly, it is difficult to distinguish tumor tissue from surrounding normal tissue by palpation and visual inspection by the surgeon. Currently, the residual tumor rate during microadenectomy exceeds 30%[369], and most patients require additional intervention[370]. Although techniques such as intraoperative MRI have been used to improve the overall total resection rate of other tumor types, it has a high incidence of false positives in patients with pituitary adenomas[371]. Intraoperative fluorescence imaging has several advantages over conventional imaging techniques, such as high contrast, high sensitivity, low cost, and tissue visualization[372]. Fluorescence-guided surgery can improve the overall resection rate of transsphenoidal sinus surgery for Cushing's disease by visualizing the tumor tissue and margins[373]. The conjugate itself had no deleterious effect on cell viability or human *POMC* promoter activity. Thus, integrins could serve as new agents for the imaging of corticotroph tumors.

4.5. Conclusion

The present study demonstrates that integrin subunits α_v, β_1, and β_5 were more highly expressed in corticotroph tumors compared to hormone inactive tumors and human normal pituitary. Silencing α_v and β_1 had a significant inhibitory effect on the corticotroph tumor cell viability, whereas silencing α_v, β_1, and β_5 suppressed human *POMC* promoter activity. Corticotroph tumor cells showed fluorescent RGD peptide uptake with no detrimental effects on cell viability and function. Integrins and their downstream signaling pathways offer potential targets for the diagnosis and treatment of corticotroph tumors.

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Affidavit

LMU LUDWIG-MAXIMILIANS-UNIVERSITÄT MÜNCHEN | Promotionsbüro Medizinische Fakultät | MMRS

Affidavit

Chen, Xiao

_____

Surname, first name

_____

Street

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Zip code, town, country

I hereby declare, that the submitted thesis entitled:

Studies on the role of integrins in corticotroph tumorigenesis

……

is my own work. I have only used the sources indicated and have not made unauthorised use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.
I further declare that the submitted thesis or parts thereof have not been presented as part of an examination degree to any other university.

Munich, 08.11.2021 — Xiao Chen

place,date — Signature doctoral candidate