Aus der Universitätsklinik und Poliklinik für Innere Medizin I

(Direktor: Prof. Dr. med. P. Michl)

Identifikation und Charakterisierung von prognostischen und prädiktiven Markern in pankreatischen neuroendokrinen Neoplasien (PanNEN)

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Pankreatische neuroendokrine Neoplasien (PanNEN) sind eine heterogene Gruppe von seltenen Erkrankungen, deren Malignitätsgrad und Prognose bislang nur durch klinische und histologische Parameter bestimmt wird, was in der WHO Klassifikation und im TNM System Ausdruck gefunden hat. Ziel dieser Arbeit war es, neue prognostische und prädiktive Biomarker aus präklinischen, translationalen und klinischen Studien zu identifizieren und zu charakterisieren. Dabei kommt der Angiogenese in PanNEN eine besondere Bedeutung zu, da deren dichte Vaskularisierung einerseits diagnostisch und therapeutisch wichtig ist, gleichzeitig aber auch einen eigenständigen prognostischen Faktor darstellt. In zwei Studien wurde der Einfluss von zwei in PanNEN funktionell aktiven Signalmolekülen, dem Tumorsuppressors Pdcd4 und dem transkriptionellen Repressor CUX1, auf die Angiogenese untersucht. Es konnte gezeigt werden, dass beide Marker durch die Modulation von Schlüsselmediatoren wie Ang-2, HIF-1a und MMP-9 die Angiogenese in PanNEN beeinflussen und Prognose-relevant sind. Durch Überexpression von CUX1 bzw. verminderte Expression von Pdcd4 wird durch die Beeinflussung weiterer, Proliferations- und Apoptose-assoziierter Signalkaskaden ein aggressiver Phänotyp vermittelt. Für CUX1 wurden diese Effekte auf Proliferation und Malignität im Xenograft- und im transgenen Mausmodell (RIP1Tag2) in vivo sowie an einer humanen Kohorte funktionell aktiver PanNEN bestätigt. Da neben Tumorzell-autonomen Signalwegen die Tumorprogression auch durch das Tumormikromilieu moduliert wird und erste Untersuchungen in PanNEN hierbei eine wichtige Rolle von Tumor-assoziierten Makrophagen (TAM) nahelegten, wurden TAM im neuroendokrinen Tumormausmodell RIP1Tag2 und in 2 großen Kohorten von funktionell aktiven und nicht aktiven PanNEN, in Metastasen und Primärtumoren genauer untersucht. Die TAM Infiltration korrelierte mit einem malignen Phänotyp. Dabei war das Ausmaß der TAM Infiltration mit gesteigerter Proliferation und Angiogenese assoziiert und eine pharmakologische Depletion von TAM konnte die Tumorprogression in frühen Stadien der Karzinogenese im RIP1Tag2 Modell verhindern.

Der Fokus des klinischen Teils der Arbeit lag auf der Charakterisierung etablierter prognostischer Marker an einer Kohorte von PanNEN Patienten unter Streptozocin-basierter Chemotherapie. Die prognostische Signifikanz des Proliferationsmarkers Ki-67 und des Gradings konnte bestätigt werden und erstmalig ein positiver Somatostatinrezeptorstatus (SSTR) als Prädiktor für das Ansprechen auf Chemotherapie beschrieben werden.

Die vorliegende Habilitationsschrift konnte einerseits in klinischen Studien etablierte prognostische Marker bestätigen und in präklinischen Arbeiten die essentielle Rolle der Angiogenese auf die Tumorprogression in PanNEN durch die Charakterisierung von Pdcd4, CUX1 und TAM molekular näher charakterisieren.

I Inhaltsverzeichnis

1 Einleitung

1.1. Definition und Nomenklatur neuroendokriner Neoplasien

Traditionell umfasst der Begriff neuroendokriner Tumor (NET) eine große Familie von Neoplasien, welche von neuralen und endokrinen Strukturen abstammen. Neuroendokrine Neoplasien (NEN) sind in Nervenplexi und extra-adrenalen Paraganglien (Paragangliome), echten endokrinen Organen wie Schilddrüse, Nebenschilddrüse und Nebennieren (medulläres Schilddrüsenkarzinom, Nebenschilddrüsenadneome, -karzinome und Phäochromozytome) und spezialisierten Strukturen des diffusen endokrinen Systems in verschiedenen Organen lokalisiert [1]. Zellen des diffusen endokrinen Systems beinhalten zwei Sekretionswege, charakterisiert durch zwei Sekretions- und Speicherungsmechanismen, genannt „large dense-core vesicles“ und „small synaptic-like vesicles“ (Abbildung 1). Beide Vesikelsysteme sind ebenso in Neuronen vorhanden. Deren Antigene und Konstituenten werden deshalb als neuroendokrin bezeichnet. Klassische echte endokrine Organe wie Schilddrüse und Nebenniere besitzen keine antigenen Strukturen. Das diffuse endokrine System ist endodermaler Herkunft, teilt sich allerdings auch neuronale Eigenschaften und besitzt daher ein einzigartiges neuroendokrines Profil, welches zusätzlich durch die Expression verschiedener Amine und Peptidhormone im gastroentero-pankreatischen (GEP) System charakterisiert ist [2].

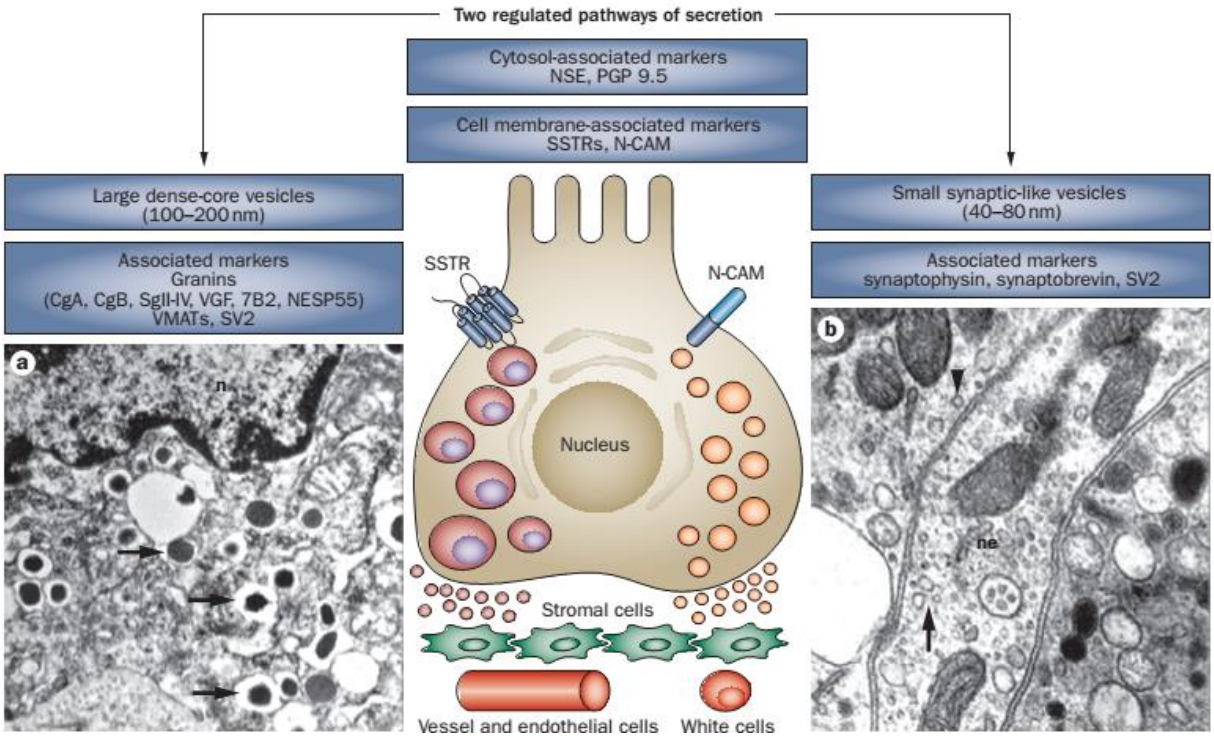

Abbildung 1: Funktionelle Charakteristika von neuroendokrinen Zellen und deren Antigene. Die Abbildung illustriert eine neuroendokrine Zelle mit dem transmembranen Somatostatinrezeptor (SSTR) und neuronalen Zelladhäsionsmolekül (N-CAM). Die Sekretion erfolgt über „large dense-core vesicles" (A) und „small synaptic-like vesicles" (B) über die Basalmembran als parakriner oder distanter endokriner Mechanismus. Elektronenmikroskopische Aufnahmen präsentieren die verschiedenen Vesikelformen (A: große Vesikel; B: kleine Vesikel)[3]

1.2. WHO-Klassifikation von 2017 und TNM-Klassifikation von PanNEN

Initial wurden neuroendokrine Neoplasien als Karzinoide (Karzinom-ähnliche Tumore) bezeichnet. Siegfried Oberndorfer präsentierte 1907 eine Fallserie von drei Patienten mit dieser neuen Entität. Die damalige tumorbiologische Einschätzung, dass NEN nicht metastasieren und von harmloser Natur sind, wurde im weiteren Verlauf durch Oberndorfer selbst 1929 revidiert [4]. Im Laufe der letzten Jahrzehnte wurde die Klassifikation von NEN zunehmend präziser. In der neusten Auflage der WHO von 2017, welche aktuell nur für das Pankreas Bestand hat (für gastrointestinale NEN hat die WHO Klassifikation von 2010 Gültigkeit), werden NEN des Pankreas morphologisch in gut differenzierte pankreatische Tumore (PanNETs, Grad 1-3), schlecht differenzierte Karzinome (PanNECs, Grad 3) und gemischte neuroendokrine

nicht-neuroendokrine Neoplasien (MiNEN) gegliedert (Tabelle 1). Diese Definition ist seit der WHO Modifikation von 2010 akzeptiert und beruht auf klinischen und prognostischen Daten [5]. Zusätzlich bedient sich die WHO Klassifikation neben der morphologischen Einschätzung einem Proliferations-basiertem Grading-System (G1-G3). Erläuterungen dazu finden Sie im Kapitel 4: Pathologie und molekular-biologische Pathogenese. Für neuroendokrine Neoplasien des Pankreas gibt es ein Staging-System der europäischen neuroendokrinen Tumorgesellschaft (ENETS) sowie der UICC/AJCC/WHO (International Union for Cancer Control/American Joint Cancer Committee/World Health Organization) (Tabelle 2) [6, 7]. Während die UICC/AJCC/WHO TNM Klassifikation von 2010 identisch zum Pankreasadenokarzinom ist und sich auf Publikationen von Krebsregisterdaten konzentriert, repräsentiert das ENETS TNM System Ergebnisse verschiedener monozentrischer Untersuchungen und multiplen Datenbanken [8, 9]. Das UICC/WHO System hat sich nach der neuesten Auflage von 2017 weitgehend dem T-Stadium der ENETS Klassifikation angepasst. Allerdings führt das Vorhandensein von zwei unterschiedlichen Staging-Systemen im Management von pankreatischen neuroendokrinen Neoplasien (PanNEN) zu Konfusionen im klinischen Alltag. Eine Studie von Rindi hat an über 1000 PanNEN Patienten beide Klassifikationen verglichen und die Nutzung des ENETS TNM Systems empfohlen, aufgrund der homogeneren Allokationen in die Stadien I-IV und daraus folgender exakter und effektiver Prädiktion des Gesamtüberlebens [5].

Tabelle 1: Präsentation der Nomenklatur neuroendokriner Neoplasien nach der WHO Klassifikation

WHO 1980	WHO 2000/2004	WHO 2010	WHO 2017
I Karzinoid	1. Gut-differenzierte endokrine Tumore (WDET)	NET G1 (Karzinoid)	NET G1
II Mukokarzinoid	2. Gut-differenzierte endokrine Karzinome (WDEC)	NET G2	NET G2
III Gemischt Karzinoid-Adenokarzinom	3. Schlecht-differenzierte neuroendokrine Karzinome/ kleinzellige Karzinome (PDEC)	NEC G3 (groß-/kleinzellig)	NET G3
IV Pseudotumoröse Läsionen	4. Gemischt endokrin-exokrine Karzinome (MEEC)	Gemisch adeno-neuroendokrines Karzinom (MANEC)	NEC G3 schlecht-differenziert (groß-/kleinzellig)
	5. Tumor-ähnliche Läsionen (TLL)	Hyperplastisch und präneoplastische Läsionen	Gemischt neuroendokrin-nicht neuroendokrine Neoplasien (MiNEN)

Tabelle 2:TNM-Klassifikation von ENETS 2006 und UICC/AJCC/WHO 2010.

Definition TNM	ENETS	UICC/AJCC/WHO
T-Stadium		
T1	Tumor begrenzt auf Pankreas <2cm	Tumor begrenzt auf Pankreas ≤2cm größter Durchmesser
T2	Tumor begrenzt auf Pankreas 2-4cm	Tumor begrenzt auf Pankreas >2 größter Durchmesser
T3	Tumor begrenzt auf Pankreas >4cm oder Duodenum/Gallengang infiltrierend	Tumor nicht begrenzt auf Pankreas, keine Infiltration der Arteria mesenterica superior
T4	Invasion in Nachbarorgane, inklusive Gefäßstrukturen	Invasion des Trunkus zöliakus und der Arteria mesenterica superior (nicht resektabel)

Stadium		
I	T1, N0, M0	
IIa	T2, N0, M0	
IIb	T3, N0, M0	
IIIa	T4, N0, M0	
IIIb	jedes T, N1, M0	
IV	jedes T, jedes N, M1	
IA		T1, N0, M0
IB		T2, N0, M0
IIA		T3, N0, M0
IIB		T1-3, N1, M0
III		T4, jedes N, M0
IV		jedes T, jedes N, M1

1.3. Epidemiologie pankreatischer neuroendokriner Neoplasien

Die Inzidenz von GEP-NET beläuft sich auf 2,5-5,0/100.000 Einwohner [10, 11]. Hinsichtlich der Verteilung der Primärtumorlokalisation wurden starke Unterschiede zwischen einzelnen geographischen Regionen publiziert (Europa versus Amerika versus Asiatisch/Pazifisch) [11]. PanNEN weisen eine Inzidenz von weniger als 1,0/100.000 Einwohner auf und umfassen 5% aller malignen Entitäten des Pankreas [10, 12].

1.4. Pathologie und molekularbiologische Pathogenese

Die histopathologische Analyse stellt den Goldstandard in der Diagnostik von NEN dar. Neben der morphologischen Graduierung an HE Schnitten ist die immunhistochemische Aufarbeitung der Marker Chromogranin A (CgA) und Synaptophysin für die Feststellung des neuroendorinen Phänotypen obligat [13]. Das Glykoprotein Synaptophysin ist der sensitivste neuroendokrine Marker und zeigt sich positiv in NET und NEC, ist allerdings wenig spezifisch, da es auch in nicht-neuroendokrinen Neoplasien positiv ausfallen kann. Immunhistochemisch findet sich eine homogene Synaptophysin-Positivität der zytoplasmatischen Mikrovesikel [14]. Die Ausprägung der CgA-Positivität als Hauptkomponente der neuroendokrinen sekretorischen Granula variiert mit der Dichte der zytoplasmatischen Granula in den Tumorzellen. In NEC zeigt sich CgA oft nur fokal und schwach positiv oder auch komplett negativ [15]. Wenn die neuroendokrine Genese der Neoplasie gesichert ist, sollte Ki-67 als Proliferationsmarker evaluiert werden. Der Ki-67 Proliferations-Index wird prozentual angegeben und sollte mittels Immunhistochemie aus einem Proliferationsherd („hotspot“) von mindestens 2000 Tumorzellen bestimmt werden. Zwar lässt sich auch an weniger Tumorzellen eine histologische Einschätzung erreichen, aber die Proliferationsaktivität wird oft unterschätzt [13]. Mittels Ki-67 Index erfolgt das Grading entsprechend der WHO Klassifikation von 2017 für PanNEN in G1 Ki-67 <3%, G2 Ki-67 3-20% und G3 Ki-67 >20% (Abbildung 2). Das Grading unterscheidet nicht automatisch in gut- und schlecht differenzierte Neoplasien. Neuere Studien zeigen, dass gut differenzierte PanNET einen Ki-67 bis 50% präsentieren können, wo hingegen schlecht differenzierte NEC sehr selten Ki-67 Werte unter 50% aufweisen [16]. Daher hat die neue WHO Klassifikation von 2017 erstmal den Begriff NET G3 in Ihre Nomenklatur aufgenommen. Das Grading kann an Metastasen und

Primärtumoren erfolgen. In der klinischen Routine zeigt sich regelmäßig eine Tumorheterogenität zwischen Primärtumor und Metastasen sowie verschiedenen Metastasen untereinander [17]. Nach der ENETs Standard of Care Leitlinie für Pathologie werden folgende, in Tabelle 3 präsentierte, Anforderungen an einen pathologischen Bericht zu PanNEN gestellt [13].

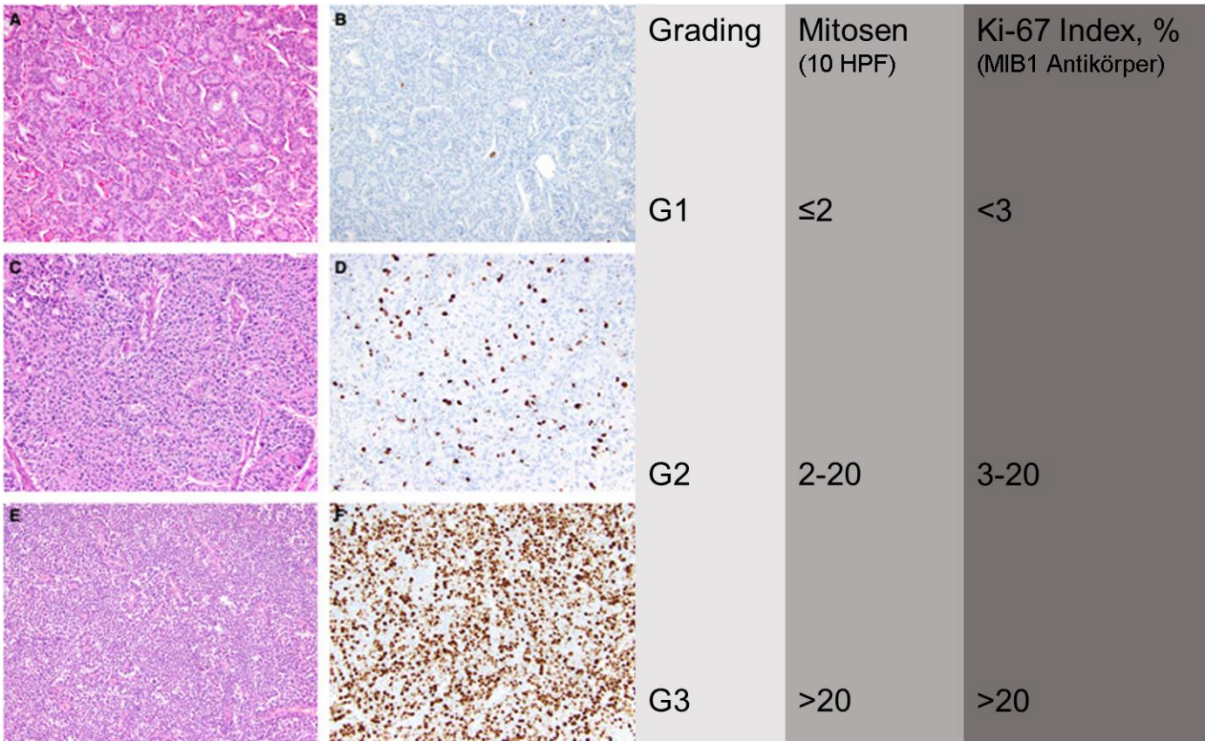

Abbildung 2: Darstellung zweier gut differenzierter pankreatischen NET morphologisch in der HE Färbung (A: G1 Tumor; C: G2 Tumor) sowie mittels Ki-67 Index (B: G1 Tumor; D: G2 Tumor). Zusätzlich zeigt sich ein PanNEC G3 in E (HE-Färbung) und F (Ki-67 Aktivität) [18]).

Tabelle 3:Darstellung der Qualitätsmerkmale für einen pathologischen Report von PanNEN.

Anforderungen an einen pathologischen Bericht zu pankreatischen NEN basierend auf der CAP (College of American Pathologists) Leitlinie [13]	
Art des Gewebes	Resektat, Stanze, Feinnadelaspiration
Tumorursprung	Pankreaskopf, -korpus, -schwanz, -uncinatus
Tumorgröße	in Zentimeter und dreidimensional
Tumorfokalität	unifokal, multifokal
Funktionalität	nicht funktionell, Art der Funktionalität
Histologische Differenzierung	gut oder schlecht differenziert
Proliferationsrate	Ki-67 Index, optional Mitosen
Tumornekrose	vorhanden, fehlend

Mikroskopische Tumorausdehnung	auf Pankreas beschränkt, peripankreatische Invasion, Organbefall
Ränder	Ränder involviert ja/nein, Abstand in cm
Lymphovaskuläre Invasion	vorhanden, fehlend
Perineurale Invasion	vorhanden, fehlend
Lymphknoten	Anzahl aller Lymphknoten Anzahl aller befallenen Lymphknoten
TNM Staging 8. Auflage	

Während die Klassifikation und Graduierung von NEN essentiell für einen einheitlichen Standard ist und partiell den klinischen Verlauf vorhersagen kann, wird die tumorbiologische Heterogenität nicht adäquat reflektiert. Spontane PanNET sind durch wenige somatische Mutationen und chromosomale Aberrationen im Vergleich zu anderen malignen Tumoren charakterisiert (Abbildung 3) [19, 20]. Zusammenfassend sind bei der neuroendokrinen Tumorprogression folgende typische Signalwege involviert: Modulation von Zellmetabolimus, Telemorlänge und -stabilität, DNA Reparatursystem, Zellwachstumsregulation und Chromatinstruktur. Dabei sind innerhalb der PanNEN die molekularen Mechanismen in PanNET deutlich besser charakterisiert worden als in PanNEC. Signalwege im Zellmetabolismus, Zellproliferation und Angiogenese betreffen Mutationen im mTOR Signalweg (Prävalenz bei ~15% der Patienten inklusive dem ESWR1 Transfusionsgen), des TSC1/TSC2 Komplex (~9%), von PTEN (~7%) und PIK3CA (1-2%) sowie aktivierende Mutationen in KIT (~3%) [20-23]. TP53 Mutationen kommen in PanNET sehr selten vor (3%) [21]. Die Serin-Threonin-Kinase ATM vermittelt Signalkaskaden, welche an der DNA-Reparatur beteiligt sind. In PanNET weisen zwischen 5-6% der Tumore Mutationen dieses Tumorsuppressors auf [23]. Veränderungen im Chromatin-Remodeling und der Telemor-Erhaltung gehören zu den häufigen Veränderungen in PanNET. Insbesondere spontane, nicht funktionelle PanNET besitzen Mutationen im MEN1 Gen (30%) sowie einen beidseitigen Verlust des Chromosom 11q [24, 25].

MEN1 kodiert für den Transkriptionsfaktor Menin, welcher die H3K4me3 Histonmethyltransferase rekrutiert und damit die Chromatinstruktur und die Expression von Genen moduliert. Einen zusätzlichen Einfluss auf das Chromatin-Remodeling haben DAXX (death-domain-associated protein) und ATRX (alpha thalassemia/mental retardation syndrome X-linked). Mutationen in DAXX/ATRX sind häufig (~40%) und korrelieren stark mit einem ALT (alternative lengthening of telomeres) Phänotyp in PanNET [26]. Ein DAXX/ATRX Verlust findet sich ausschließlich in PanNET >2cm und ist assoziiert mit einer chromosomalen Instabilität (CIN). Hieraus resultieren biologisch aggressivere Verläufe und eine schlechtere Prognose [20, 27, 28]. Das Mutationsspektrum scheint PanNET in verschiedene Subgruppen zu diversifizieren. Diese wurden in der Arbeit von Scarpa et al. basierend auf whole-genome Sequenzierung systematisch identifiziert und charakterisiert (Abbildung 4) [20].

Die beschriebenen humanen Daten werden unterstützt durch präklinische Evidenz am genetischen Mausmodell von Douglas Hanahan (RIP1Tag2). Das RIP1Tag2 Modell reflektiert die humane Tumorprogression partiell und bietet eine elegante Plattform für präklinische Studien [29]. Basierend auf Transkriptomanalysen wurden drei biologische Subtypen im RIP1Tag2 Modell verifiziert: gut differenzierte Tumore (IT), schlecht differenzierte Tumore mit häufiger Metastasierung (MLP) sowie eine intermediäre Gruppe (MEN1 mutierte Tumore). Mutationen im mTOR Signalweg sowie von ATM waren hauptsächlich in IT Tumoren zu finden, während Veränderungen von Genen des Chromatin-Remodeling sehr präsent im MLP und MEN1 Phänotyp waren [30]. Chromosomale Aberrationen sind ebenso in spontanen nicht-funktionellen PanNET vorhanden. Neben dem schon beschriebenen Chromosom 11q Verlust, auf dem die Gene von MEN1, BRCA und ATM lokalisiert sind (Mutationen bei 13-39% der Patienten), fehlen häufig auch 6q und11p, während ein Zugewinn von 17q, 7q und 20q Gene von NEU/ERB2, AURKA und cMET involviert [31]. Die Publikation von Scarpa

et al. hat für die chromosomalen Rearrangements ebenso eine Klassifikation beschrieben: Gruppe 1 (häufig G2 PanNET) zeigte ein Muster mit Verlust vieler Chromosomen; Gruppe 2 präsentierte exklusiv Aberrationen im Chromosom 11; Gruppe 3 war charakterisiert durch den Zugewinn an Chromosomen (polyploide Tumore), inklusive der höchsten somatischen Mutationsrate; Gruppe 4 zeigte eine Aneuploidie [20].

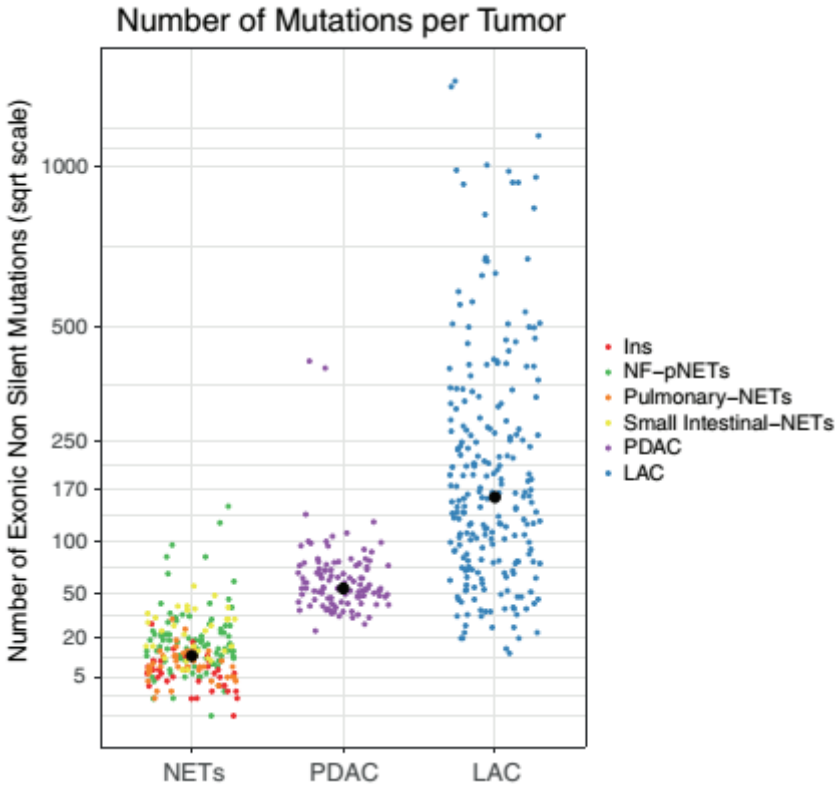

Abbildung 3: Mutationsrate von neuroendokrinen Tumoren. Präsentation von Mutationsmustern in Exonen in Insulinomen (Ins), nicht-funktionellen PanNET (NF-pNETs), pulmonalen NET, Dünndarn-NET, Pankreasadenokarzinomen (PDAC) sowie Adenokarzinomen der Lunge (LAC)[20] .

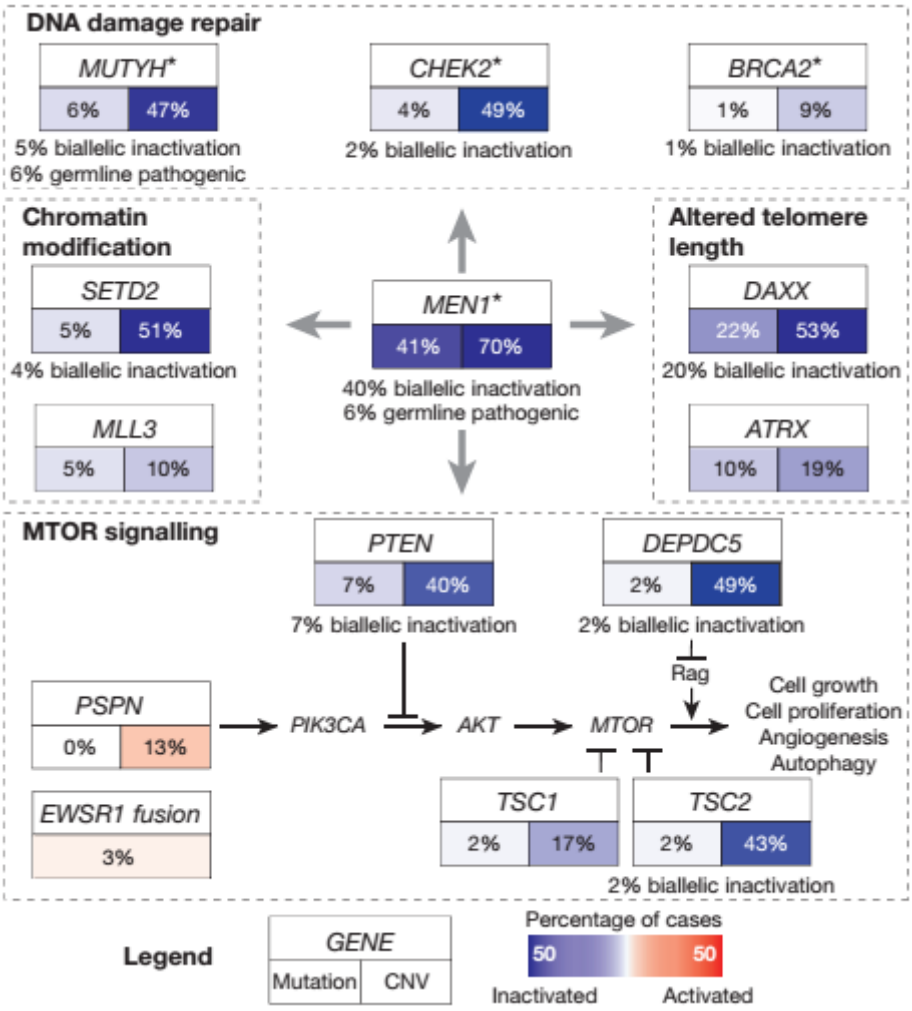

Abbildung 4: Zentrale Signalwege in PanNET. Die Häufigkeit von somatischen Mutationen und Kopiezahlvariationen (copy number variants) für die Hauptgene im mTOR Signalweg, der Histonmodifikatoren, Telomerstabilität und DNA Reparatur wurden charakterisiert in dieser Arbeit von Scarpa et al. 2017.

1.5. Etablierte klinisch-pathologische prognostische Faktoren in neuroendokrinen Neoplasien

1.5.1. Klinische und bildgebende Biomarker

Das mediane Überleben von Patienten mit pankreatischen NEN variiert stark und steht unter Beeinflussung multipler Faktoren. Neben dem Alter ist der Performance Status ein wichtiges Kriterium [32]. In funktionell nicht aktiven (FNA) PanNETs untersuchte eine Studie den Einfluss von Symptomen auf die Prognose in der metastasierten Situation. Inzidentelle PanNETs weisen zwar auch in 30% ein lokal fortgeschrittenes

Stadium auf, scheinen aber mit einem wenig aggressivem Verlauf assoziiert zu sein [33]. Unter klinischen Gesichtspunkten sind Beschwerden ein Kriterium für den Einsatz einer intensivierten Therapie mittels Chemotherapie [34]. Basierend auf vielen monozentrischen Studien wurde ein Staging System entwickelt, welches das Ausmaß der Erkrankung und damit das Patientenmanagement und die Prognose bestimmt. Entsprechend der aktuellen ENETs und UICC-TNM Klassifikation reduziert sich das Gesamtüberleben mit zunehmender Primärtumorgröße und dem Auftreten von Lymphknotenmetastasen, was zusätzlich mit einer Fernmetastasierung korreliert. Ab einer Primärtumorgröße von über 2cm steigt die Lymphknotenmetastasierungsrate von funktionell nicht aktiven PanNETs deutlich an und liegt zwischen 10-40% [5]. Dabei korreliert sowohl der Lymphknotenbefall per se, aber auch die Anzahl von betroffenen Lymphknoten mit dem Gesamtüberleben [35, 36]. Eine zusätzliche Fernmetastasierung, vorrangig in die Leber (80-90%), ist ein weiterer schlechter prognostischer Faktor, der unabhängig von der Therapie den Krankheitsverlauf beeinflusst. Das Ausmaß der Lebermetastasierung wirkt sich auch auf die Prognose aus [37]. Liegt zusätzlich eine extrahepatische Metastasierung vor (bis zu 40% ossäre Metastasen bei PanNET), zeigt sich eine weitere Verschlechterung der Prognose [38, 39]. Das TNM-basierte Staging System wurde im europäischen (ENETs) und amerikanischen (AJCC) Raum in mehreren großen Kohortenstudien untersucht und bestätigte den großen Einfluss auf die Prognose von NEN Patienten [8, 40-42].

Das Vorhandensein von Kalzifikationen im Primärtumor ist ein CT-morphologisches Zeichen, welches in 15-20% vorkommt und Ausdruck einer höher proliferativen und metastasierten Erkrankung ist [43, 44]. Mittels MRT (T1 Wichtung und SPIR Sequenzen (Spectral Presaturation with Inversion Recovery)) können vor allem kleine PanNEN unter 2cm gut detektiert werden. Eine ausgeprägte Vaskularisation mit hohem Diffusionskoeffizienten und eine runde/ovale Form sind dabei mit einem

benignen Verhalten assoziiert [45]. Klassischerweise zeigen vor allem Lebermetastasen von NET eine starke früharterielle Kontrastmittelaufnehme. Eine Arbeit der Berliner Arbeitsgruppe von Marianne Pavel untersuchte das Kontrastmittelverhalten von Lebermetastasen bei Patienten mit G1/G2 Tumoren. Ein Hypoenhancement in der früharteriellen Phase stellte einen unabhängigen negativen Prädiktor dar und war mit einer frühen Tumorprogression assoziiert [46].

Die meisten NET exprimieren Somatostatinrezeptoren (SSTR), insbesondere den SSTR2, welcher dadurch für funktionelle Analysen mittels ^{111}InOctreoscan und ^{68}Ga-DOTA–PET zur Verfügung steht [47]. In den aktuellen Leitlinien wird der Einsatz der SSTR2 basierten PET/CT als vorteilhaft gegenüber der Szintigraphie angesehen, weil hierdurch eine bessere Darstellbarkeit der Läsionen und das Erkrankungsausmaß akkurater wiedergegeben werden kann[48]. Das PET-CT als Goldstandard kann als ^{68}Ga-DOTATATE/TOC/NOC–PET, je nach Verfügbarkeit, durchgeführt werden. Der SUV_{max} des PET/CT korreliert dabei mit der Expression des SSTR2 und spiegelt daher ein gut-differenziertes biologisches Verhalten und eine bessere Prognose wieder [49, 50]. Eine Abnahme des SUV während des Krankheitsverlaufs und/oder unter Therapie kann Rückschlüsse über ein Ansprechen der Erkrankung (auf z.B. PRRT) oder eine Entdifferenzierung der Erkrankung geben. Allerdings ist hierzu die Datenlage deutlich spärlicher. Zeigt sich pathologisch ein schlecht differenziertes neuroendokrines Karzinom ist die SSTR Expression sehr gering und bildgebend nicht darstellbar. Dabei kann das FDG PET die starke metabolische Aktivität der Erkrankung reflektieren. Die deutsche Leitlinie hat eine kann Empfehlung ausgesprochen, wenn die Durchführung einer FDG PET Untersuchung in Patienten mit neuroendokrinen Karzinomen therapeutische Konsequenzen hat. Das FDG PET korreliert mit der Proliferationsaktivität in NEC Patienten und das Auftreten von FDG PET positiven Läsionen während der Tumorprogression von gut-differenzierten NEN stellt einen

ungünstigen prognostischen Verlauf dar [51, 52]. Neben dem prognostischen hat die funktionelle Bildgebung auch einen prädiktiven Stellenwert. Eine SSTR Positivität ist Voraussetzung für die Bindung von Somatostatinanaloga (SSA). Sowohl in der CLARINET (SSA) als auch NETTER-1 (PRRT) Studie war die SSTR2 Expression ein Einschlusskriterium; beide Studien zeigten einen deutlichen Nutzen der SSTR-bedingten Therapie [53, 54]. Eine Studie von Kratochwil analysierte den Einfluss des SUV_{max} im ^{68}Ga-DOTA–PET und den Verlauf des SUV_{max} vor und nach einer PRRT. Hohe SUV_{max} Werte (>15) und ein Abfall der absorbierten Dosis innerhalb einer Läsion korrelierten mit dem Ansprechen auf eine PRRT [55, 56].

1.5.2. Tumor-basierte Biomarker

Die Anzahl an Mitosen in NEN wurde schon sehr lange als Marker für die Proliferationsaktivität benutzt. Dabei werden HPF (high-power-field = 2cm^2) von mindestens 40 Gesichtsfeldern in markanten Proliferationsabschnitten ausgezählt und dann werden die Mitosen pro 10 Gesichtsfelder angegeben [13]. Mit der WHO Klassifikation 2010 wurde der Ki-67 Index eingeführt, welcher heute der Standard Proliferationsmarker bei GEP-NEN weltweit ist. Ki-67 ist präsent in allen Zellen während der Zellteilungsphasen G1, S, G2 und Mitose, allerdings nicht im G0 Abschnitt [57]. Die Anzahl aller positiven Zellen an der gesamten Zellpopulation ergibt einen Index. Dadurch liefert der Ki-67 Index mehr Informationen als die Anzahl der Mitosen [58]. Ki-67 besitzt eine starke Assoziation zur Prognose von Patienten mit GEP-NEN und vor allem auch PanNEN [6, 59, 60]. Mit der neuen WHO Klassifikation von 2017 erfolgte eine Anpassung des G1 Bereichs auf Ki-67 Werte <3% und damit eine Aufwertung um 1%. Die optimale Grenze zwischen G1 und G2 wird allerdings weiterhin diskutiert. Einige Studien haben eine bessere prognostische Stratifizierung gezeigt, wenn sich der cut-off bei 5% bewegt [9, 34, 59]. Ein weiterer Kritikpunkt des

Ki-67 basierten Grading Systems ist die große Fraktion der G3 Neoplasien, welche sich von 20-100% erstreckt. Neben der schon oben erwähnten Diskussion zwischen NET G3 und NEC G3 hat eine skandinavische Studiengruppe (NORDIC study group) mit dem evaluierten Grenzwert von Ki-67 55% innerhalb der NEC eine zusätzliche Stratifizierung auf das Gesamtüberleben postuliert [61]. Ki-67 ist auch als prädiktiver Biomarker untersucht worden. Die zugelassenen Therapie mit Sunitinib in PanNET zeigt bei Ki-67 <5% eine bessere HR (0.38) als bei über 5% (0.63) [62]. Patienten mit PanNET unter Temozolomid und Capecitabin mit einem Ki-67 >5% sprachen besser auf die zytotoxische Chemotherapie und die schon erwähnte NORDIC NEC Studie belegte eindeutig ein besseres Ansprechen auf eine Platin-haltige Therapie bei einer Proliferationsfraktion >55% [61].

1.5.3. Biomarker im Blut und Urin

Chromogranin A (CgA) ist nicht nur ein essentieller immunohistochemischer Marker zur Diagnosestellung einer NEN, sondern wird auch in relevanten Konzentrationen ins Blut sezerniert. CgA wird unabhängig von der Funktionalität sezerniert mit Sensitivitäten von 76-87% [63]. Erhöhte CgA Werte finden sich vor allem bei Patienten mit hepatischer Tumorlast. Dabei kann CgA mit dem Ausmaß der Lebermetastasierung korrelieren[64] und erhöhte CgA Werte waren mit einem schlechteren Überleben assoziiert [40]. Allerdings ist die Interpretation von CgA durch die Beeinflussung verschiedener Faktoren schwierig und die Inter-Assay Variabilität führt zur einer geringen Standardisierbarkeit [48]. Neben CgA steht NSE (Neuronen-spezifische Enolase) bei NEC Patienten zur Verfügung. Zumindestens die einmalige Bestimmung während der initialen Diagnose wird empfohlen. Falls positiv, eignet sich NSE zur Verlaufsbeurteilung. Ein erhöhtes NSE reflektiert ein fortgeschrittenes Krankheitsstadium und eine schlechte Prognose in NEC Patienten [65]. 5-

Hydroxyindolessigsäure (5-HIES) ist das Abbauprodukt von Serotonin und die Exkretion findet über den Urin statt. Serotonin-produzierende Tumore befinden sich häufig im Dünndarm (20-30%) und seltener im Pankreas (<5%) und können das sogenannte Karzinoid-Syndrom verursachen. Zumindest in Dünndarm-NET wird bei Diagnosestellung auch bei asymptomatischen Patienten die Bestimmung der 5-HIES empfohlen [48, 66]. Während die Aussagefähigkeit von 5-HIES für die Therapie das Karzinoid-Syndroms relevant ist, existieren widersprüchliche Daten zum Einfluss von 5-HIES auf die Gesamtprognose [67, 68]. Allerdings kann in Folge eines nicht behandelten Karzinoid-Syndroms eine Karzinoid-Herzerkrankung (Hedinger-Syndrom) auftreten, die als Komplikation mit einem sehr schlechten Überleben vergesellschaftet ist [69].

2 Hypothesen und Ziele

1. Die Angiogenese ist ein zentraler Mediator der Tumorprogression in NEN. Ziel ist es, neue Mediatoren der Angiogenese zu identifizieren und zu modulieren sowie deren Einfluss auf weitere Tumorzell-spezifische Eigenschaften wie Proliferation und Migration zu untersuchen.
2. Der Einfluss der unter 1. charakterisierten Zielgene sollen in-vivo mittels Xenograft Studie und am genetisches Mausmodell sowie an klinischen Kohorten validiert werden.
3. Neben Tumorzell-autonomen Signalkaskaden sind Signalwege in nicht-neoplastischen Stroma-infiltrierenden Zellen, wie den Tumor-assoziierten Makrophagen (TAM), an der neoplastischen Transformation beteiligt. Die Charakterisierung von Tumor-assoziierten Makrophagen in funktionellen und nicht funktionellen PanNEN und deren Assoziation zur Angiogenese und Tumorprogression im genetischen Mausmodell sollen evaluiert werden.
4. An einer definierten Patientenkohorte mit metastasierten PanNET, die mit Streptozocin-basierter Chemotherapie behandelt wurden, sollen neue prognostische und prädiktive klinisch-pathologische Charakteristika identifiziert werden.

3 Publikationen

Krug S, Huth J, Göke F, Buchholz M, Gress TM, Göke R, Lankat-Buttgereit B: Knock-down of Pdcd4 stimulates angiogenesis via up-regulation of angiopoietin-2. Biochim Biophys Acta 2012, 1823(4):789-799. [70]

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Knock-down of Pdcd4 stimulates angiogenesis via up-regulation of angiopoietin-2

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ABSTRACT

The tumor suppressor Pdcd4 is involved in multiple pathways. Considering its biological action conflicting data in the literature exist and, consequently, our own studies point to a cell type specific action of Pdcd4. In the present study, using several Pdcd4 knock down cell lines we succeeded to identify angiopoietin-2 (Ang-2) as a gene up-regulated on the mRNA and protein level. The subsequent enhanced peptide secretion forced wild type Bon-1 cells in a neoplastic direction demonstrated by increased proliferation and colony formation while cell adhesion was decreased. Most likely, the stimulation of Ang-2 is in part mediated by increased activation of AP-1 but different signal transduction pathways may also be involved since we found opposite activation of PI3K/Akt/mTOR and MAPK7ERK pathways (both known to regulate in Ang-2 expression). Ang-2 is a modulator of vascular remodeling. Therefore, we analyzed the effect of supernatants from Pdcd4 knock-down cell lines on endothelial cells. Again, we detected reduced cell adhesion and increased colony formation. Probably, the most impressive effect was described on tube formation in a model for angiogenesis. Tube length and junctions of endothelial cells treated with conditioned medium from Pdcd4 knock-down cells were considerably increased. Both, up-regulation of Ang-2 and down-regulation of Pdcd4 are described for many tumors. However, this is the first study showing a direct impact of Pdcd4 on Ang-2 levels and, thereby, angiogenesis. Our data suggest a completely new mechanism for Pdcd4 to act as a tumor suppressor rendering Pdcd4 an attractive target for new therapeutic strategies in cancer treatment.

1. Introduction

Pdcd4 (programmed cell death 4) is a well established tumor suppressor and is regulated by different pathways (for review [1]). Meanwhile, it became clear that miR-21 (microRNA-21) is a major regulator of Pdcd4 and, to date, numerous publications describe the inverse correlation of Pdcd4 and miR-21 levels with an increase of miR-21 and a decrease of Pdcd4 in a wide variety of tumors (for review see [2, 3]). The protein contains two α-helical MA-3 domains mediating binding of Pdcd4 to the RNA helicase eIF4A, thereby inhibiting helicase activity and subsequently translation [4]. Consistently, translational repression was demonstrated for carbonic anhydrase II and IL-10 [5, 6]. Further target molecules of Pdcd4 involved in cell cycle progression, proliferation and invasion were identified on a pre-translational level such as HPK1 (hematopoietic progenitor kinase 1), uPAR (urokinase receptor), and dUTPase [7-9]. Additionally, secretion of TIMP-2 (tissue inhibitor of metalloproteinase-2), chromogranin A and secretogranin II [10, 11] was shown to be affected by Pdcd4 levels in different cell lines (for review see [1]). Pdcd4 acts via multiple signal, transduction pathways. However, conflicting data exist depending on the cell lines used.

In a neuroendocrine cell line elevated levels of Pdcd4 induced $p21^{Waf1/Cip1}$ [12]. In contrast to these data the knockdown of Pdcd4 just as well induced $p21^{Waf1/Cip1}$ in HeLa and HCT116 cells [13], while in AML (acute myeloid leukemia) cells reduced Pdcd4 levels did not alter $p21^{Waf1/Cip1}$ [14]. These findings are well in line with other data showing no correlation of Pdcd4 levels and the expression of different proteins associated with cell cycle and apoptosis in various tumor cell lines [7]. Moreover, for several proteins opposite effects mediated by Pdcd4 were found in the neuroendocrine Bon-1 and the colon carcinoma HCT116 cell lines stably over-expressing Pdcd4 including dUTPase, pMEK1/2, caspases 3, 7 and 10, cdc2 and $p21^{Waf1/Cip1}$ [7]. These data suggest to a cell-type specific action of Pdcd4. Therefore, we performed a cDNA microarray analysis with RNA from stably transfected shPdcd4 (knockdown) Bon-1 and HCT116 cell lines in comparison to RNA from mock transfected cells. Interestingly, in both cell lines we found a significant increase of angiopoietin-2 (Ang-2) mRNA while levels of Pdcd4 are clearly reduced. The angiopoietin family of growth factors was identified around 1995 (for review see [15]). Four members are known: angiopoietins 1 to 4. The best characterized proteins are Ang-1 and Ang-2 and these proteins have a role in blood vessel formation. Angiopoietins are secreted glycosylated proteins with a molecular weight of

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approximately 75 kDa. Ang-1 and Ang-2 bind with similar affinity to the Tie2 receptor, but only Ang-1 binding induces autophosphorylation and activation of the receptor [16]. Ang-2 acts as an antagonistic ligand and inhibits Ang-1/Tie2 signaling [17]. However, other data showed an agonistic function of Ang-2 [18, 19]. Tie2 activation promotes vessel assembly and maturation (for review see [15]) and Tie2 deficient mice die early at E10.5 due to vessel remodeling defects [20, 21]. In a similar manner, Ang-1 deficiency in mice leads to death at E11–E12.5 due to severe vascular defects [21]. For Ang-2 deficient mice the lethality seems to depend on the genetic background [22, 23]. Surviving mice show minor defects, but disorganized lymphatic vessels. These contradictory results may reflect a dual role of Ang-2 during remodeling and vascular regression during physiological and pathological processes. In the presence of VEGF, Ang-2 induces angiogenesis, whereas in the absence of VEGF vessel regression is triggered (for review see [24]). In addition, Ang-2 is strongly expressed in many tumors and may promote tumor associated vascularization. Moreover, elevated levels of Ang-2 were detected in the circulation of cancer patients and possibly the increase in Ang-2 concentration correlates with tumor progression (for review see [25]).

In this study, we evaluated the regulation of Ang-2 by Pdcd4 knock-down in different cell lines and the implications of elevated Ang-2 concentrations on endothelial and tumor cells.

2. Materials and methods

2.1. Cell culture

All media contained 10% fetal bovine serum and 40 μg/ml gentamicin. All cell lines were from human origin and cultured in the indicated media. Bon-1 carcinoid [26], Hek293 embryonic kidney cells: DMEM/HAM's F12 medium; HCT116 colon carcinoma, HT29 colorectal adenocarcinoma cells: McCoy's 5A medium; HepG2 hepatocellular carcinoma cells: RPMI1640 medium; MCF-7 breast adenocarcinoma cells: RPMI1640 with 1 mM sodium pyruvate, 1× non essential amino acids and 10 μM insulin; Capan-1 pancreatic adenocarcinoma from metastatic site liver, Imim PC1 pancreatic adenocarcinoma (courtesy of Prof. Paco Real, CNIO, Madrid, Spain), MDA-231 ductal breast adenocarcinoma, Panc-1 pancreatic adenocarcinoma and Pa-Tu8988T pancreatic adenocarcinoma cells from metastatic site liver (courtesy of Prof. H.-P. Elsässer, Department of Cytobiology, Philipps-University, Marburg, Germany): DMEM High Glucose with L-Glutamine; HMEC-1 immortalized microvascular endothelial cells (courtesy of PD Dr. R. Köhler, Department of Internal Medicine—Nephrology, Philpps-University, Marburg, Germany [27]): DMEM High Glucose with L-Glutamine and 1 mM sodium pyruvate. Capan-1, HCT116, Hek293, Hep G2, HT29, MCF-7, MDA-231 and Panc-1 cells were purchased from ATCC. Cell number was analyzed by counting the cells in a Neubauer chamber. Conditioned media were obtained from stably shPdcd4 transfected HCT116 and Bon-1 cells cultured for 3 days to a confluency of about 70%. The amount of medium used for these experiments was sufficient for growth of the cells for 9–10 days, therefore, enough nutrients should be left to ensure growth of other cells for several days. HCT116 cells showed no alteration in growth by transfection with shPdcd4 and the 20% increase in growth of shPdcd4 Bon-1 cells at day 3 was adjusted by plating different densities.

2.2. Transfections, MTT-assay, Western Blot and antibodies

Stably shPdcd4 transfected Bon-1 and HCT116 cells, transient transfections, MTT-assays, protein extraction, Western Blots and immunodetection were performed as described in Lankat-Buttgereit et al. [10]. Down-regulated expression of Pdcd4 and Ang-2 was confirmed by Western Blotting using an antibody directed against Pdcd4 [7]. Polyclonal antibodies to Ang-2 were from Alpha Diagnostic International, USA, antibodies directed against Akt, pAkt, pMek1/2 and c-jun were from Cell Signaling, pErk1/2, pc-jun and actin-HRP antibodies from Santa Cruz. Transient transfections for knockdown of Ang-2 were performed with 3 different FlexiTube siRNAs (Qiagen, Germany).

2.3. RNA isolation, microarray analysis and semi-quantitative RT-PCR

Total RNA isolation, microarray analysis with the Human Genome Survey Microarray V2.0 and semiquantitative RT-PCR were performed as described in Lankat-Buttgereit et al. [10]. Primers for amplification of Ang-2 were synthesized yielding a 274 bp product (Ang-2 sense: GTC CAA TGC TGT GCA GAG GGA CG; Ang-2 antisense: CTG ATT TAA TAC TTG GGC TTC CAC). As an internal control β- actin primers were used yielding a 332 bp product (actin sense: AGG CCA ACC GCG AGA AGA TGA CC; actin antisense:GAA GTC CAG GGC GAC GTA GCA C). After 40 cycles, aliquots from each sample were removed and analyzed on a 2% (w/v) agarose gel.

2.4. Tube formation assays

BD Matrigel™Basement Membrane Matrix Growth Factor Reduced (BD Bioscience) was thawed and plated according to the supplier's protocol. 20,000 HMEC-1 cells suspended in the appropriate conditioned media or media containing recombinant Ang-2 (Biomol, Germany), respectively, were plated onto the Matrigel. After an incubation for 18 h at 37 °C the wells were photographed at a 4× magnification with a Canon 450 EOS camera connected to the microscope (Olympus IMT2). Length and junctions of the generated tubes were analyzed with the TimeLapseAnalyzer program [28]. Each experiment was performed in duplicates and repeated twice with at least 4 independently obtained conditioned media.

2.5. Cell migration and wound healing experiments

Cell migration and wound healing were performed as described previously [29]. Briefly, 40,000 cells in 500 μl per inset of a 24-well plate were used and incubated for 4 h. Insets were then rinsed with PBS, dried at room temperature and fixed with methanol. Migration was measured by counting 10 fields of view under a microscope covering about 70–80% of the inset area of the fixed and stained cells (0.2% crystal violet) migrating towards the conditioned media and normalized to the number of migrated cells towards mock conditioned media. For wound healing cells were plated in 12 well plates and grown to 90% confluency. For 3 h 10 μg/ml mitomycin was added to inhibit proliferation. A linear scratch wound was made on the plate, cells were rinsed with PBS and conditioned media were added. The cells were incubated at 37 °C and 5% CO_2 under a Zeiss Axiovert 200M for 24 h. The ratio cell/wound was calculated using the TimeLapseAnalyzer program [28]. Each experiment was performed in duplicates and repeated twice. At least 3 different independently obtained conditioned media were used for each experiment.

2.6. Adhesion assays

Adhesion assays were performed as described earlier [30]. Briefly, conditioned media were added to 12 well plates and incubated at 4 °C for 24 h. Media were removed and 1% BSA was added for 1 h at 4 °C to block unspecific binding. After removal of the BSA 80,000 cells were plated and incubated for 2 h at 37 °C. Unadherent cells were removed by repeated washing with PBS and finger tapping to agitate the wells. Adherent cells were stained with MTT. Each experiment was performed in duplicates and repeated twice. At least 3 different independently obtained conditioned media were used for each experiment.

2.7. Colony formation

Colony formation assays were performed as described in Buchholz et al. [31]. Per well 10,000 cells were suspended in 0.7 ml conditioned media, 50 µl 5% bacto-agar was added, plated and incubated at 37 °C for 10 days. Colonies containing more than 5 cells were counted in an Olympus IMT-2 microscope and averaged over 10 fields of view. Each experiment was performed in duplicates and repeated twice. At least 3 different independently obtained conditioned media were used for each experiment. Approximately 4000 colonies for Bon-1 and 800 colonies for HMEC-1 cells were formed per 10,000 cells.

2.8. Statistics

All data is presented as mean ± standard error. Two tailed paired Student's *t* test was used for statistical evaluation of the data. A p value < 0.05 was considered significant.

3. Results

3.1. Knockdown of Pdcd4 enhances Ang-2 expression

In order to further elucidate the role of Pdcd4, we stably knocked-down Pdcd4 in two cell lines, neuroendocrine Bon-1 and colorectal carcinoma HCT116 cells, by transfection with shPdcd4 plasmid as described earlier [10] and isolated RNA from at least two different clones each. With this RNA we performed cDNA microarray analysis using the Human Genome Survey Microarray V2.0 (32,878 probes for detection of more than 27,000 genes) in comparison to RNA from mock-transfected cells (see supplemental material). Whereas only a small number of similar deregulated genes in both cell systems were uncovered by microarray analysis, we detected a prominent increase in Ang-2 mRNA in both stably shPdcd4 transfected cell lines. The expression of other molecules involved in vascular remodeling like VEGF-A and Ang-1 remained unchanged in Bon-1 cells and even decreased in HCT 116 cells (−1.6 for Ang-1 and −2.6 for VEGF-A, data not shown). Consistent with this data, over-expression of Pdcd4 slightly reduced Ang-2 (0.9 for HCT116 and 0.6 for Bon-1 cells), induced levels of Ang-1 (1.38 in HCT116 and 4.9 in Bon-1 cells) and revealed slightly higher values for VEGF-A (1.9 and 1.8) in microarray analysis (MWG Biotech, Germany, data not shown). To verify the data concerning Ang-2 on the RNA level as well as on the protein level, we transiently transfected the corresponding wild type cell lines (Bon-1 and HCT116) with siPdcd4-RNA and compared the results with the data obtained from two stably transfected clones to exclude integration artifacts. After isolation of RNA and protein, semi-quantitative RT-PCR and Western Blot were performed. Semi-quantitative RT-PCR demonstrated the increased amounts of Ang-2 mRNA in cells with reduced Pdcd4 expression (Fig. 1A). The knock-down of Pdcd4 in stably as well as in transiently transfected cell lines was proven by Western Blot (Fig. 1B). Furthermore, the increased Ang-2 mRNA levels were reflected by elevated Ang-2 protein amounts in both cell lines (Fig. 1B). An opposed effect was observed by over-expression of Pdcd4 in Bon-1 and HCT116 cells: Ang-2 was down-regulated demonstrated by microarray analysis (see above) and Western Blot analysis (supplemental Fig. 1A and 1B) of stably transfected cell lines [7]. The weakened effect in comparison to knockdown cell lines is most probably due to endogenous levels of Pdcd4 which was a reason to change to knockdown cell lines.

Since we had found a similar regulation of a protein by Pdcd4 levels in Bon-1 and HCT116 cells for the first time, we investigated Ang-2 levels in a variety of other cell lines transiently transfected

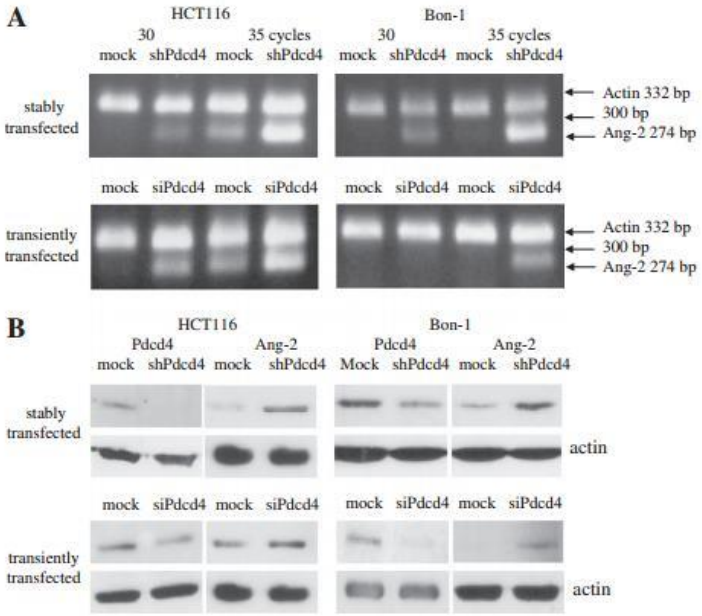

Fig. 1. Regulation of Ang-2 by reduced Pdcd4 levels. A: Semi-quantitative RT-PCR of RNA obtained from stably and transiently transfected HCT116 and Bon-1 cells (siPdcd4) in comparison to mock transfected cells (mock). Actin amplification served as an internal control for equal RNA amounts as templates. B: To verify the knockdown of Pdcd4 (Pdcd4) and induction of Ang-2 (Ang-2) protein levels Western Blot analysis was used. Actin served as a control for equal protein loading. mock: mock transfected cells; siPdcd4: stably or transiently shPdcd4 and siPdcd4 transfected cell lines.

with siPdcd4 RNA. The efficiency of the knockdown of Pdcd4 levels in siPdcd4 transfected cells in comparison to mock transfected cells by Western Blot (Fig. 2). Additionally, Ang-2 expression was analyzed on the same blots. Even though the Pdcd4 levels were rather low in some cell lines (Panc-1, Hep G2 and MDA-231) and hard to verify by Western Blot, elevated Ang-2 protein amounts in transiently transfected cells could be detected. For some selected cell lines semi-quantitative RT-PCR were conducted to verify the Ang-2 regulation at the mRNA level (Fig. 3A). In every case reduced Pdcd4 levels resulted in increased Ang-2 expression. Digital quantification of the signals of at least 3 independent experiments showed a significant 3 to 19-fold increase of Ang-2 mRNA in almost all cell lines (Fig. 3B).

To confirm that the enhanced expression of Ang-2 resulted in increased secretion we performed Western Blot analysis of the supernatants of stably (HCT116, Bon-1) siPdcd4 transfected cell lines (Fig. 2 in supplement). A clearly intensified signal is visible for Ang-2 in the medium of shPdcd4 transfected Bon-1 as well as HCT116 cells in comparison to mock transfected cells, pointing to an enhanced release of the protein. No quantification was performed since the different cell lines release different amounts of Ang-2 (the neuroendocrine cell line Bon-1 can secrete higher amounts than the colon carcinoma cell line HCT116, clearly visible in supplemental Fig. 2). The change in relative levels of Ang-2 is crucial for the question addressed in these experiments.

Ang-2 expression is induced at least in part via AP-1 [32]. Since Pdcd4 influences AP-1 transactivation, we investigated the effect of Pdcd4 levels on activation of c-jun. Whereas we found inconsistent results for c-jun proteins levels in stably transfected cell lines (supplemental Fig.1A), an increase in phosphorylated c-jun is found in both Pdcd4 knockdown Bon-1 and HCT116 cells (digital scan in supplemental Fig. 1B). Therefore, activation of AP-1 is one probable mechanism for increased expression of Ang-2 in different cell lines. However, Ang-2 expression and secretion are regulated by multiple stimuli activating different signal transduction pathways [33]. One of these is the MAPK/ERK pathway. In human breast cancer cells and endothelial cells ERK kinase activity regulates Ang-2 expression [32, 34]. This mechanism seems unlikely for all cell types since by knock-down of Pdcd4 in Bon-1 and HCT116 cells we found no regulation of pERK1/2 and opposite effects on pMEK1/2 and c-jun ([7, 10] and supplemental Fig. 2). Likewise, conflicting data exist concerning the involvement of the PI3K/Akt/mTOR pathway in the regulation of Ang-2. In endothelial cells an activation of Akt down-regulated Ang-2 whereas in breast cancer cells enhanced pAkt levels lead to an up-regulation of Ang-2 [34, 35]. Consistent with these hints for a cell-type dependent regulation of Ang-2 expression we found opposite effects on pAkt levels by modulating Pdcd4 in Bon-1 and HCT116 cells (supplemental Fig. 1A and 1B). Most likely, Ang-2 expression is regulated in a cell-type specific manner.

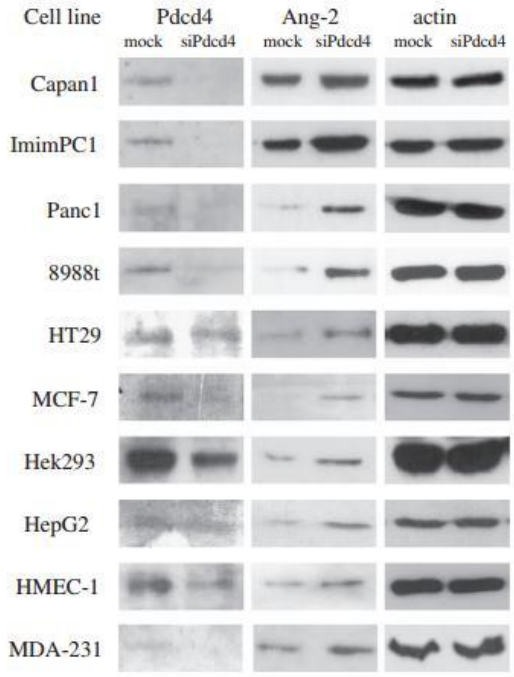

Fig. 2. Regulation of Ang-2 by reduced Pdcd4 levels in different transiently siPdcd4 transfected cell lines. Western Blot with Pdcd4 antibody (Pdcd4) was used to verify Pdcd4 knockdown (siPdcd4) in comparison to mock transfected cells (mock). An increase of Ang-2 was found in every cell line. At least 3 independent transient transfections were performed for each cell line and representative experiments are shown. Actin served as a control for equal protein loading.

3.2. Supernatant from shPdcd4 transfected Bon-1 cells stimulates proliferation and colony formation of non-transfected wild type Bon-1 cells while reducing cell adhesion

Loss of Pdcd4 results in an increased expression and secretion of Ang-2 as well as of other peptides in Bon-1 cells [10]. In further experiments, we tested if conditioned media can influence the non-transfected neuroendocrine cell line Bon-1. Treatment of these cells for 7 days with media obtained from shPdcd4 transfected Bon-1 cells showed a clear increase in proliferation in comparison to the treatment with conditioned medium from mock-transfected cells (Fig. 4A). Whereas only minor and non significant effects could be detected after 1 and 3 days, significantly enhanced proliferation is seen after 7 days. Viability of the cells seemed not to be influenced since doubling times were comparable to Bon-1 cells growing in the regular medium (data not shown).

Multiwell plates coated with conditioned medium served for adhesion assays. Again, a clear effect of shPdcd4 conditioned medium from transfected Bon-1 and HCT116 cells was observed: adhesion was clearly reduced in comparison to medium from mock-transfected cells (Fig. 4B). These experiments could also be performed with conditioned medium from HCT116 also, since the cells can grow in their optimal medium. The reduced cell adhesion in presence of conditioned medium from shPdcd4 transfected cells may refer to an augmented metastatic potential of the cells.

In addition, we found a small but significant increase in colony formation of wild type Bon-1 cells by application of conditioned medium in soft agar assays suggesting an enhanced carcinogenesis (Fig. 4C). In wound healing experiments the effect of conditioned medium was not significantly depicted ($p = 0.163$) but a tendency to faster closing of the wound was visible (data not shown). All the experiments show a clear effect of the conditioned medium on non-transfected wild type Bon-1 cells.

3.3. Impact of conditioned medium from shPdcd4 transfected cells on the endothelial cell line HMEC-1

Ang-2 is involved in angiogenesis and the effect of conditioned medium from cells with reduced Pdcd4 levels should be most pronounced on endothelial cells. As a model system, we utilized the endothelial cell line HMEC-1 for different assays [27]. One of the most important functions of endothelial cells is the generation of vessels. Therefore, we established a tube formation assay in matrigel to investigate the influence of conditioned medium on the ability of

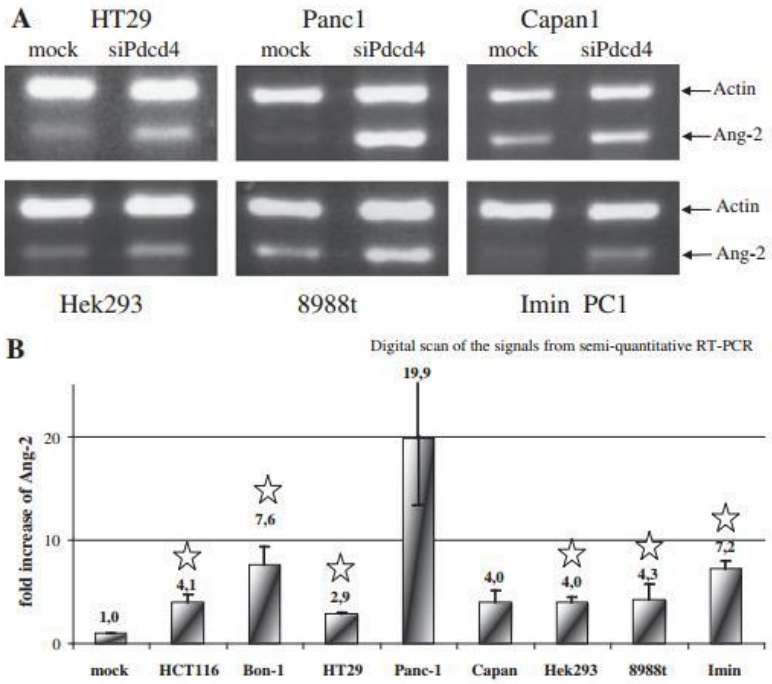

Fig. 3. A: Semi-quantitative RT-PCR of Ang-2 mRNA in transiently siPdcd4 (siPdcd4) transfected in comparison to mock (mock) transfected cell lines. B: Digital scans of signals from semi-quantitative RT-PCR from at least 3 different experiments. The signal from mock transfected cells was set to 1 for every cell line. Except for Panc-1 ($p = 0.07$) and Capan ($p = 0.1$) cells all values were significant ($p < 0.05$) indicated by stars (☆).

endothelial cells to generate vessels. As shown in Fig. 5A, the supernatant from shPdcd4 transfected Bon-1 as well as HCT116 cells induced tube formation (open arrows tubes, solid arrows junctions). Analysis of the assays with the TimeLapseAnalyzer program (example in Fig. 6A) from at least 7 independent experiments revealed a striking increase in tube length and junctions (Fig. 6B). To verify that these effects were caused by Ang-2, tube formation assays were performed with medium containing 50–100 ng Ang-2/ml (Fig. 5B). Again, an increase in length and junctions of the tubes could be detected (Fig. 6C). All these experiments were done with medium containing 10% FBS since the function of Ang-2 depends on the presence of growth factors. To further prove that the effects observed are definitely due to Ang-2, we transiently transfected the stably Pdcd4 knockdown HCT116 cells with three different siRNAs directed against Ang-2, isolated protein extracts and collected the supernatants of the cells two days post transfection. Knockdown of Ang-2 was verified by Western Blot (Fig. 7A). All three siRNAs resulted in a comparable decrease of Ang-2 protein (data not shown). Again we performed tube formation analysis and found that increased length and junctions of the tubes were reversed by knockdown of Ang-2 (Fig. 7B and C). The smaller effect on tube formation in comparison to only stably transfected cell lines is most probably due to the fact that the media were already collected at day 2. Clearly, these results demonstrate the importance of Pdcd4 for regulated angiogenesis and, thereby, for tumor progression.

Cell migration capability was examined with the Boyden Chamber assay and just as well, migration of the endothelial cells increased significantly by use of shPdcd4 conditioned medium from Bon-1 and HCT116 cells as attractant (Fig. 8A). The sole exception in significance was obtained with conditioned medium from shPdcd4 transfected Bon-1 cells containing 1% FBS ($p = 0.073$). In general, the HMEC cells grow poorly in medium containing 1% FBS or less most likely explaining non significant results. Clear results were obtained with soft agar assays for the colony formation ability of HMEC-1 cells as well. The number of colonies increased significantly ($p < 0.05$) by the treatment of the cells with conditioned medium from shPdcd4 transfected Bon-1 cells in comparison to mock-transfected cells (Fig. 8B).

For adhesion assays the cells were cultured in the appropriate medium, and a reduced adhesion of the HMEC-1 cells could be observed on multiwell plates coated with conditioned medium from both shPdcd4 transfected Bon-1 and HCT116 cells in comparison to mock-transfected cells (Fig. 8C). Though the effect was not significant at the 0.05 level ($p = 0.082$ for Bon-1 and $p = 0.054$ for HCT116 conditioned medium) a tendency to reduced adhesion is visible. The HMEC-1 cells adhered to the coated wells very fast (in 15 min or less) which might explain the low significance. Proliferation of the endothelial cells determined using the MTT assay was induced at day 3 but not to a significant level (data not shown). Unfortunately, the HMEC-1 cells started to die at day 2, probably due to the fact that the cells were not cultured in the optimal medium, but in conditioned medium obtained from transfected Bon-1 and HCT116 cells, respectively. The same problem was observed for wound healing experiments and no significant results were obtained (data not shown).

4. Discussion

Tumor growth often requires vascular remodeling including vessel regression and neovascularization and vascularization influences the resistance of tumors to chemotherapeutic agents. Therefore, the

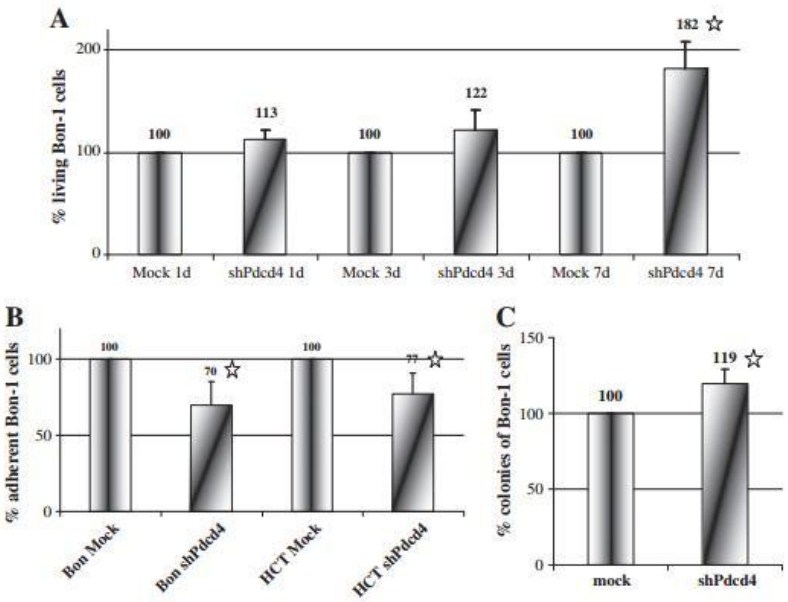

Fig. 4. Impact of conditioned medium from stably shPdcd4 transfected Bon-1 cells (shPdcd4) in comparison to mock transfected (mock) cells on wild type Bon-1 cells. A: MTT assay for examination of proliferation was performed. Treatment of the cells with the supernatant from mock transfected cells was set to 100%. Even if the values for 1 and 3 days were not significant ($p = 0.07$ and 0.15) after 7 days increased proliferation was significant ($p = 0.012$). B: Bon-1 cell adhesion is significantly reduced to plates coated with the supernatant from either Bon-1 of HCT116 cells transfected with shPdcd4 in comparison to mock transfected cells ($p < 0.05$). C: Colony formation of Bon-1 cells in soft agar assays is significantly increased ($p < 0.05$) by treatment with conditioned medium from shPdcd4 transfected Bon-1 cells. Stars (☆) indicate significant p-values ($p < 0.05$).

vascular signaling pathways gain attention as an aim for targeted therapies. Interestingly, Ang-2 of the angiopoietin family is strongly expressed in the vasculature of many tumors (for review see [15]). However, the role of Ang-2 in angiogenesis is complex since Ang-2 acts context dependent as an agonist or an antagonist of the Tie-2 receptor: in the presence of VEGF angiogenesis is induced, whereas an opposite effect is observed in the absence of VEGF (for review see [15]).

Pdcd4 was first identified as a protein involved in apoptosis but soon a function as a tumor suppressor emerged. There is evidence that the effects of Pdcd4 are cell type specific [7]. In this study, we identified Ang-2 to be regulated by Pdcd4 levels in many if not all cell lines which might explain the tumor suppressor function of Pdcd4 in a variety of tumors. In addition, we showed that the enhanced expression and secretion of Ang-2 by reduced Pdcd4 levels can affect surrounding cells especially tube formation by endothelial cells.

Data from earlier studies indicated that the function of Pdcd4 may be cell type specific [7]. However, Pdcd4 acts as a tumor suppressor in different tumor cells and is down-regulated in a wide variety of cancers (for review see [1]). In order to elucidate an overall function of Pdcd4, we stably knocked-down Pdcd4 expression in Bon-1 and HCT116 cells and performed a cDNA microarray analysis. The most prominent regulated gene in both cell lines was identified as Ang-2, whereas Ang-1 and VEGF-A were not influenced. The enhanced Ang-2 expression was confirmed on the RNA and protein level by semi-quantitative RT-PCR and Western Blot in two clones of each stably transfected cell line as well as in a variety of different transiently siPdcd4 transfected cell lines. Moreover, we could show that the increased expression of Ang-2 resulted in enhanced release of the protein. Noteworthy, we previously found that Pdcd4 levels regulated secretion of chromogranin A (CgA) and secretogranin II (Sg II) in the neuroendocrine cell line Bon-1 via activation of the protein kinase Akt [10]. These results may now be of special relevance since secretoneurin, a Sg II derived peptide acts as a proangiogenic cytokine [36]. The complex action of Pdcd4 is highlighted by the fact that CgA and Sg II release is at least in part due to activation of Akt by low levels of Pdcd4 in Bon-1 cells, whereas in HCT116 cells no regulation of pAKT neither by over-expression of Pdcd4 [7] nor by silencing Pdcd4 levels was found (supplemental Fig. 2). Interestingly, in lung microvascular endothelial cells a stimulation of Ang-2 secretion is described by inhibition of Akt [35] which is in contrast to our data in Bon-1 cells showing enhanced Ang-2 release in combination with raised levels of activated Akt by silencing Pdcd4. Therefore, Pdcd4 is likely to regulate Ang-2 expression in Bon-1 cells by other mechanisms than the Akt pathway. In accordance with a cell-type specific regulation, in breast cancer cells human epidermal growth factor receptor 2 (HER2) activity correlated with Ang-2 expression and Akt activity was necessary for the up-regulation of Ang-2 which is in contrast to the data obtained in endothelial cells [34]. However, these opposing results are in good agreement with our findings that knock-down of Pdcd4 induced pAkt in Bon-1 cells and decreased pAkt in HCT116 cells even though both cell lines showed increased expression of Ang-2. Another mechanism for regulation of Ang-2 expression displays the MAPK/ERK pathway. Even if enhanced ERK kinase activity resulted in stimulation of Ang-2 levels in breast cancer and endothelial cells [32, 34] our data point to a cell-type dependent regulation of Ang-2. We found no effects on the levels of pERK1/2 and an opposite impact on pMEK1/2 and c-jun levels by modulating Pdcd4 amounts in Bon-1 and HCT116 cells but an increased phosphorylation of c-jun providing a mechanism for enhanced Ang-2 expression in both HCT116 and Bon-1 cell lines. Therefore, stimulated

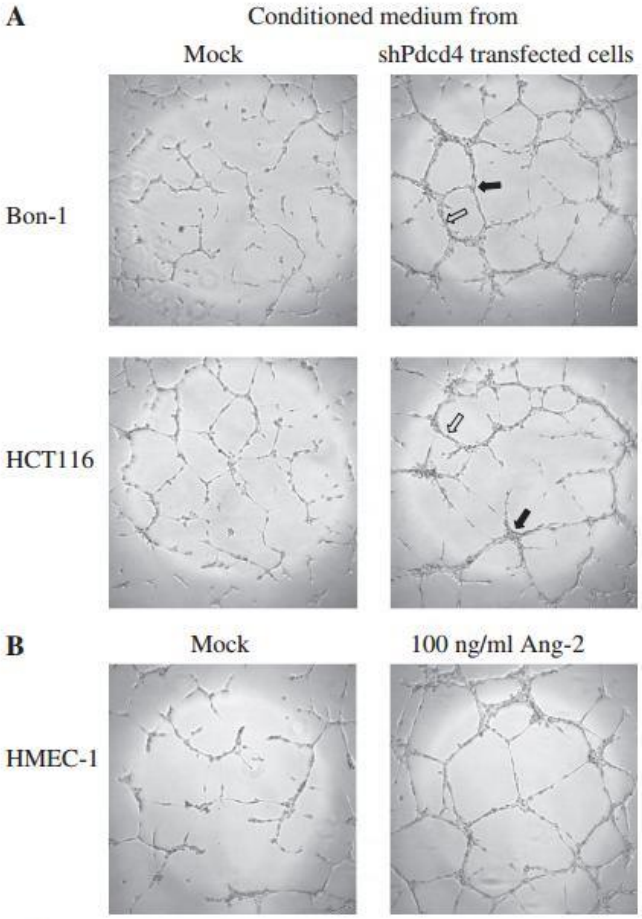

Fig. 5. A: Tube formation of HMEC-1 cells in matrigel upon treatment with conditioned medium from mock and shPdcd4 transfected Bon-1 and HCT116 cells. Photographs were taken with 4× magnification. The open arrow indicates an example of a tube, the solid arrow of a junction. B: Addition of recombinant Ang-2 resulted in tube formation comparable to treatment with shPdcd4 conditioned medium.

AP-1 transactivation may at least in part be responsible for the increased Ang-2 levels by knockdown of Pdcd4. Anyhow, considering the multiple stimuli influencing Ang-2 expression most likely different pathways contribute to variable parts to the regulation of Ang-2.

In addition to regulation of Ang-2 we show that cells with depletion of Pdcd4 secrete peptides affecting surrounding cells. This effect is at least in part due to Ang-2 release. This is of particular interest for the development of new therapeutic strategies fighting neuroendocrine tumors which are often resistant to radiation and chemotherapeutic agents especially since we previously demonstrated that Pdcd4 acts as a tumor suppressor in neuroendocrine cells [5, 37]. Investigating the effect of medium containing the secreted peptides (conditioned medium from shPdcd4 transfected cell lines) on neuroendocrine wild type Bon-1 cells we found increased proliferation, colony formation and wound healing, but decreased adhesion of the cells. Consistent with our data epithelial enhanced expression of Ang-2 was found in neuroendocrine tumors, and recombinant expression of Ang-2 in orthotopic Bon-1 xenografts led to increased microvessel density and spread of tumor cells into lymph nodes showing again the impact of secreted Ang-2 on tumor formation [38]. Moreover, circulating Ang-2 levels correlated with metastatic disease in patients with neuroendocrine tumors. Additionally, Ang-2 expression corresponded to lymph node invasion in patients with non-small cell lung cancer and breast cancer, respectively [39, 40]. Even if down-regulation of Pdcd4 and increased levels of Ang-2 were demonstrated in a variety of tumors, we have shown this correlation in the same cells for the first time. All this data stress the importance of Ang-2 regulation in tumor progression, angiogenesis and metastasis.

A tumor can only proliferate up to the size of 1–2 mm without vascularization. Neoangiogenesis promotes tumor growth in two ways: firstly by supply of nutrients and removal of metabolic waste products and, secondly, by the accession of neoplastic cells to the blood vessels, thereby facilitating metastasis. Neovascularization is tightly regulated and depends on the balance of proangiogenic and antiangiogenic factors (inducers and suppressors) and deregulation

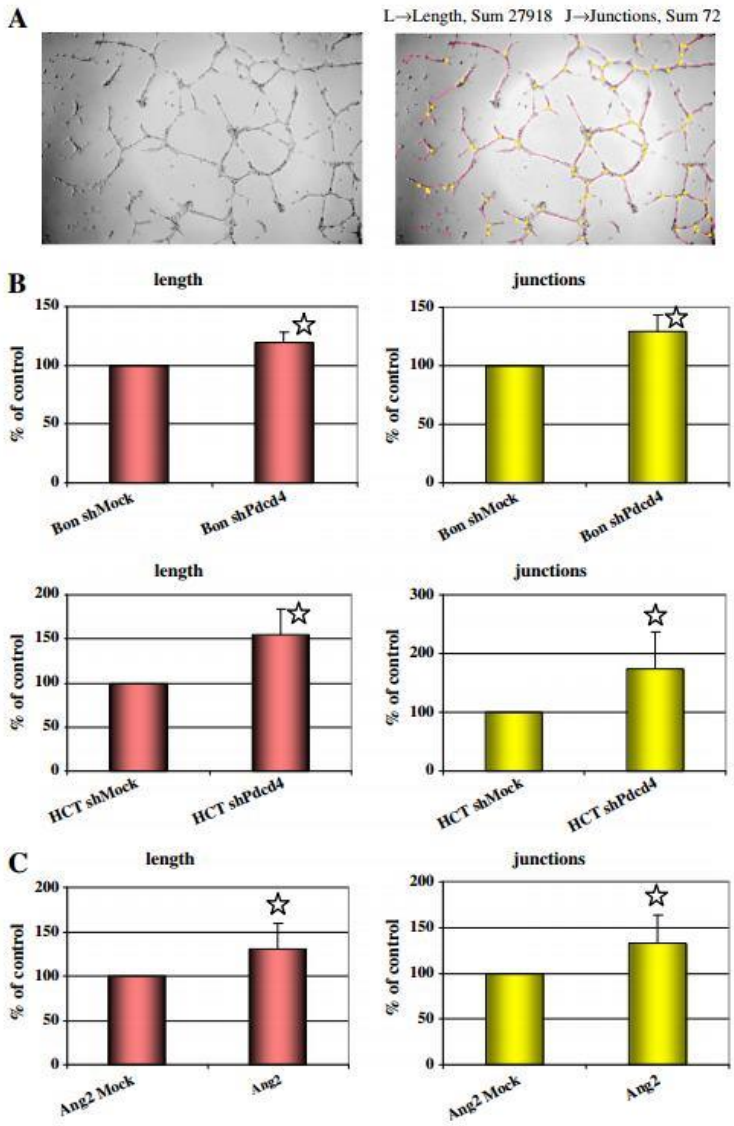

Fig. 6. Analysis of tube formation assay by determining length and junctions of tubes. A: Example of analysis by the TimeLapseAnalyser program. B: Calculation of length and junctions of pictures from 5 to 7 independent experiments. Tube formation was performed with conditioned medium from shPdcd4 transfected Bon-1 and HCT116 cells (shPdcd4) in comparison to mock (shMock) transfected cells. Stars (☆) indicate significant p-values ($p<0.05$). C: Calculations of length and junctions of 7 tube formation experiments with medium containing 50–100 ng/ml Ang-2 in comparison to medium without Ang-2. Stars (☆) indicate significant p-values ($p<0.05$).

of the angiopoietin-Tie2 system represents an angiogenic switch in tumor formation (for review see [41]). Ang-2 is an interesting modulator of angiogenesis since in the presence of VEGF induction of vessel formation proceeds whereas in the absence of VEGF vessel regression occurs (for review see [15]). In addition, Bon-1 cells are known to produce and secrete high levels of VEGF and, therefore, provide the growth factors necessary for the proangiogenic effects of Ang-2 [42]. Therefore, we investigated the influence of conditioned medium not only on Bon-1 cells but also on an endothelial cell line (HMEC-1). Though no effect could be shown on proliferation of HMEC-1 cells most likely due to the fact that the conditioned medium obtained from transfected Bon-1 and HCT116 cells is improper for growth of endothelial cells, a slight decrease in adhesion to multiwell plates coated with conditioned medium was observed. It is well established that Ang-2 induces a loosening of perivascular and endothelial cells from the extracellular matrix, thereby disturbing vascular integrity.

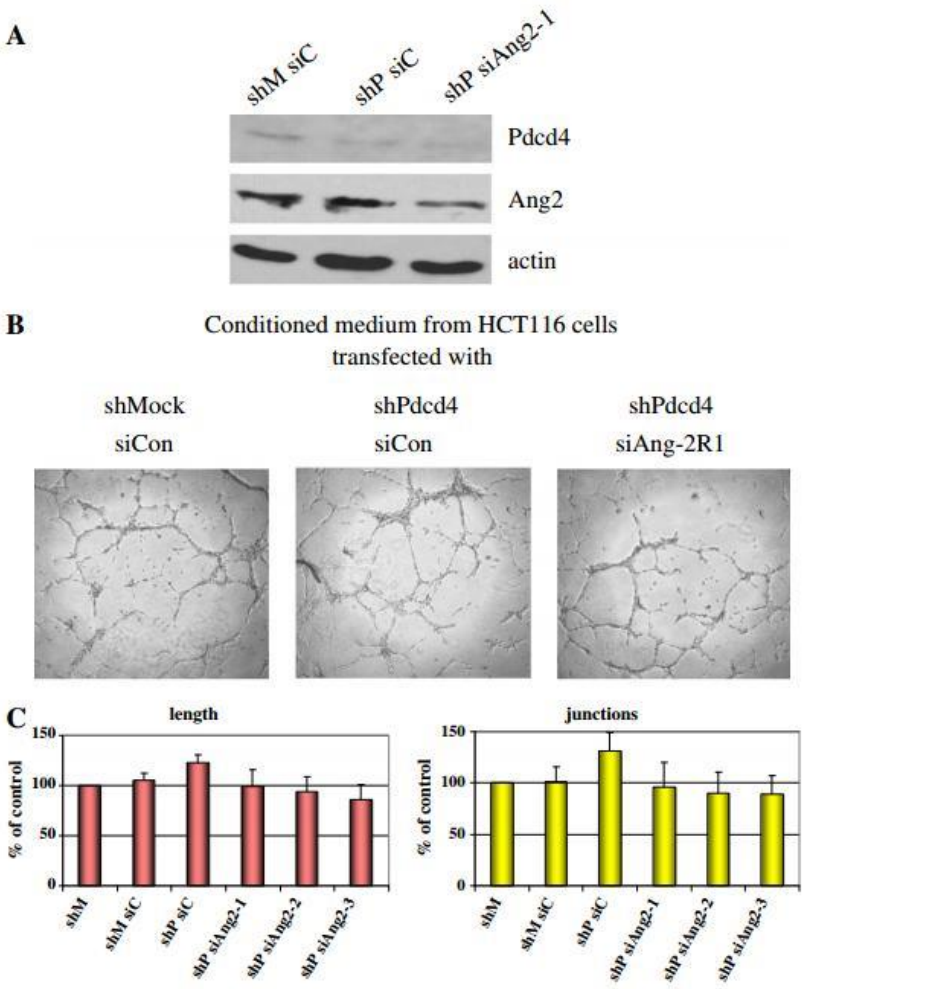

Fig. 7. Knockdown of Ang-2 in shPdcd4 HCT116 cells by transient transfection with siRNAs. A: Western Blot to verify the knockdown of Ang-2 (shMsiC: shMock transfected HCT116 cells transiently transfected with siControl RNA, shPsiC: shPdcd4 transfected cells transiently transfected with siControl RNA, shPdcd4siAng2-1: shPdcd4 transfected cells transiently transfected with siAng-2 RNA1 as an example). All 3 siRNAs resulted in a comparable knockdown of Ang-2. B: Examples of the tube formation of HMEC-1 cells upon treatment with conditioned medium obtained from double transfected cells (see panel A). C: Analysis of the tube formation assays by determining length and junctions of tubes. The increase of tube length and junctions by treatment of the HMEC-1 cells with conditioned medium of shPdcd4 transfected cells was reversed by transient knockdown of Ang-2. P-values of shPdcd4siControl cells in comparison to shMock transfected cells and for shPdcd4siAng2-1-3 in comparison to shPdcd4siControl were significant ($p<0.05$) for both graphs.

However, a significant increase in colony formation and cell migration induced by the supernatant of shPdcd4 transfected Bon-1 and HCT116 cells was found.

The observation that the endothelial cell line was affected by the conditioned medium obtained from Pdcd4 depleted cells prompted us to study the influence of this medium on angiogenesis. In fact, we found enhanced tube formation by treatment of endothelial cells with conditioned medium from shPdcd4 transfected Bon-1 and HCT116 cells which was reversed by treatment of the endothelial cells with medium obtained from cells additionally transfected with siAng-2 RNA. These data suggests an involvement of Pdcd4 expression in abnormal vascularization of tumors by affecting Ang-2 expression and release. Hence, induction of Pdcd4 may offer a new therapeutic possibility to inhibit cancer growth by suppression of Ang-2. Supporting this assumption, Ang-2 levels are discussed as an interesting therapeutic target and as a useful clinical marker for progression of human cancers (for review see [33]). However, the mechanisms of tumor angiogenesis are not completely understood and, furthermore, the Ang-2 function is cell context dependent. Pdcd4 acts tumor suppressive by affecting not only angiogenesis but also transcription and translation of proteins involved in cell cycle, differentiation and apoptosis (for review see [1]). Therefore, it certainly represents an attractive target for future cancer therapy. There is emerging evidence that Pdcd4 impacts angiogenesis. Aerosol delivery of Pdcd4 into non-small cell lung cancer model mice (K-ras null) inhibited angiogenesis and both proangiogenic factors VEGF and

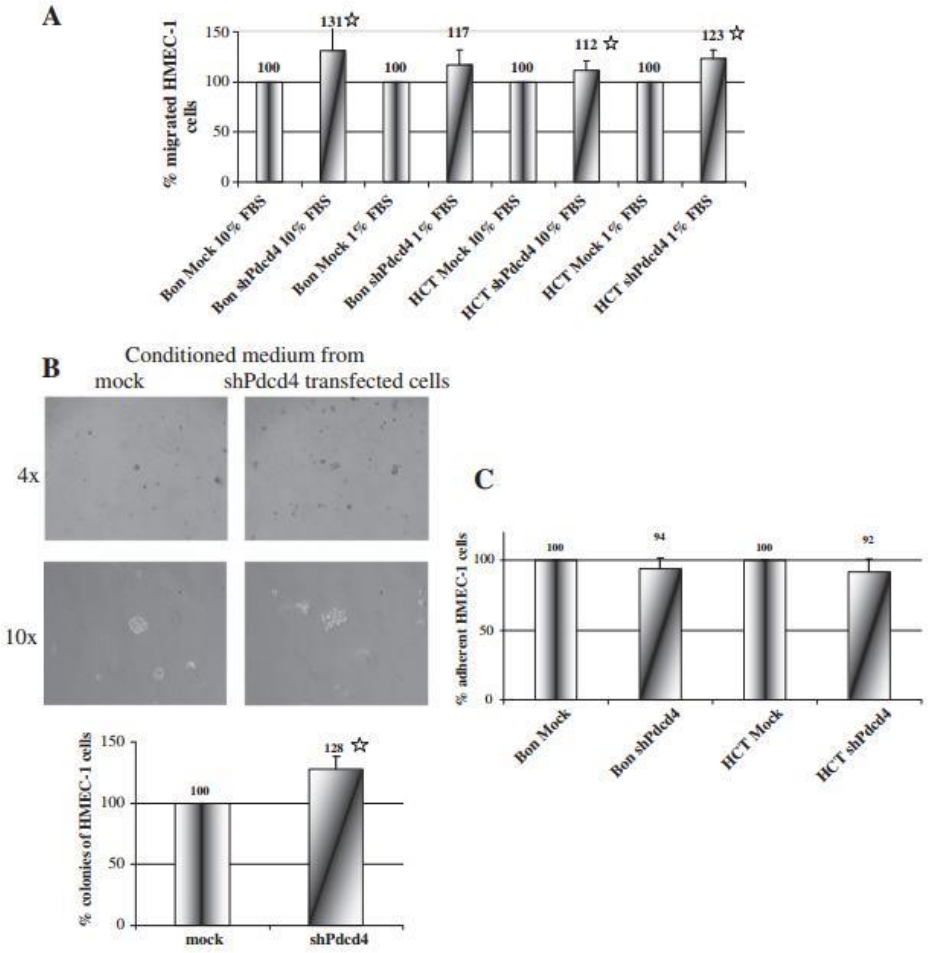

Fig. 8. Impact of conditioned medium from shPdcd4 transfected Bon-1 and HCT116 cell lines on endothelial HMEC-1 cells. A: Cell migration of HMEC-1 cells in Boyden Chamber assays. Conditioned medium from mock and shPdcd4 transfected Bon-1 and HCT116 cells containing 1% and 10% FBS was used. Stars (☆) represent significant p-values ($p<0.05$). B: Colony formation of HMEC-1 cells in soft agar assays and treatment with conditioned medium from mock and shPdcd4 transfected cells. As an example two photographs were shown with a 4× magnification and the centers with a 10× magnification. C: HMEC-1 adhesion to plates coated with conditioned shPdcd4 medium in comparison to conditioned medium from mock transfected Bon-1 and HCT116 cells. Reduced adhesion was not significant ($p=0.082$ and 0.054) but a trend was visible.

FGF-2 were suppressed by Pdcd4 [43]. Although Ang-2 was not examined it maybe speculated that its levels were as well reduced by high amounts of Pdcd4. Furthermore, another study demonstrates that Ang-2 was over-expressed in non-small cell lung cancer and was associated with high vascularization [40]. Previously, we have shown that Pdcd4 knockdown results in enhanced release of secretogranin II [10]. The Sg II derived peptide secretoneurin can function as a proangiogenic factor, indicating that Pdcd4 alters angiogenesis by regulating different proteins.

In the present study, we show for the first time that Ang-2 expression is controlled by Pdcd4 in a wide variety of cell types. This provides a new mechanism for regulation of Ang-2 and, thereby, for an influence on vessel formation. In this context it is of special interest, that over-expression of Ang-2 promoted metastasis in breast cancer cells and leads to suppression of E-cadherin, induced Snail expression and phosphorylation of GSK-3β [44]. Therefore, Ang-2 is not only able to boost cancer progression by stimulating tumor vessel formation, but also to convey metastasis. Taken together, even if the functional network of Ang-2 and tumor microenvironment is not fully understood yet, the regulation of Ang-2 expression by Pdcd4 may provide a promising target for cancer therapy. It will be most fascinating to further investigate the complex functions of the tumor suppressor Pdcd4.

5. Conclusions

The tumor suppressor Pdcd4 influences cancer development, growth and metastasis by multiple mechanisms. A completely new mode is the regulation of Ang-2 expression and secretion by Pdcd4

levels. Enhanced Ang-2 release enables neovascularization of tumor tissues and, thereby, a stimulation of tumor growth can occur.

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Zusammenfassung: Biochim Biophys Acta 2012, 1823(4):789-799

Der Tumorsuppressor Pdcd4 (programmed cell-death 4) zeigte in Vorarbeiten der Arbeitsgruppe auf verschiedenen Ebenen eine Beeinflussung von Schlüsselfunktionen in neuroendokrinen Tumoren u.a. eine Regulation der Expression und Sekretion der Glykoproteine Chromogranin A und Sekretogranin II (Chromogranin C) in der neuroendokrinen Tumorzelllinie Bon1. Daraufhin führten wir in Bon1 Zellen einen stabilen knockdown von Pdcd4 durch und konnten in cDNA Mikro-Array Analysen eine deutliche Regulation von Angiopoietin-2 (Ang-2) mRNA detektieren. Western Blot Untersuchungen zeigten die Hochregulation von Ang-2 unter Reduktion von Pdcd4 auf Proteinebene. Mittels siRNA Technologie und transienter Transfektion unter Repression von Pdcd4 wurden diese Ergebnisse in Bon1 und weiteren nicht neuroendokrinen Tumorzelllinien bestätigt. Neben der gesteigerten endogenen Expression zeigte sich zusätzlich auch eine vermehrte Sekretion von Ang-2 in das Medium von Bon1 Zellen. Die Stimulation von Ang-2 wurde vorzugweise durch die Induktion von AP-1 vermittelt, während unsere Analysen des PI3K/Akt/mTOR und MAPK/ERK Signalweges differente Ergebnisse präsentierten. In funktionellen Studien untersuchten wir den Effekt von Überstand der shPdcd4 Bon1 Zellen auf Wildtyp Bon1 Zellen. Dabei zeigte sich in MTT und Soft Agar Assays eine gesteigerte Proliferation und Kolonieformationsbildung, während Untersuchungen auf Adhäsionsebene, auf mit konditioniertem Medium beschichteten Multiwell Platten, eine verringerte Bon1 Adhäsion präsentierte und ein Hinweis auf ein gesteigertes Metastasierungspotential darstellt. Neben den parakrinen Mechanismus untersuchten wir den Effekt von konditioniertem Medium auf die humane Endothelzelllinie HMEC-1 (human immortalized microvascular endothelial cell line). Der Einfluss auf Zelladhäsion und Kolonieformation wurde ebenso in HMEC-1 Zellen validiert. Beeindruckende

Ergebnisse wurden allerdings in Tube Formation Assays gesehen. Mittels Software-basierter Auswertung zeigte sich eine signifikant gesteigerte Angiogenese von HMEC-1 Zellen in Matrigel durch die Ausbildung von größeren und verzweigteren Gefäßen. Unter Anwendung von rekombinantem Ang-2 auf HMEC-1 Zellen wurde der exklusive Einfluss von Ang-2 aus dem Mediumüberstand von shPdcd4 Bon1 Zellen bestätigt, da sich ähnliche Effekten auf die Gefäßausbildung präsentierten. Neben der Gefäßneubildung wurde in Migrations-Assays ebenso ein promigratorischer Effekt der HMEC-1 Zellen festgestellt. Zusammenfassend beeinflusst Pdcd4 die Tumorentwicklung, das Wachstum und die Metastasierung über multiple Mechanismen. Neu ist allerdings die Regulation der Expression und Sekretion von Ang-2 durch Pdcd4. Die Ang-2-VEGF Achse ist beschrieben als wichtiger Regulator der Tumorangiogenese. Die gesteigerte Ang-2 Bildung ist damit ein Mediator der Neoangiogenese und Tumor-fördernder Mechanismen in neuroendokrinen Tumorzellen.

Research | S Krug et al. | Role of CUX1 in pancreatic neuroendocrine neoplasms | 21:6 | 879–890

CUX1: a modulator of tumour aggressiveness in pancreatic neuroendocrine neoplasms

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Abstract

Pancreatic neuroendocrine neoplasms (PNENs) constitute a rare tumour entity, and prognosis and treatment options depend on tumour-mediating hallmarks such as angiogenesis, proliferation rate and resistance to apoptosis. The molecular pathways that determine the malignant phenotype are still insufficiently understood and this has limited the use of effective combination therapies in the past. In this study, we aimed to characterise the effect of the oncogenic transcription factor Cut homeobox 1 (*CUX1*) on proliferation, resistance to apoptosis and angiogenesis in murine and human PNENs. The expression and function of *CUX1* were analysed using knockdown and overexpression strategies in Ins-1 and Bon-1 cells, xenograft models and a genetically engineered mouse model of insulinoma (RIP1Tag2). Regulation of angiogenesis was assessed using RNA profiling and functional tube-formation assays in HMEC-1 cells. Finally, *CUX1* expression was assessed in a tissue microarray of 59 human insulinomas and correlated with clinicopathological data. *CUX1* expression was upregulated during tumour progression in a time- and stage-dependent manner in the RIP1Tag2 model, and associated with pro-invasive and metastatic features of human insulinomas. Endogenous and recombinant *CUX1* expression increased tumour cell proliferation, tumour growth, resistance to apoptosis, and angiogenesis *in vitro* and *in vivo*. Mechanistically, the pro-angiogenic effect of *CUX1* was mediated via upregulation of effectors such as HIF1α and MMP9. *CUX1* mediates an invasive pro-angiogenic phenotype and is associated with malignant behaviour in human insulinomas.

Key Words
- CUX1
- pancreatic neuroendocrine neoplasms
- angiogenesis
- RIP1Tag2
- insulinomas

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Endocrine-Related Cancer

Introduction

Gastroenteropancreatic neuroendocrine neoplasms (GEP–NENs) represent a heterogeneous group of solid cancers, on the basis of on embryogenic, morphological and biochemical findings (Modlin *et al.* 2008, Rindi & Wiedenmann 2012). The incidence and prevalence of neuroendocrine neoplasms (NENs) are constantly increasing, with an estimated number of 5.76/100 000 and 35/100 000 respectively (Yao *et al.* 2008, Lawrence *et al.* 2011). The subgroup of pancreatic NENs (PNENs) is one of the top three entities in NEN, with a reported prevalence

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of between 6 and 25% (Pape *et al.* 2008, Lawrence *et al.* 2011). The prognosis, time to progression and response to treatment of PNENs largely depend on tumour-mediating hallmarks such as proliferation rate and resistance to drug-induced apoptosis. Metastasised PNENs show a poor 5-year survival rate of 37.6% with a median overall survival (OS) of 18.9 months (Panzuto *et al.* 2011, 2012, Falconi *et al.* 2012, Larghi *et al.* 2012).

The most frequent genetic alterations in PNENs are inactivating mutations of the *MEN1* and *DAXX/ATRX* genes, dysregulation of the PI3K/AKT/mTOR signalling pathway and overactivation of growth factors and their receptors, such as VEGF PDGF, IGF and c-KIT (Grothey & Galanis 2009, Missiaglia *et al.* 2010, Jiao *et al.* 2011). Increased tumour angiogenesis is a hallmark histological feature of PNENs and is generally correlated with aggressive tumour biology and disease progression (Grothey & Galanis 2009). Preclinical data for an established genetically engineered mouse model of insulinomas (RIP1Tag2) revealed promising activity of the multi-tyrosine-kinase inhibitor sunitinib, which has been approved by the FDA for the treatment of progressive, unresectable, locally advanced or metastatic PNENs (Olson *et al.* 2011, Raymond *et al.* 2011). However, although sunitinib significantly prolonged the progression-free survival (PFS) in metastasised PNENs, it failed to improve the OS, and surrogate markers that predict the response to treatment are currently not available (Raymond *et al.* 2011). Therefore, further molecular characterisation and functional validation of novel targets are urgently needed and hold promise of classifying PNENs into several prognostic and therapeutic subgroups that can be employed for combined and personalised treatment strategies in the future (Capurso *et al.* 2009, 2012).

The transcription factor Cut homeobox 1 (*CUX1*), also called CCAAT-displacement protein (*CDP*) or Cut-like1 (*CUTL1*), is located on chromosomal band 7q22.1 and is expressed in multiple isoforms. Hence, *CUX1* is involved in repressive and activating transcriptional processes depending on the promoter regulation. Results described in recent reports have indicated an important role for *CUX1* in tumourigenesis and tumour progression (Hulea & Nepveu 2012). We have shown that *CUX1* directly mediates tumour cell proliferation, migration and invasiveness and also acts via the target genes *GRIA3* and *WNT5A* in the development of pancreatic ductal adenocarcinomas (PDACs; Aleksic *et al.* 2007, Ripka *et al.* 2007, 2010*a*, *b*, Griesmann *et al.* 2013). Moreover, *CUX1* expression is strongly associated with a less differentiated phenotype and decreased survival in patients with breast cancer (Michl *et al.* 2005). So far, the expression and function of *CUX1* in PNENs have not, to our knowledge, been investigated. In this study, we aimed to elucidate the effects of *CUX1* on tumour growth and progression in neuroendocrine tumour cell lines as well as in a xenografts and the RIP1Tag2 model *in vitro* and *in vivo*. In addition, we investigated potential mechanisms by which *CUX1* mediates angiogenesis, and assessed *CUX1* expression in a series of human insulinomas.

Materials and methods

Material and cell lines

All media contained 10% fetal bovine serum and 40 μg/ml gentamicin. The cell lines were cultured in the indicated media: human Bon-1 carcinoid cells (a kind gift of R Göke, University of Marburg, Germany) (Parekh *et al.* 1994, passage 10–30) were cultured in DMEM/HAM's F12 medium; rat Ins-1 insulinoma cells (donated by C B Wollheim, University of Geneva, Switzerland) (Asfari *et al.* 1992, Lankat-Buttgereit *et al.* 2004, passage 80) were cultured in RPMI1640 medium supplemented with additional 1 mM sodium pyruvate, 10 mM HEPES and 0.05 mM 2-β-mercaptoethanol; HMEC-1 human immortalised microvascular endothelial cells (courtesy of Dr R Köhler, Department of Internal Medicine – Nephrology, Philipps-University, Marburg, Germany (Ades *et al.* 1992)) were cultured in DMEM high-glucose with L-glutamine and 1 mM sodium pyruvate. All cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C. The cell number was analysed by counting the cells in a Neubauer chamber. Thereafter, in our laboratory, the cells were used at passages 8–11. The conditioned media were obtained from stably *CUX1*-expressing Bon-1 cells cultured for 3 days with approximately 70% confluency. The amphotropic packaging cell line LinX was maintained in DMEM, 10% fetal bovine serum, 250 μg/ml gentamicin and 100 μg/ml hygromycin B (Roth, Karlsruhe, Germany). 5-Fluorouracil (5-FU) was purchased from Sigma-Aldrich and tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) was obtained from Lilly (Bad Homburg, Germany). Synchronization of cells was not performed before functional assays.

Plasmids, siRNA and retroviral infection

CUX1 was transiently suppressed by using two different oligonucleotides (*CUX1_1* and *CUX1_2*) as described previously (Michl *et al.* 2005). All assays were confirmed using two different siRNA sequences to minimise the risk

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of possible off-target effects. siRNA oligonucleotides were purchased from Ambion (Austin, TX, USA). Both silencing sequences resulted in a knockdown efficiency of over 70% (Ripka *et al.* 2010*a*). Stable Bon-1 and Ins-1 cells were generated using retroviral systems. For retroviral expression, *CUX1* was amplified and cloned into the pENTR-vector using the pENTR/D-TOPO Cloning Kit (Invitrogen) and recombined into a Gateway competent pQCXIP (a gift from T Stiewe). To produce retroviruses, we transfected LinX packaging cells with 5 μg of retroviral vectors; 48 and 72 h after transfection, the retrovirus-containing supernatant was harvested, filtered and supplemented with 8 μg/ml polybrene (Sigma). The target cells were transduced by spin infection at 252 ***g***, at 37 °C for 1 h and selected with puromycin and G418.

RNA isolation, cDNA synthesis and real-time PCR

Murine RIP1Tag2 RNA isolation was done as previously described (Fendrich *et al.* 2011). The RNA was extracted using the RNeasy Mini Kit (Qiagen), and first-strand cDNA was synthesised using random hexamer primers and Superscript II reverse transcriptase (Invitrogen). Quantitative RT-PCR analysis was carried out using an Applied Biosystems 7500 Fast Real-time PCR machine using the SYBR Green PCR Master Mix Kit (Applied Biosystems) according to the manufacturer's instructions. Sequence-specific primer pairs were designed using the Primer Express Software (Applied Biosystems). RPLP0 was used as an internal standard (XS-13: forward, 5′-GTC GGA GGA GTC GGA CGA G-3′ and reverse, 5′-GCC TTT ATT TCC TTG TTT TGC AAA-3′). The primer sequences for *CUX1* are forward 5′-GAC GTG TTG CGC ACT TAA CG-3′ and reverse 5′-ACG ACA TAG ATT GGG CTT AAT GCT-3′. Human angiogenesis-pathway-focused gene expression profiling comprising 84 genes was performed using the RT^2 Profiler PCR Array System (Qiagen) according to the manufacturer's instructions. A list of the genes analysed in this profiler is available online (http://sabiosciences.com/Manual/1062756).

Immunoblotting

The cells were lysed in RIPA buffer supplemented with complete protease inhibitor cocktail (Roche). Nuclear and cytoplasmic fractions were obtained using the ProteoJET Cytoplasmic and Nuclear Protein Extraction Kit (Fermentas, St Leon-Rot, Germany). Immunoblottings were carried out as described previously (Ripka *et al.* 2010*a*).

Viability, proliferation and apoptosis assays

Bromodeoxyuridine (BrdU) incorporation was measured by using the colorimetric BrdU Cell Proliferation ELISA (Roche Diagnostics) according to the manufacturer's instructions. Histone-associated DNA fragments were quantified using Cell Death Detection ELISA PLUS (Roche) according to the manufacturer's instructions. Cell viability was determined by using the MTT Cell Proliferation Kit (Roche Diagnostics) according to the manufacturer's instructions.

Cell migration experiments

The cell migration experiments were carried out as described previously (Michl *et al.* 2005). Briefly, 40 000 cells in 500 μl/insert on a 24-well plate were used and incubated for 4 h. The inserts were then rinsed with PBS, dried at room temperature and fixed with methanol. Migration was measured by counting ten fields of view under a microscope covering about 70–80% of the insert area of the fixed and stained cells (0.2% crystal violet) migrating towards the conditioned media and normalised to the number of cells that migrated towards mock conditioned media. The number of migrated cells was also validated using the via TimeLapseAnalyzer program (Huth *et al.* 2010).

Tube formation assays

BD Matrigel Basement Membrane Matrix Growth Factor Reduced (BD Bioscience, Heidelberg, Baden-Württemberg, Germany) was thawed and plated according to the supplier's protocol. Briefly, 20 000 cells of the human microvascular endothelial cell line HMEC-1 suspended in the appropriate conditioned media of *CUX1* overexpressing Bon-1 cells were plated onto the Matrigel. After incubation for 24 h, at 37 °C, the wells were photographed at a fourfold magnification using a Canon 450 EOS camera connected to the microscope (Olympus IMT2). The length and junctions of the generated tubes were analysed using the TimeLapseAnalyzer program (Huth *et al.* 2010). Each experiment was carried out at least in triplicate with independently obtained conditioned media.

Immunohistochemistry, construction of tissue arrays and evaluation

In brief, paraffin sections were stained after antigen retrieval (microwave in antigen-unmasking solution, Vector Laboratories, Burlingame, CA, USA) with rabbit polyclonal anti-CUX1 (1:200), as described previously

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(Michl *et al.* 2005). In addition, CD31 (1:20; Dianova; Hamburg, Germany) and Ki-67 (1:200; Clone SP6; Thermo Fischer Scientific, Fremont, CA, USA) antibodies were used. The binding of antibody was visualised using a biotinylated secondary antibody, avidin-conjugated peroxidase (ABC method; Vector Laboratories), 3,30-diaminobenzidine tetrachloride (DAB) as a substrate and H&E as a counterstain. The insulinoma tissue samples (FFPE) were obtained from the neuroendocrine tumour archives of the Departments of Pathology in Zürich (Switzerland), Düsseldorf (Germany) and Kiel (Germany) from 1975 to 2006 according to the guidelines of the local ethics committee. The 1.5-mm tissue cores were taken from representative areas and inserted into four paraffin blocks. The four MTAs contained a total of 286 cores and additional human tonsil orientation/control cores. The construction was performed using MTA1 tissue arrayer equipment (Beecher Instruments, Sun Prairie, WI, USA). The MTAs were routinely processed for paraffin sectioning. The paraffin sections were deparaffinised and rehydrated and immunohistochemically stained according to routine methods. The analysis was performed by a pathologist with expertise in endocrine and pancreatic tumours (B S) in a blinded fashion regarding tumour parameters. The intensity of the reactions was scored as mild, moderate or strong (score 1, 2 or 3 respectively). The proportion of the positive cells in ducts and tumour areas was estimated as percentage and divided into scores (<10%, 1; 10–50%, 2; 51–80%, 3 and >80%, 4). The final score was determined as a product of the intensity of the staining and the proportion of positive cells (minimum 0 and maximum 12; Remmele *et al.* 1986). The scores for cores derived from the same tissue were averaged.

Animals and xenografts

Athymic female nude mice (nu/nu mice) were purchased from Charles River (Sulzfeld, Germany). The mice were housed in a climate-controlled SPF facility. All animal experiments were approved by the local government authorities and were carried out according to the guidelines of the animal welfare committee. The nude mice received subcutaneous injections of 1×10^6 CUX1-overexpressing or WT Bon-1 cells (10^6 cells of each clone) dispersed in 0.1 ml of normal saline at each flank. Six mice per group received injections of cells stably transfected with either CUX1 or empty vector. The size of the tumours was measured during the course of observation (twice a week) and after the animals had been killed.

Statistical analyses

All data are presented as mean±S.E.M. Two-tailed paired Student's *t*-test was used for statistical evaluation of

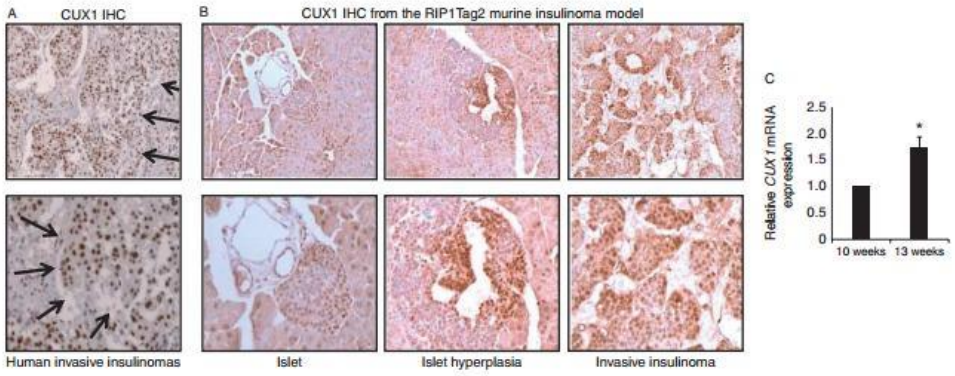

Figure 1

CUX1-expressing human and murine insulinoma tissues. (A) Representative sections of human invasive insulinomas immunohistochemically stained for CUX1 demonstrating a prominent expression of the target (upper row ten× and bottom row 40× magnifications; $n=10$). Arrows indicate the invasive front and highly proliferative areas (B). Increasing *CUX1* expression in correlation with the islet tumour stage ranging from normal islets, hyperplastic islets to invasive insulinomas, depicted at two different magnifications (upper row 10× and bottom row 20×; $n=10$). (C) *CUX1* mRNA levels as measured by RT-PCR of five 10-week-old RIP1Tag2 mice (islet hyperplasia stage) compared with five 13-week-old mice (invasive insulinoma stage). Data were normalised to RPLP0 and are representative of at least five independent experiments and presented as relative expression levels±S.D. (mean: 1.0 vs 1.74; $n=5$, S.D.=0.2; $P=0.011$). *$P<0.05$; IHC, immunohistochemistry.

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the data. The one-way and non-parametric ANOVA test was used to calculate the *P* value for more than two groups. A *P* value <0.05 was considered statistically significant.

Results

CUX1 is expressed in human insulinomas and is upregulated during tumour progression in the RIP1Tag2 model

Immunohistochemistry revealed that *CUX1* is strongly expressed in a series of human insulinomas and shows a particular intense immunoreactivity at the invasive front and in highly proliferative areas (Fig. 1A). To assess *CUX1* expression during tumour progression, we employed an established genetically engineered mouse model of insulinoma (RIP1Tag2 model). The RIP1Tag2 model is a tumour progression model, which has been generated to develop invasive neuroendocrine pancreatic islet neoplasms via hyperplastic and angiogenic precursor stages that closely recapitulate the development of human disease (Hanahan 1985). In this murine model, *CUX1* mRNA and CUX1 protein were increasingly expressed during tumour progression, with the highest levels in invasive insulinomas in comparison with islet hyperplasia and native islets (Fig. 1B and C: statistical analysis of relative *CUX1* mRNA expression: control vs CUX1, mean 1.0 vs 1.7, $P=0.011$). These results indicate that *CUX1* is highly expressed in murine and human PNENs and that *CUX1* may play a role in tumour progression.

CUX1 modulates proliferation, basal and TRAIL-induced apoptosis in neuroendocrine tumour cell lines

In order to study the functional role of CUX1 in neuroendocrine tumour cells, CUX1 was genetically ablated in Ins-1 and Bon-1 cells by transient transfection with two different siRNAs. Knockdown of *CUX1* resulted in a significant reduction in cell viability as well as proliferation rate in both cell lines (Fig. 2A, B and C, Supplementary Figure 1A, B and C, see section on supplementary data given at the end of this article: statistical analysis for siCUX1 versus siC in Ins-1 for MTT, mean: 65% versus 100%, $P=0.013$ and BrdU: mean: 81% versus 100%, $P=0.023$ and Bon-1 for MTT: mean: 74% versus 100%, $P=0.027$ and BrdU: 68% versus 100%, $P=0.012$). Next, we overexpressed *CUX1* by stable transfection of Ins-1 and Bon-1 cells using two different clones with an appropriate empty vector control for each cell line (Fig. 2D and Supplementary Figure 1D). MTT *in vitro* assays (Fig. 2E and Supplementary Figure 1E: statistical analysis for Mock versus *CUX1*-overexpressing after 72 h in Ins-1: mean: 461% versus 657%, $P=0.002$ and Bon-1: mean: 609% versus 741%, $P=0.023$) and BrdU experiments (Fig. 2F and Supplementary Figure 1F: Mock versus *CUX1* in Ins-1: mean: 100% versus 123%, $P=0.0003$ and Bon-1: mean: 100% versus 123%, S.D. 19; $P=0.009$) unequivocally showed robust and statistically significant increase in proliferation in both cell lines compared with the relevant mock control group.

Next, we investigated the effect of *CUX1* on basal and TRAIL-induced apoptosis in Ins-1 and Bon-1 cells using the same experimental setting. Knockdown of *CUX1* induced basal apoptosis in both cell lines, as determined by cell death detection assay (Fig 3A and Supplementary

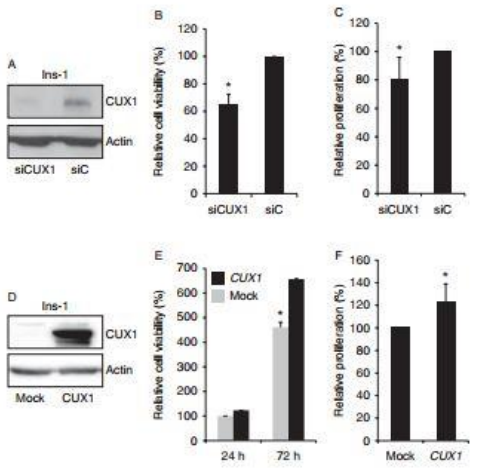

Figure 2
CUX1 affects proliferation in insulinoma cells. (A) Ins-1 cells were transiently transfected with *CUX1* siRNA (siCUX1) or control siRNA (siC). Protein isolation and western blotting analysis were carried out demonstrating the *CUX1* knockdown. (B) Relative cell viability was measured via MTT after 24 h ($n=4$; mean: 65% versus 100%; S.D. 7.5; $P=0.013$). (C) Proliferation after suppression of CUX1 in Ins-1 cells after 24 h was assessed by BrdU assay ($n=4$; mean: 81% versus 100%; S.D. 14.9, $P=0.023$). (D) Confirmation of stable overexpression of CUX1 in Ins-1 cells by western blot analysis. (E) The relative cell viability of *CUX1* overexpressing (CUX1) and empty vector control-transfected cells (Mock) was detected after 24 h ($n=6$; mean: 100% versus 123%, S.D. 4) and 72 h ($n=6$; mean: 461% versus 657%, S.D. 13, $P=0.002$) via MTT assay. (F) Similarly, the relative proliferation of *CUX1*-overexpressing cells in comparison with mock-transfected cells was quantified by BrdU assay ($n=5$; mean: 100% versus 123%, S.D. 16, $P=0.0003$). Averages of the two stable clones each were evaluated and presented. Data are representative of at least three independent experiments and are shown as mean ± S.D. $*P<0.05$ as compared with control cells.

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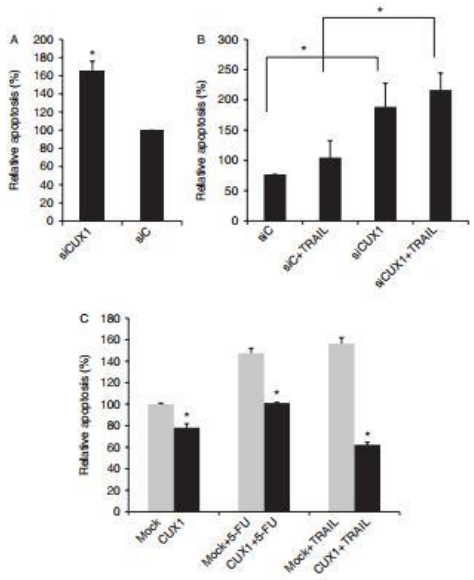

Figure 3
CUX1 overcomes basal and drug-induced apoptosis in insulinoma cells. (A) Knockdown of *CUX1* in Ins-1 cells via siRNA (siCUX1) in comparison with empty vector (siC) ($n=3$; mean: 166 vs 100, s.d. 10, $P=0.001$). Apoptosis was determined by using a specific cell death detection ELISA quantifying the histone-bound DNA fragmentation. (B) Apoptosis after knockdown of *CUX1* by siRNA and apoptosis induced by TRAIL (100 ng) after 24 h, as assessed by a caspase-3/7 activation assay ($n=4$; mean: 100% versus 200% and 124% versus 227%; s.d. 26, 35, 24; siC/ siCUX1, $P=0.019$; siC+TRAIL/siCUX1+TRAIL, $P=0.0043$). (C) Apoptosis in Ins-1 cells stably overexpressing *CUX1* and mock-transfected control cells +/− TRAIL and +/− 5-FU (0.2 mM) for 24 h as assessed by quantification of histone-bound DNA fragmentation ($n=3$; mean: 100 vs 78; 147 vs 101; 156 vs 62; s.d. 1 vs 3.7; 4.6 vs 0.6; 5.7 vs 2.3; Mock/CUX1, $P=0.04$; Mock/CUX1+5-FU, $P=0.011$; Mock/CUX1+TRAIL, $P=0.0023$). Data are representative of at least three independent experiments and are shown as mean±s.d. *$P<0.05$ as compared with control cells.

Figure 2A: statistical analysis siCUX1 versus siC in Ins-1: mean: 166% versus 100%, $P=0.001$ and Bon-1: mean: 133% versus 100%; $P=0.021$). Interestingly, Ins-1 cells were more sensitive than Bon-1 cells to the suppression of *CUX1*, resulting in a significant increase in apoptosis without any apoptotic stimulus. In addition, knockdown of *CUX1* significantly enhanced TRAIL-induced apoptosis, whereas treatment with TRAIL alone had only a minor effect on apoptosis induction, as measured by effector caspase-3/7 activity (Fig. 3B and Supplementary Figure 2B: statistical analysis for Ins-1: mean: 100% versus 200% and 124% versus 227%, siC/siCUX1, $P=0.019$; siC+TRAIL/siCUX1+TRAIL, $P=0.0043$ and Bon-1: mean: 100% versus 298% and 110% versus 413%, siC/siC+TRAIL, $P=0.001$; siCUX1/siCUX1+TRAIL, $P=0.0031$). TRAIL-induced apoptosis was more pronounced in Bon-1 cells, probably due to the fact that these cells were highly proliferative and less differentiated. To confirm the anti-apoptotic effects of CUX1, we additionally carried out western blotting for cleaved caspase-3: knockdown of *CUX1* increased cleaved caspase-3 levels (Supplementary Figure 3A).

To exclude the possibility that only siRNA-mediated suppression of endogenous CUX1 affects apoptosis due to off-target effects, we assessed whether overexpression of *CUX1* is able to protect cells from TRAIL- and 5-FU-induced apoptosis. Upon *CUX1* overexpression, basal apoptosis rates were strongly reduced, as determined by cell death detection assays (Fig. 3C and Supplementary Figure 2C, see section on supplementary data given at the end of this article: statistical analysis for Ins-1: Mock/*CUX1*, Mock/*CUX1*+5-FU and Mock/*CUX1*+TRAIL: mean: 100% versus 78%, 147% versus 101%, and 156% versus 62%; $P=0.04$, $P=0.011$, and $P=0.0023$, respectively and Bon-1: Mock/*CUX1*, Mock/*CUX1*+5-FU and Mock/*CUX1*+TRAIL: mean: 100% versus 43%, 30% versus 32%, and 167% versus 129%; $P=0.003$, $P=0.8$, and $P=0.024$, respectively). Furthermore, *CUX1* expression protected Ins-1 and Bon-1 cells from TRAIL-induced apoptosis (Fig. 3C and Supplementary Figures 2C and 3B). Interestingly, while CUX1 affected cell viability and protected cells from TRAIL-induced apoptosis in both cell lines, it affected 5-FU-induced apoptosis only in Ins-1 cells (Fig. 3C and Supplementary Figure 2C). Taken together, these results indicate that *CUX1* mediates pro-proliferative and anti-apoptotic effects in neuroendocrine tumour cell lines.

CUX1 increases tube formation in HMEC-1 cells via tumour cell-derived secreted pro-angiogenic mediators

One of the most important features of NENs is the high vascularisation that is established and maintained by a complex molecular and cellular crosstalk between neoplastic cells and endothelial cells. To assess the potential angiogenic effects of *CUX1*, we used an established tube formation assay. The conditioned medium from CUX1-overexpressing neuroendocrine tumour cells was used to stimulate human immortalised microvascular endothelial cells (HMEC-1) in order to assess the ability of endothelial cells to generate vessels. After 24 h stimulation with Ins-1 and Bon-1 cell supernatants, HMEC-1 cells displayed

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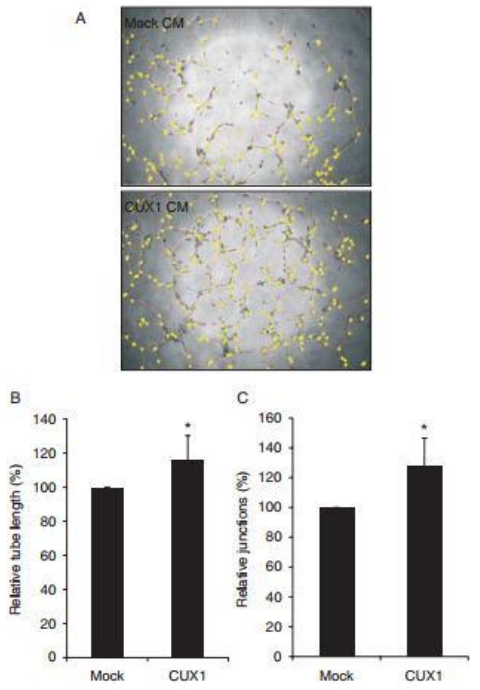

Figure 4
CUX1 augments angiogenesis in tube formation assays. (A) Representative pictures of HMEC-1 cells incubated with mock or CUX1-conditioned medium from Bon-1 cells. Analysis of tube formation assay by determining length (red lines) and junctions (yellow dots) of tubes evaluated using TimeLapseAnalyser program (B and C). Calculation of length (mean: 100% versus 116%; s.d. 14.6; $P=0.044$) and junctions (mean: 100% versus 128%; s.d. 18.9; $P=0.022$) from pictures were done from at least nine independent experiments. Photographs were taken at 4× magnification. Tube formation was performed with conditioned medium from Ins-1 cells stably transfected with CUX1 compared with mock-transfected cells. *$P<0.05$ as compared with control cells.

significant induction of tube formation in a CUX1-dependent manner (Fig. 4A, B and C, Supplementary Figure 4A and B, see section on supplementary data given at the end of this article: statistical analysis of Ins-1 Mock CM and CUX1 CM: length: mean: 100% versus 116%; $P=0.044$ and junctions: mean: 100% versus 128%; $P=0.022$ and Bon-1: length: mean: 100% versus 115%; $P=0.012$ and junctions: mean: 100% versus 132%; $P=0.006$). Analysis of the assays, using the TimeLapseAnalyzer program, from at least five independent experiments demonstrated a remarkable increase in tube length and junctions (Fig. 4A, B and C and Supplementary Figure 4A and B). In contrast to tube length and formation of junctions, HMEC-1 migration was not altered significantly (Supplementary Figure 5: statistical analysis of Ins-1-CM: mean: 100% versus 118%; $P=0.08$ and Bon-1-CM: mean 100% versus 120%; $P=0.06$).

To elucidate the transcriptional alterations underlying this *CUX1*-dependent pro-angiogenic phenotype, we used a human angiogenesis-pathway-focused gene expression profiler comprising 84 genes to examine potential CUX1-regulated targets at the mRNA level in *CUX1*-overexpressing Bon-1 cells (Supplementary Table 1, see section on supplementary data given at the end of this article). Among others, the hypoxia-induced transcription factor

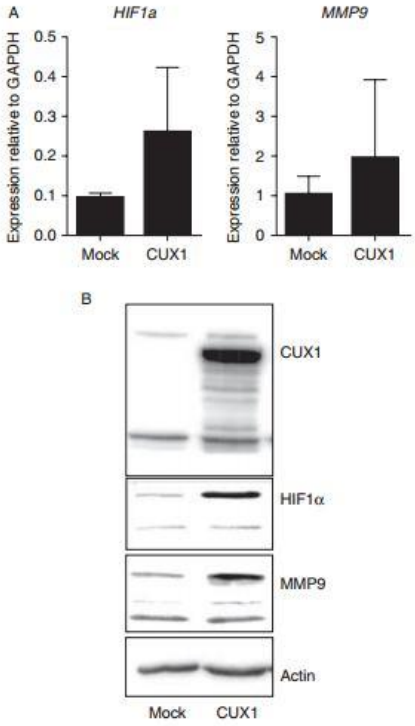

Figure 5
CUX1 modulates the expression of angiogenic markers in neuroendocrine cells. (A) QRT-PCR analysis for *HIF1α* and *MMP9* mRNA in CUX1-over-expressing and mock transfected Bon-1 cells. *HIF1α*: $n=3$; mean: 0.09 vs 0.26; s.d. 0.009 vs 0.11; $P=0.1$ and *MMP9*: $n=3$; mean: 1.0 vs 1.9; s.d. 0.23 vs 1.2 $P=0.05$. (B) Confirmation of the CUX1-dependent regulation of HIF1α and MMP9 at the protein level by western blotting analysis. Actin served as a control for equal protein loading.

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HIF1α as well as the matrix metalloproteases *MMP9* were consistently induced by CUX1. Both genes could be confirmed to be modulated by CUX1 at the mRNA and protein level (Fig. 5A and B: statistical analysis for mRNA expression relative to GAPDH in Bon-1 Mock and CUX1-overexpressing cells: *HIF1α*: mean: 0.09 vs 0.26; $P=0.1$ and *MMP9*: 1.0 vs 1.9; $P=0.05$). These results indicate that *CUX1* expression stimulates angiogenesis in endothelial cells *in vitro*, with a number of differentially regulated pro-angiogenic effector molecules indicating an important mechanistic crosstalk between the secretome of neuroendocrine tumour cells and endothelial cells during angiogenesis (Supplementary Figures 6, 7 and 8).

CUX1 expression correlates with tumour-mediating hallmarks in murine and human insulinomas

To validate our *in vitro* findings on the role of CUX1 as a regulator of proliferation and angiogenesis, xenograft experiments were carried out on nude mice using CUX1-transfected Bon-1 cells. Expression of CUX1 was confirmed by western blotting analysis in $n=4$ mice each at endpoint (Fig. 6B). *CUX1* tumours showed a marked trend towards increased tumour volumes 7 weeks after implantation which, however, did not reach statistical significance due to the limited number of animals (Fig. 6A: statistical analysis: mean: 134 mm versus 226 mm; $P=0.26$).

In line with our *in vitro* data, *CUX1* expression *in vivo* caused a significant increase in tumour cell proliferation (Mock versus CUX1: mean: 14.6% versus 37.5%; $P=0.0045$), as assessed by Ki-67 immunohistochemistry, but failed to increase mean vessel density (Mock versus CUX1: mean: 26.4 versus 38.6 positive cells per visual field; $P=0.074$), as determined by CD31 immunohistochemistry (Fig. 6C and E). Despite the fact that grafting of *CUX1*-expressing Bon-1 cells resulted in larger and more proliferative xenograft tumours, systematic histological analysis of H&E sections showed a significant reduction in the necrotic areas of *CUX1*-expressing xenografts (Fig. 6D: statistical analysis Mock versus CUX1: mean: 49.7% versus 24.8%; $P=0.027$).

As *CUX1* is involved in the tumour progression *in vitro* and *in vivo*, we sought to elucidate the clinical relevance of *CUX1* in tumour progression and metastasis by studying *CUX1* expression in human insulinoma samples. To this end, we used MTAs containing 59 different human insulinomas tissues for immunohistochemical analysis. In the entire cohort, samples from 39 female and 20 male patients were included (Supplementary Table 2, see section on supplementary data given at the end of this article): 52 primary tumours, three lymph nodes and four liver metastases were immunohistochemically investigated. Overall, 45 tumours exhibited a benign behaviour in comparison with 14 malignant tumours. CUX1 showed a distinct nuclear expression in islets, as well as benign and malignant insulinomas. Out of 59 insulinomas, 58 were CUX1 positive. Importantly, malignant tumours revealed significantly increased immunoreactivity for CUX1 compared with those with benign behaviour (immunoreactivity score (IRS) 8.8 versus 5.8, $P=0.002$; Table 1, Fig. 7A and B). Metastases scored higher for CUX1 positivity than primary tumours ($P=0.018$), independently of their localisation (lymph node or liver). In addition, a significant correlation between CUX1 expression and G1/G2 grading was observed ($P=0.038$), indicating a connection between CUX1 and proliferation, because tumour grading is based on the Ki-67 proliferation index. Among the 14 patients with malignant insulinoma,

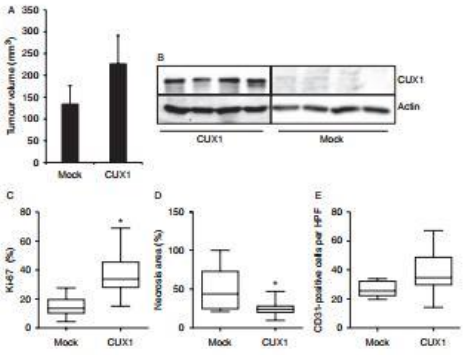

Figure 6
CUX1 influences tumour growth via acceleration of proliferation and neovascularisation *in vivo*. (A) Nude mice received subcutaneous injections of 1×10^6 CUX1-overexpressing or mock-transfected Bon-1 cells (10^6 cells of each clone). Six mice per group received injections. Tumours were measured *ex vivo* after the animals had been killed (mean: 134 mm versus 226 mm; s.d. 42 vs 65; $P=0.26$). (B) Representative expression of CUX1 in protein lysates of CUX1 overexpressing or mock-transfected Bon-1 xenografts, for which material for protein lysates was available. (C, D and E) Immunohistochemistry of Ki-67 and CD31 as well as H&E evaluation in tumour tissues +/− CUX1 overexpression. (C) The proliferation index of the xenograft tumours was estimated using the antibody against Ki-67 ($n=8$; mean: 14.6% versus 37.5%; s.d. 3.1 versus 4.8; $P=0.0045$). (D) Necrosis areas were quantified and evaluated comparing CUX1- and mock-transfected xenografts ($n=8$; mean: 49.7% versus 24.8%; s.d. 12.2 versus 3.1; $P=0.027$). (E) Proportion of endothelial cells per visual field in sections of CUX- or mock-tumours as measured using the CD31 antibody which detects endothelial cells ($n=8$; mean: 26.4 versus 38.6 CD31-positive cells per visual field; s.d. = 2.4 vs 4.6; $P=0.074$). Data are shown as mean ± s.d. *$P<0.05$ as compared with mock plasmid.

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Table 1]*CUX1* expression is increased in malignant insulinomas. *CUX1* expression was evaluated using the Remmele and Stegner immunoreactive score as described in 'Materials and methods'

	CUX1 score (0–12)		
WHO 2010	Mean	S.E.M.	**ANOVA**
Grade 1 ($n=43$)	5.93	3.42	
Grade 2 ($n=12$)	8.07	2.76	$P=0.038$
Site			
Primary benign ($n=45$)	5.84	3.1	
Primary malignant ($n=7$)	8.42	3.91	
Node metastasis ($n=3$)	10	1.73	
Liver metastasis ($n=4$)	9.25	3.77	$P=0.018$
Behaviour			
Benign ($n=45$)	5.84	3.1	
Malignant ($n=14$)	9.0	3.35	$P=0.002$

Data are shown as mean ± S.E.M.

five disease-related deaths occurred, none in the benign group (Supplementary Table 2). The OS of 36.0 months in patients with malignant insulinomas demonstrated the poor prognosis of this tumour entity.

In summary, our *in vivo* data indicate that *CUX1* mediates tumour progression and angiogenesis in murine neuroendocrine tumours and is associated with malignant behaviour in human insulinomas.

Discussion

PNENs represent the second most common malignant disease of the pancreas with the majority of patients presenting at a metastasised and unresectable stage. The mTOR inhibitor everolimus and the multi-target anti-angiogenic agent sunitinib have recently been introduced for the treatment of progressive, unresectable, locally advanced or metastatic PNENs, but still fail to prolong OS (Raymond *et al.* 2011, Yao *et al.* 2011). Therefore, novel therapeutic approaches and predictive biomarkers are needed to classify patients into subgroups to better predict treatment success and to guide treatment by molecular rationales. Furthermore, molecular and biochemical characterisation of novel targets in PNENs may aid in devising new treatment approaches and treatment combinations that can be deployed upon primary and secondary chemoresistance.

CUX1 has been identified as a transcriptional activator as well as a transcriptional repressor, and its activity has been associated with tumour progression and shortened survival in several tumour entities (Michl *et al.* 2005, Ripka *et al.* 2010*a*).

Based on several studies on PDAC, CUX1 was found to co-opt Src and other downstream signalling molecules such as WNT5A to induce an invasive phenotype in pancreatic cancer (Aleksic *et al.* 2007, Ripka *et al.* 2007, 2010*b*, Griesmann *et al.* 2013). Moreover, *CUX1* has been described as transcriptional target of TGFB, mediating cell motility via influencing migration and invasion in diverse *in vitro* and *in vivo* systems (Michl *et al.* 2005). Previous results indicated that *CUX1* is also a major mediator of PI3K/AKT-induced tumour cell survival (Ripka *et al.* 2010*a*).

Although PDAC and PNENs are distinctly different in terms of molecular evolution, clinical behaviour, prognosis and treatment options, there seems to be a considerable molecular overlap in terms of dysregulated pathways in PDAC and PNENs. For instance, the PI3K/AKT/mTOR pathway, the TGFB pathway and Src family kinase activity have been implicated in the pathogenesis of PNENs (Di Florio *et al.* 2007, 2011, Ghayouri *et al.* 2010, Missiaglia *et al.* 2010, Capdevila &

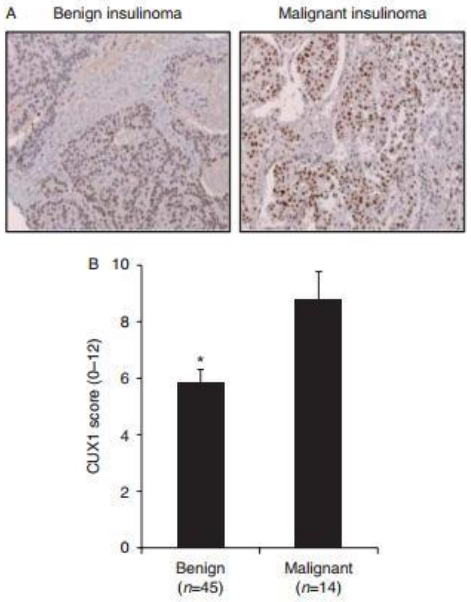

Figure 7
CUX1 expression is increased in malignant insulinomas. (A) Comparison of representative CUX1 IHC in benign and malignant insulinomas at 10× magnification. (B) CUX1 score assessed in human benign and malignant insulinomas (mean: 5.8 versus 8.8; S.E.M. 0.47 versus 0.98; $P=0.002$). Data are shown as mean ± S.E.M. (B). *$P<0.05$ as compared with benign insulinomas.

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Tabernero 2011). Therefore, we aimed to investigate the expression and molecular function of the oncogenic transcription factor *CUX1* in neuroendocrine tumours.

We showed that *CUX1* is a mediator of tumour progression in two neuroendocrine tumour cell lines and xenograft tumours by promoting proliferation, tumour growth and resistance to apoptosis. Importantly, 5-FU-containing regimens represent a current standard of care treatment in combination with streptozocin (Moertel *et al.* 1992, Falconi *et al.* 2012), and in fact CUX1 protected cells against 5-FU-induced apoptosis in the insulinoma cell line thus indicates the potential therapeutic relevance of our findings.

Interestingly, in xenograft experiments, *CUX1* overexpression reduced necrotic areas which might be attributable to *CUX1*-induced improvements in perfusion and angiogenesis in this experimental setting.

Furthermore, we identify *CUX1* as novel modulator of tumour angiogenesis *in vitro* and *in vivo* by paracrine stimulation of endothelial cells via a CUX1-dependent, pro-angiogenic secretome in neuroendocrine tumour cells. Surprisingly, the classic mediators of angiogenesis, VEGF, FGF and PDGF, revealed no differences in response to *CUX1* expression. However, we found that MMP9 and HIF1α were both upregulated in *CUX1*-overexpressing cells, and both proteins have been implicated in tumour progression and angiogenesis (Couvelard *et al.* 2008). For instance, MMP9 has been identified as critical modulator of the angiogenic switch in the RIP1Tag2 model (Bergers *et al.* 2000). Interestingly, MMP9 does not only promote angiogenesis but also interacts with VEGF, facilitating its expression and release (Bergers *et al.* 2000).

Finally, to evaluate the clinical significance of *CUX1* in human PNENs, we carried out *CUX1* immunohistochemistry on a large cohort of human insulinomas. Our results indicate that *CUX1* is strongly expressed in the majority of insulinoma tissues. Moreover, malignant invasive insulinomas expressed more *CUX1* compared with those with benign behaviour, and metastatic tumours were slightly more positive for CUX1 than primary tumours. In analogy to our findings, CUX1 has been reported to be overexpressed in breast and pancreatic PDAC, and inversely correlated with the patients' outcome in breast cancer (Michl *et al.* 2005, Ripka *et al.* 2010*a*). Data on the clinical follow-up of our patients confirmed that surgical cure is feasible in most cases of patients with benign insulinomas. However, malignant insulinomas presented with an aggressive behaviour associated a shortened OS of approximately 35 months. Based on our data on insulinomas, we are currently planning to investigate and correlate *CUX1* expression with clinicopathological features and outcome in patients with human non-functional pancreatic neoplasms.

In summary, our results indicate for the first time, to our knowledge, an involvement of the transcription factor *CUX1* in NENs. CUX1 promotes carcinogenesis via regulation of growth, apoptosis and angiogenesis by multiple mechanisms. Mechanistically, we found that *CUX1* promotes tumour angiogenesis via paracrine stimulation of endothelial cells. Tumour angiogenesis represents a hallmark feature of PNENs, and anti-angiogenic drugs are currently the most promising candidates for treatment of PNENs. Therefore, CUX1-dependent pathways may constitute attractive targets for future therapeutic strategies for PNENs.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-14-0152.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Der Transkriptionsfaktor CUX1 wurde schon im Mamma- und Pankreaskarzinom dezidiert charakterisiert und ist als transkriptioneller Repressor an der Tumorprogression beider Entitäten beteiligt. Der Einfluss von CUX1 auf die Tumorprogression in neuroendokrinen Neoplasien wurde bislang nicht erforscht. Präliminäre immunhistochemische Arbeiten an PanNEN habe eine starke CUX1 Lokalisation an der Infiltrationsfront von Insulinomen dargestellt, weshalb eine dezidiertere Evaluation von CUX1 in PanNEN erfolgte. An einer Kohorte von 59 Insulinompatienten haben wir mittels Immunohistochemie CUX1 gefärbt und mit klinisch-pathologischen Daten korreliert. Die Expression von CUX1 zeigte sich in malignen (metastasierten) Insulinom deutlich verstärkt und bot eine Assoziation zur Proliferation (G1 vs. G2). Die höchsten Expressionsraten wurden in Metastasen von Lymphknoten und Leber festgestellt. Die humanen Ergebnisse von CUX1 wurden im genetischen Mausmodell, genannt RIP1Tag2 ebenso repliziert. Im Rahmen der Tumorprogression dieses Mausmodells entstehen aus präinvasiven Vorstufen maligne Insulinome. Sowohl auf mRNA, als auch auf Proteinebene korrelierte die CUX1 Expression mit der Tumorprogression. Basierend auf diesen Vordaten führten wir weitere Arbeiten zur Charakterisierung der Funktion von CUX1 in-vitro durch. Dazu verwendeten wir die neuroendokrinen Zelllinien Bon1 und Ins-1. Unter Verwendung stabiler Überexpressionskonstrukte und transienter Transfektionen mittels siRNA wurde endogenes CUX1 moduliert. Hierbei zeigt sich ein signifikanter proproliferativer und antiapoptotischer Phänotyp in Bon1 und Ins-1 Zellen. Die Proliferationsassays wurden mit MTT und BrdU durchgeführt, während die Apoptose mittels 5-FU und TRAIL induziert und durch DNA Fragmentation validiert wurde. Der Einfluss von konditioniertem Medium CUX1 überexprimierender Bon1 und Ins-1 wurde in den

endothelialen Zellen HMEC-1 untersucht und ausgewertet. Das Medium beider Zelllinien führte zu einer deutlichen Steigerung der Gefäßlänge und Gefäßverzweigungen. Mittels einem auf Angiogenese fokussiertem RNA Profiler untersuchten wir 84 Gene, um CUX1 induzierte Effekte mechanistisch genauer zu evaluieren. HIF-1a und MMP-9 waren die am stärksten regulierten Gene und diese konnten auf Proteinebene ebenso validiert werden. Um die Rolle von CUX1 als Regulator von Proliferation/Apoptose und Angiogenese zu verifizieren, führten wir Xenograft-Experimente durch. Stabil überexprimierende Bon1 Zellen (2 Klone) wurden jeweils in 4 Nacktmäuse appliziert im Vergleich zu einer Kontrollgruppe. Nach 7 Wochen wurden die Mäuse geopfert und immunhistochemisch aufgearbeitet. Während sich ein nicht statistisch signifikanter Trend in Bezug auf das Tumorwachstum präsentierte, sahen wir deutliche Unterschiede bei der Auswertung von Ki-67 als Marker der Proliferation und der Nekrose als Marker der Antiapoptose. Zusammenfassend präsentierte sich CUX1 als starker Mediator der Tumorprogression in neuroendokrinen Neoplasien in verschiedenen Tumormodellen (Xenograft und genetisches Mausmodell) sowie in in-vitro und an einer großen Kohorte von humanen Insulinomen. CUX1 beeinflusst durch die Regulierung von HIF-1a und MMP-9 die Proliferation, Apoptose und Angiogenese signifikant.

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RESEARCH ARTICLE

Streptozocin-Based Chemotherapy in Patients with Advanced Neuroendocrine Neoplasms – Predictive and Prognostic Markers for Treatment Stratification

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Abstract

Background and Aim

Chemotherapy with streptozocin (STZ) in combination with 5-FU or doxorubicin (Dox) represents a standard of care for patients with metastatic pancreatic neuroendocrine neoplasms (pNEN). However, predictive markers for patient selection are still missing. The aim of this study was a retrospective evaluation of the clinicopathological characteristics of pNEN patients receiving STZ-based chemotherapies and to identify predictive and prognostic markers.

Patients and Methods

We retrospectively analyzed 77 patients treated at our center between 1995 and 2013. The median overall survival (OS) and progression-free survival (PFS) were calculated using Kaplan-Meier and Cox regression methods, respectively. Uni- and multivariate analyses were performed.

Results

The median PFS (mPFS) in patients receiving STZ/5-FU/Dox was 16 months with a median OS (mOS) of 28 months. Objective response rate (ORR) and disease control rate (DCR) were 34% and 72%, respectively. Biochemical response and positive octreotide scintigraphy predicted objective response. Univariate analysis revealed Ki-67 > 10% and the absence of biochemical or objective response by imaging as independent risk factors for shorter PFS. Additionally, performance status (PS) and resection of the primary tumor were observed to influence mOS. Treatment was well tolerated with less than 10% grade 3 and 4 toxicities.

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Conclusions

STZ-based chemotherapy is an effective and well-tolerated treatment option in patients with well differentiated neuroendocrine neoplasms. Positive octreotide scintigraphy and biochemical response predict objective response.

Introduction

Neuroendocrine tumors (NETs) are a heterogeneous group of neoplasms with increasing incidence [1] originating from endocrine cells in different anatomic locations. Pancreatic NETs differ from intestinal NETs in many aspects including clinical presentation with distinct hormone syndromes, genetic findings (e.g. mutations in the Menin gene [2]), a more aggressive course of disease resulting in worse prognosis [1], and responsiveness to treatment modalities such as molecular targeted agents and chemotherapy.

While the results of chemotherapy in patients with intestinal NETs are disappointing resulting in objective response rates of less than 20% in most trials, pancreatic NETs were shown to be chemosensitive. The combination of streptozocin (STZ) and fluorouracil (5-FU) is recommended as standard treatment for metastatic pancreatic NETs in European guidelines [3, 4].

STZ is available since the early 80ies and approved for the treatment of pancreatic NETs in several countries. The early prospective randomized trials by Moertel reported high response rates (RR) of STZ-based combinations exceeding 60% [5, 6]. However, two subsequent retrospective series failed to confirm these results, which was attributed the definition of response [7, 8]. More recently, larger retrospective studies using standardized radiological response criteria repeatedly reported RR´s ranging between 30 and 40% for STZ-based combination treatments [9–11].

A variety of prognostic factors has been described for patients with NETs including age, performance status, stage according to ENETS [12, 13] and AJCC [14], tumor load, levels of chromogranin A (CgA) [15], presence of circulating tumor cells [16] and grading based on the proliferation marker Ki-67.

The latest WHO classification [17] of NETs is based on Ki-67 values and the prognostic relevance of this grading system has been validated in several studies [12, 18–20]. In contrast, the predictive value of Ki-67 is less clear. To date, no established predictive markers are available to facilitate treatment decisions. The ESMO guideline recommends the use of STZ-based chemotherapy in patients with pancreatic NETs and a proliferation rate between 5 and 20% [4]. However, this represents an expert opinion which is not evidence-based, since most chemotherapy trials in pNEN published so far did not assess the role of Ki-67 as predictive marker. Only in one study by O´Toole and co-workers an association between Ki-67 levels >5% and lack of response to systemic chemotherapy was reported [21]. It was thus the aim of our study to identify prognostic and predictive markers for pNEN-patients treated with STZ-based chemotherapy at our center.

Patients and Methods

Patients

77 consecutive patients with histologically confirmed pancreatic neuroendocrine tumors who received STZ-based chemotherapy between 1995 and 2013 were retrospectively identified from a database at the comprehensive cancer center at the University Hospital of Marburg.

This study was conducted in accordance with the Declaration of Helsinki. Collection, storage, and evaluation of patient-related information in our NEN database were performed with the approval of the local ethics committee at the University of Marburg and after obtaining the patients informed consent. The initial statement of the local ethics committee was that a formal approval and written informed consent for collection and analysis of data arising from the routine clinical evaluation within the own hospital was not required. Therefore, patients who had their last visit/ died before 2004 only were asked for verbal consent (with approval of the ethics committee of this consent procedure). In 2004 the German NET registry was built up and for transmission of pseudonymized data a written approval of the ethics committee was obtained. Since then all patients additionally gave a written consent for data collection and analysis.

Protocol treatment and toxicity assessment

All patients received STZ-containing chemotherapy in combination with Doxorubicin (Dox) or 5-FU. For patients who initially received chemotherapy with STZ/Dox, Dox was replaced by 5-FU before the cumulative cardiotoxic dose of 550mg/m^2 Dox was reached. The chemotherapeutic STZ/Dox regimen included STZ at a dose of 500 mg/m^2 on day 1–5 and Dox at a dose of 50 mg/m^2 on day 1 and 22. The regimen was repeated every 6 weeks. The STZ/5-FU regimen included short-term infusion of 5-FU at a dose of 400 mg/m^2 on day 1–5, in addition to STZ at a dose of 500 mg/m^2 on day 1–5 every 6 weeks. In case of impaired performance status or toxicity a delay of 2 weeks was allowed. Concomitant treatment with somatostatin analogs were allowed for patients suffering from significant hormone excess syndromes.

Adverse effects were recorded during each cycle of chemotherapy, and reported using CTCAE version 3.0 (http://ctep.cancer.gov).

Follow up and evaluation of tumor response

Follow-up investigations were scheduled after 3 completed treatment courses including history, physical examination, laboratory investigations and imaging (CT or MRI scan). Biochemical evaluation included chromogranin A (CgA) measurements. In addition, the surveillance schedule included a somatostatin receptor scintigraphy every 18–24 months in the majority of cases.

Response to treatment was evaluated in this study using the international criteria proposed by the Response Evaluation Criteria in Solid Tumors (RECIST) Committee [22]. The biochemical response was defined as a reduction of more than 30% compared to baseline levels.

Immunohistochemistry

The Ki-67 proliferation index was evaluated using a standardized protocol with the Leica-Bond-Max-Autostainer and the Dako antibody in a 1:1000 dilution. Classification was done according to the revised WHO classification of tumors of the digestive system 2010 [23]. Ki-67 values found in the pathologic reports and in some cases when the Ki-67 value was not available in the reports, from new Ki-67 stainings of archival tissue specimens were used. Tumors were graded using the proposed grading system of Rindi et al [23, 24]. The analyses were performed by one pathologist with expertise in endocrine and pancreatic tumors (W.N.) in a blinded fashion regarding tumor parameters.

Statistical design and analysis

The comparisons between response and tumor characteristics, disease extension, or laboratory features were based on Chi-square and Fisher's exact tests, as appropriate. PFS was measured from the beginning of treatment to progression, death, or last follow-up. OS was measured

from the beginning of treatment to the time of last follow-up or death. Actuarial survival was measured by the method of Kaplan and Meier [25]. The statistical differences in PFS between groups of patients were estimated by the log-rank test [26]. The statistical independence between prognostic variables was evaluated by multivariate analysis using the Cox proportional hazard model [27]. All statistical calculations were performed using SPSS (IBM SPSS Statistics). Differences were considered statistically significant when the P value was less than 0.05.

Results

Patient characteristics

We identified 77 patients who received STZ in the time period between 1995 and 2013. Baseline characteristics are listed in Table 1. The majority of patients had pancreatic neuroendocrine neoplasms (n = 65, 84.4%), while 12 (15.6%) patients had NEN from non-pancreatic origin, among them 8 bronchial NEN (10.4%), 3 CUPs (3.9%) and 1 duodenal (1.3%) NEN. There were 55 (71.5%) non-functioning and 21 (27.3%) functioning tumors. Information about tumor differentiation and grading were available in 70 (90.9%) and 67 (87%) patients, respectively. Among these, 68 tumors (88.3%) were well differentiated including 9 (11.7%) G1 and 51 (66.2%) G2 tumors, and 5 well differentiated tumors albeit with Ki-67 proliferation rates >20% (so-called NET G3). For 3 older samples of well differentiated tumors grading and Ki-67 were not documented and could not be determined since no tissue was available anymore. Finally, two neuroendocrine carcinomas (2.6%) were documented. 88.3% patients had liver (n = 68) and 66.2% lymph node (n = 51) metastases. Bones represented the third most common site of metastases (n = 30, 39%). Somatostatin receptor status determined by octreotide scintigraphy was positive in 56 patients (72.7%) and negative in 14 patients.

Treatment details are listed in Table 2. 23 patients (29.9%) underwent primary tumor resection and 14 patients (18.2%) received synchronous resection of liver metastases. Only 15 patients (19.5%) were treatment-naive when chemotherapy was started. Most patients had one (n = 32, 41.6%) or two (n = 30, 39%) prior systemic or liver directed treatments. Therefore, median time to STZ-chemotherapy was 33 months (range 1–181). In our cohort, 31 patients received STZ and Dox (40.3%), 30 patients received STZ and 5-FU (39%) and 13 patients received STZ and 5-FU (16.9%) after STZ and Dox in a sequential approach. The median number of cycles administered was 3 (range 1–6) and 4 (range 1–12) for STZ/ Dox and STZ/ 5-FU, respectively.

Safety

Sixty-six patients were assessable for toxicities (Table 3). Altogether, 228 events were documented during and after chemotherapy including 20 grade 3 and 4 adverse events (8.8%). In brief, most common adverse events comprised grade 1 or 2 hematological (n = 67, 29%), hepatological (n = 40, 17.5%), gastrointestinal (n = 33, 14.5%) and renal (n = 31, 13.6%) toxicity. Less than 10% grade 3 or 4 toxicities occurred including leukopenia (n = 3, 1.3%) and nausea or vomiting (n = 5, 2.2%). A total number of 20 events (8.8%) occurred due to hair loss mostly following Dox treatment. In contrast, renal adverse events including proteinuria and/or renal failure were typical side effects of STZ infusion. Other adverse events (n = 22, 9.6%) included infectious, neurological and cardiac toxicities of which 5 were grade 3 cardiac toxicities (2.2%).

Therapy efficacy, predictive and prognostic indicators for PFS and OS

For 64 patients (83.1%) the radiologic response was assessable (Table 4). 22 patients (34.4%) experienced an objective response including 2 (3.1%) complete (CR) and 20 (31.3%) partial

Table 1. Clinicopathological features of patients (N = 77).

Characteristics	No. of patients	%
Gender		
male	39	50.6
female	38	49.4
Age at diagnosis in years		
median	53	
range	25–79	
WHO PS		
0	38	49.4
1	32	41.6
unknown	7	9.1
Primary tumor location		
Pancreas	65	84.4
Non-Pancreas	12	15.6
Bronchus	8	10.4
CUP	3	3.9
Duodenum	1	1.3
Tumor type		
Functioning	21	27.3
Insulinoma	7	9.1
Gastrinoma	6	7.8
Glucagonoma	2	2.6
VIPoma	4	5.2
ACTHoma	2	2.6
Nonfunctioning	55	71.5
unknown	1	1.3
Differentiation		
NET	68	88.3
NEC	2	2.6
unknown	7	9.1
Grading		
G1	9	11.7
G2	51	66.2
G3	7	9.1
unknown	10	13.0
Sites of metastases		
Lymph Node	51	66.2
Liver	68	88.3
Bone	30	39.0
Others	31	40.3
Octreotide scintigraphy		
positive	56	72.7
negative	14	18.2
unknown	7	9.1

Abbreviations: PS = performance status, CUP = carcinoma of unknown primary, G = grading, VIP = vasoactive intestinal polypeptide, ACTH = adrenocorticotropic hormone, NET = neuroendocrine tumor, NEC = neuroendocrine carcinoma.

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Table 2. Clinicopathological features of patients (N = 77).

Characteristics	No. of patients	%
Primary tumor resection		
yes	23	29.9
no	48	62.3
unknown	6	18.2
synchronic liver metastases resection	14	7.8
Age at CTx in years		
Median	56	
Range	27–77	
Time to CTx in months		
Median	33	
Range	1–181	
Chemotherapy No.	**No. of patients**	
STZ/Dox	31	40.3
STZ/5-FU	30	39
sequential approach	13	16.9
Median No. of cycles administered		
STZ/Dox		
Median	3	
Range	1–6	
STZ/5-FU		
Median	4	
Range	4–12	
No. prior systemic / liver directed therapies	**No. of patients**	
0	15	19.5
1	32	41.6
≥2	30	39.0

Abbreviations: CTx = chemotherapy, Dox = doxorubicin, STZ = streptozocin.

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Table 3. Toxicities of chemotherapy (in 66 evaluable patients).

Toxic reaction	Grade 1	%	Grade 2	%	Grade 3	%	Grade 4	%
Hematologic								
Leukopenia	20	30.3	18	27.3	3	4.5	0	0
Thrombocytopenia	6	9.1	2	3.0	0	0	0	0
Anemia	15	22.7	6	9.1	0	0	0	0
Gastrointestinal								
Nausea or Vomiting	12	18.2	8	12.1	5	7.6	1	1.5
Diarrhea/Obstipation	7	10.6	6	9.1	0	0	0	0
Mucositis	0	0	0	0	1	1.5	0	0
Hepatologic	31	47.0	9	13.6	2	3.0	1	1.5
Renal	27	40.9	4	6.1	2	3.0	0	0
Hair loss	4	6.1	16	24.2	0		0	0
Other	10	15.2	7	10.6	5	7.6	0	0

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Table 4. Response and predictive markers (in 64 evaluable patients).

Predictor	CR	PR	SD	PD	ORR %	DCR %	Fisher's exact test		X2-test for trend	
Number of patients	2	20	24	18	34.4	71.9	OR	DC	OR	DC
Primary tumor location							P value		P value	
Pancreas	2	17	23	14	33.9	75.0	1	0.095	0.89	0.076
Non-Pancreas		2	1	4	28.6	42.9				
Site of metastases										
Liver+Lymphnode	1	10	10	6	40.7	77.8	0.26	0.4	0.21	0.3
Liver+LN+other		9	14	12	25.7	65.7				
Ki-67 in %										
<2		3	5	1	33.3	88.9	G1 vs G2/3		0.28	0.058
2–20	2	16	17	13	37.5	72.9	1	0.42	0.85	0.22
>20		1		3	25.0	25.0				
Age before CTx										
<60	1	6	12	6	28.0	76.0	0.59	0.77	0.42	0.62
>60	1	13	12	11	37.8	70.3				
PS										
0	2	13	12	10	40.5	73.0	0.105	0.78	0.089	0.67
≥1		5	12	8	20.0	68.0				
Octreotide scintigraphy										
positive	2	18	18	12	40.0	76.0	0.046	0.28	0.037	0.22
negative		1	6	5	8.3	58.3				
Biochemical response										
yes	1	13	5	10	48.3	65.5	0.052	0.082	0.027	0.066
no	1	5	18	5	20.7	82.8				
Chromogranin A										
>50 U/l	1	15	15	14	35.6	68.9	1	0.42	0.9	0.22
≤50 U/l	1	2	5	1	33.3	88.9				
Primary tumor operation										
yes	1	7	5	8	38.1	61.9	0.58	0.25	0.57	0.24
no	1	12	19	10	31.0	76.2				

Abbreviations: CR = complete response, PR = partial response, SD = stable disease, PD = progressive disease, ORR = objective response rate, DCR = disease control rate, LN = lymph node, others = bone, lung, cerebral, peritoneal, lienal and adrenal gland, CTx = chemotherapy, PS = performance status.

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responses (PR). Disease control was achieved for a high proportion (71.9%) of patients comprising patients with response (PR and CR) and with stable disease (n = 24, 37.5%). Progressive disease was documented in 18 patients (28.1%). Median PFS and median OS were calculated as 16 and 28 months, respectively (Fig 1).

A panel of clinicopathological parameters was evaluated for their association to objective response (OR) as putative predictive markers (Table 4). Out of these, only a positive SMS receptor status, (positive vs. negative: 40.0% vs. 8.3%, P = 0.037) as well as biochemical response (positive vs. negative: 48.3% vs. 20.7%, P = 0.027) correlated with objective response as determined by imaging.

Univariate analyses were performed evaluating Ki-67, performance status (PS), primary tumor resection, objective response (OR), disease control (DC), biochemical response and

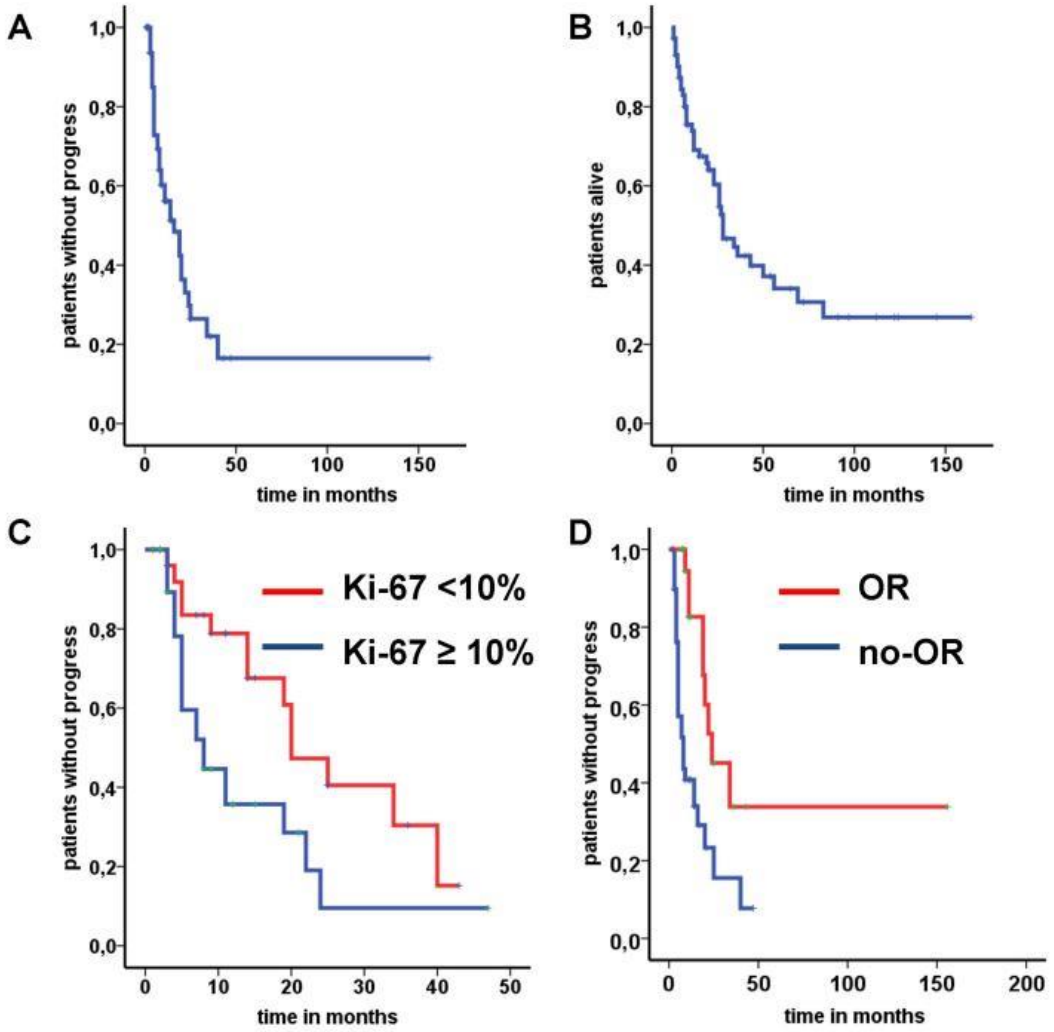

Fig 1. Kaplan Meier survival analyses in patients treated with streptozocin. (A) Progression-free survival (mPFS = 16 months) for the entire group of patients (n = 64). (B) Overall survival (mOS = 28 months) for the entire group of patients (N = 64). (C) Association between Ki-67 and mPFS (cut-off 10%; 20 vs. 8 months, P = 0.015; N = 60) (D) Association between objective response (OR) and mPFS (20 vs 4 months, P < 0.001; N = 62).

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prior therapies to identify the correlation to PFS and OS (Table 5). Statistically significant risk factors for disease progression included Ki-67 ≥10% (HR 2.3, 95% CI 1.1–4.8, P = 0.022), whereas OR (HR 0.3, 95% 0.2–0.7, P = 0.002) and presence of a biochemical response (HR 0.4, 95% CI 0.2–0.9, P = 0.035) significantly decreased the risk for disease progression. There was

Table 5. Univariate analysis for prognostic indicators.

Variable	PFS HR	95% CI	P-value	OS HR	95% CI	P-value
Ki-67						
<10%	1		**0.022**	1		**0.024**
≥10%	2.3	1.1–4.8		2.3	1.1–4.7	
PS						
0	1		0.17	1		**< 0.001**
≥1	1.6	0.8–3.1		3.5	1.8–6.9	
Primary tumor resection						
no	1		0.72	1		**0.034**
yes	1.1	0.6–2.2		0.5	0.2–0.9	
OR						
no	1		**0.002**	1		0.11
yes	0.3	0.2–0.7		0.5	0.3–1.1	
DC						
no				1		**0.01**
yes				0.4	0.2–0.8	
Biochemical response						
no	1		**0.035**	1		0.13
yes	0.4	0.2–0.9		0.5	0.2–1.2	
Prior therapies						
≤1	1		0.12	1		0.72
>1	0.5	0.3–1.2		0.9	0.4–1.8	

Abbreviations: PS = performance status, OR = objective response, DC = disease control, HR = Hazard ratio, mPFS = median progression-free survival, mOS = median overall survival, CI = confidence interval.

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no statistical association between PFS and performance status, primary tumor resection or prior therapies.

Prognostic indicators for reduced OS were Ki-67 ≥10% (HR 2.3, 95% CI 1.1–4.7, P = 0.024) and PS ≥1 (HR 3.5, 95% CI 1.8–6.9, P = < 0.001). In contrast, primary tumor resection (HR 0.5, 95% CI 0.2–0.9, P = 0.034) and radiological disease control (HR 0.4, 95% CI 0.2–0.8, P = 0.01) were identified as relevant favourable prognostic indicators.

Interestingly, there was no correlation between OS and OR or biochemical response. In addition, multivariate analysis revealed that only a Ki-67-value ≥10% (HR 2.9, 95% CI 1.1–7.8, P = 0.034) was an independent prognostic marker for PFS (Table 6). However, no association between the risk factors used for univariate analyses and OS was found.

Representative Kaplan-Meier curves for Ki-67 (mPFS 20 vs. 8 months, P = 0.015) and OR (mPFS 24 vs. 8 months, P = 0.001) are shown in Fig 1C and 1D to visualize the impact of these parameters on PFS.

Discussion

In this study, we confirmed that STZ-based regimens in combination with Dox or 5-FU are safe and effective treatment options in a large, retrospective cohort of patients mostly with advanced pancreatic neuroendocrine neoplasms. Consistent with prior studies, chemotherapy induced an ORR and DCR of 34% and 72%, respectively, translating into a mPFS of 16 months and mOS of 28 months. Beyond well-characterized prognostic indicators including Ki-67 and

Table 6. Multivariate analysis for prognostic indicators.

	PFS			OS		
Variable	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
Ki-67						
<10%	1		**0.034**	1		0.11
≥10%	2.9	1.1–7.8		2.3	0.8–6.2	
PS						
0	1		0.72	1		0.076
≥1	1.2	0.5–2.6		2.6	0.9–7.3	
Primary tumor resection						
no	1		0.24	1		0.15
yes	0.6	0.2–1.4		0.4	0.1–1.4	
OR						
no	1		0.08	1		0.65
yes	0.4	0.1–1.1		0.8	0.2–2.5	
DC						
no				1		0.2
yes				0.5	0.1–1.5	
Biochemical response						
no	1		0.69	1		0.22
yes	1.3	0.4–4.4		0.5	0.1–1.6	
Prior therapies						
≤1	1		0.5	1		0.9
>1	0.7	0.3–1.9		1	0.3–3.1	

Abbreviations: PS = performance status, OR = objective response, DC = disease control, HR = Hazard ratio, PFS = progression-free survival, OS = overall survival, CI = confidence interval.

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biochemical response for PFS, we identified biochemical response and positive SMS scintigraphy as predictive markers for response to STZ-based chemotherapy. In the analysis of the RADIANT-1 trial for predictive biomarkers Yao and colleagues suggested that an early decrease of CgA and NSE may serve as a marker of response to everolimus [28]. As the elevation of biomarkers is correlated with tumor load which is an important prognostic marker [29] it appears feasible that a biochemical response predicts OR and correlates with PFS. The observed correlation of a positive SMS-receptor scintigraphy signal indicating a better differentiation with response is intriguing, and it may be argued that SMS-positivity represents a prognostic rather than a predictive marker. However, our study showed a statistically significant association of SMS-positivity with objective response, which may be in line with the observation of O´Toole and co-workers that less well differentiated pNENs with Ki-67 levels >5% lack response to systemic chemotherapy [21].

For advanced, well differentiated pancreatic neuroendocrine tumors chemotherapy represents the therapeutic standard of care. A limited number of cytotoxic drugs have been evaluated in this context. So far, STZ represents the backbone for combination chemotherapy regimens. Former randomized trials revealed superiority of STZ given in combination with Dox or 5-FU as compared to STZ-monotherapy [30, 31]. Due to the cardiotoxicity of Dox the ENETS guidelines favor the administration of STZ in combination with 5-FU [32]. The ORR and DCR and the resulting mPFS and mOS observed in our study are consistent with previous publications. However, there has been considerable controversy regarding the efficacy of STZ-

based chemotherapy in pNEN patients. Response rates published so far have ranged between 6% and 69% [5, 7, 8, 30, 33]. The large variability in response rates is thought to be at least partly explained by different standards in assessment of response. As such, in the initial trials the assessment of response was not based on RECIST criteria and in addition to morphological criteria also clinical and biochemical criteria of response were included. This may have resulted in an overestimation of ORR. In our series, a standardized response evaluation by radiological cross-sectional imaging was used.

Our results are comparable with the study of Kouvaraki et al., that reported an ORR of 39% and a mPFS of 18 months using the combination of STZ+Dox+5FU [9]. Furthermore, in the trials mentioned above the different treatment arms were not homogeneous concerning clinicopathological parameters such as age, performance status, grading, tumor load and number of prior treatments factors which are of prognostic relevance and may thus have influenced the outcome to chemotherapy in the different treatment arms. In the study of Kouvaraki et al. a high hepatic tumor load exceeding 75% and the use of chemotherapy as second line treatment was associated with shorter PFS in multivariate analysis. In comparison to this trial, only 20% of our patients received chemotherapy as first-line treatment. Nevertheless, from a clinical point of view chemotherapy remains the first choice in patients in whom response is required e.g. in patients with symptoms due to high tumor mass or in patients who are borderline resectable and may become resectable after induction chemotherapy. Predictive markers as described in our study may help to identify those patients that have the highest benefit from STZ-based chemotherapy. Lack of a biochemical response may thus help in the decision whether to discontinue or proceed with a STZ-based chemotherapy.

In the palliative setting toxicity profile and quality of life in patients undergoing chemotherapy are of utmost importance. In our series, we documented less than 10% grade 3/4 toxicities. In particular, renal failure was one of the limiting toxicities [30, 31], however, occurring in only 3% of the cases, thus confirming the safety of STZ. In addition, the emetogenic and myelosuppressive potential of the combination treatment was less frequent in our patients than reported in prior trials [10, 33, 34] possibly as a result of better standardized supportive management protocols.

With the advent of sunitinib and everolimus, the era of targeted therapies was recently introduced for pancreatic neuroendocrine neoplasms. By improving median PFS from 6 to approximately 12 months, both sunitinib and everolimus were approved in Europe and North America for therapy of metastatic pancreatic NEN [35, 36]. When considering the different therapeutic options, two main questions remain to be elucidated. First, the best choice and sequence of targeted therapies and STZ-based chemotherapy remain to be defined. The ORR of sunitinib and everolimus were only 9% and 5%, respectively, as compared to response rates around 30–40% for STZ-based chemotherapy regimens. At present no comparative trials of chemotherapy versus molecular targeted treatments are available. Trials on the best sequence of treatments, e.g. the SEQTOR trial, a European randomized phase III study investigating STZ +5-FU followed by everolimus versus the reverse sequence, are ongoing and results have to be awaited. Second, so far no means are available for a personalized approach by selecting the most effective therapy for individual patients at the right time by using predictive biomarkers. Up to date mainly prognostic biomarkers such e.g. Ki-67 [24, 37, 38] have been established. However, the role of the proliferation marker Ki-67 as predictor of response to treatment is less well defined. In patients with neuroendocrine carcinoma a Ki-67 value above 55% was associated with better response to platinum based chemotherapy [39] but poorer OS. In patients with well differentiated pNEN it has also been hypothesized that tumors with high Ki-67 may show a better response to chemotherapy [40]. In our study the OR was similar in patients with G1 and G2 tumors although the data on G1 tumors are limited to only 9 G1 cases. Ki-67 values

>10% were associated with a shorter mPFS of only 8 months versus 20 months in patients whose tumor had Ki-67 values <10%, however, this was not associated with response to chemotherapy, indicating that in our study the Ki-67 values were prognostic rather than predictive as previously suggested [21].

Finally, our data suggest that objective response (OR) is a favourable prognostic marker for PFS which, however, was not translated into prolonged OS. There are limited data about the relevance of OR as prognostic marker. In fact, our results are in accordance with the PRRT study of Kwekkeboom et al. In this study there were no differences in the survival of patients that revealed either PR or SD [41]. In contrast, PD after PRRT was strongly associated with a poor survival. Patients with a rapid progression after the first reevaluation may thus have a poorer outcome than those with a PD after initial SD or PR, which may partly be explained by a more aggressive biological phenotype as well as by primary or acquired mechanisms of treatment resistance. In this line, Perren and coworkers have shown that loss of DAXX or ATRX is associated with chromosomal instability and shorter survival times in patients with pNENs [42]. In the same way loss of MGMT protein was associated with an adverse outcome in pNEN patients, this prognostic value, however, was not independent from grade and stage in multivariate analysis [43]. These results suggest, that among well differentiated pNENs subtypes exist that show a more aggressive and unfavourable course, such those that are DAXX- and ATRX-negative or show a loss of MGMT-protein expression.

In conclusion, our data support the use of STZ-based chemotherapy in patients with advanced pancreatic NENs. Prognostic and predictive subtypes of pNENs exist and our data based on standard clinicopathological characteristica suggest that positivity of SMS-receptor scintigraphy as a surrogate parameter of better differentiation and biochemical response may be useful to predict response to therapy and thus contribute to personalize treatment. However, based on the retrospective nature and the limited number of included patients in this study, our findings need to be validated in well-designed prospective clinical trials.

Supporting Information

S1 Table. Patient data for statistical analyses.
(XLS)

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Author Contributions

Conceived and designed the experiments: SK AR PM TG. Performed the experiments: SK MB WN. Analyzed the data: SK HD. Contributed reagents/materials/analysis tools: SK MB HD AR. Wrote the paper: SK MB AR PM TG WN. Quality control of data and algorithms: SK MB DM.

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In dieser retrospektiven Analyse wurden alle Patienten ausgewertet, die eine Streptozocin (STZ) basierte Therapie mit 5-FU oder Doxorubicin am Universitätsklinikum Marburg erhielten. Die aktuellen deutschen und europäischen Leitlinien empfehlen diese Kombinationschemotherapie bei metastasierten gut-differenzierten pankreatischen neuroendokrinen Neoplasien, wenn Patienten einen Ki-67 Index >10% haben, symptomatisch sind und eine hohe Tumorlast aufweisen. In dieser monozentrischen Kohorte waren 84% (65 von 77 Patienten) pankreatischen Ursprungs. 88% (n=65) waren G1/G2 Tumore und über 70% zeigten kein hormonelles Syndrom. Die häufigsten Metastasierungsorte waren Leber (88%), Lymphknoten (66%) und Knochen (39%). 73% der Patienten zeigten eine positive SSTR Bildgebung, hauptsächlich ermittelt durch den OctreoScan (^{111}Indium-Szintigraphie). Insgesamt präsentierte die Therapie eine gute Verträglichkeit mit weniger als 10% Grad 3/4 Nebenwirkungen betreffend vor allem hämatologische und renale Komplikationen. Basierend auf den RECIST Kriterien 1.1 waren 2 (3.1%), 20 (31.3%) und 24 (37,5%) Patienten in kompletter/partieller Remission sowie in einer stabilen Erkrankung als bestes Ansprechen. Das mPFS und mOS belief sich auf 16 und 28 Monate. Ein positiver SSTR Status sowie ein biochemisches Ansprechen (definiert als eine Reduktion des Tumormarkers um mehr als 30%, meisten Chromogranin A) zeigten sich als prädiktive Marker für das Ansprechen auf die Therapie. Zusätzlich wurden prognostische Faktoren mit dem Cox Regressions Modell auf uni- und multivariater Ebene untersucht. Ein Ki-67 Index über 10% war der stärkste Prädiktor für eine Tumorprogression in uni- und multivariaten Analysen bezogen auf das PFS. Während sich der Profilerationsindex Ki-67 (cut-off 10%), der Performance Status (PS) sowie die Primärtumorresektion in der univariaten Analyse als prognostisch erwiesen, konnte

dies in der multivariaten Analyse nicht bestätigt werden. Hier zeigte sich ein nicht signifikanter Trend des PS (P=0.076). Die STZ-basierte Chemotherapie sollte in metastasierten PanNET eingesetzt werden, da sie gut verträglich ist und adäquate Ansprechraten erreicht. Das Nebenwirkungsprofil ist akzeptabel. Ein positiver SSTR Status sowie ein biochemisches Ansprechen präsentierten sich als prädiktive Marker, während einzig Ki-67 ein robuster Anhaltspunkt für einen prognostisch schlechteren Verlauf darstellt.

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Therapeutic targeting of tumor-associated macrophages in pancreatic neuroendocrine tumors

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Pancreatic neuroendocrine tumors (PNETs) represent a heterogeneous group of neuroendocrine neoplasms with varying biological behavior and response to treatment. Although targeted therapies have been shown to improve the survival for patients at advanced stage, resistance to current therapies frequently occurs during the course of therapy. Previous reports indicate that the infiltration of tumor-associated macrophages (TAMs) in PNETs might correlate with tumor progression and metastasis formation. We aimed to evaluate the prognostic and functional impact of TAMs in human PNETs *in vitro* and *in vivo* and to investigate the effect of therapeutic targeting TAMs in a genetic PNET mouse model. TAM expression pattern was assessed immunohistochemically in human PNET tissue sections and a tissue-micro-array of PNET tumors with different functionality, stage, and grading. The effect of liposomal clodronate on TAM cell viability was analyzed in myeloid cell lines and isolated murine bone macrophages (mBMM). *In vivo*, RIP1Tag2 mice developing insulinomas were treated with liposomal clodronate or PBS-Liposomes. Tumor progression, angiogenesis and immune cell infiltration were assessed by immunohistochemistry. In human, insulinomas TAM density was correlated with invasiveness and malignant behavior. Moreover, TAM infiltration in liver metastases was significantly increased compared to primary tumors. *In vitro*, Liposomal clodronate selectively inhibited the viability of myeloid cells and murine bone macrophages, leaving PNET tumor cell lines largely unaffected. *In vivo*, repeated application of liposomal clodronate to RIP1Tag2 mice significantly diminished the malignant transformation of insulinomas, which was accompanied by a reduced infiltration of F4/80-positive TAM cells and simultaneously by a decreased microvessel density, suggesting a pronounced effect of clodronate-induced myeloid depletion on tumor angiogenesis. Concomitant treatment with the antiangiogenic TKI sunitinib, however, did not show any synergistic effects with liposomal clodronate. TAMs are crucial for malignant transformation in human PNET and correlate with metastatic behavior. Pharmacological targeting of TAMs via liposomal clodronate disrupts tumor progression in the RIP1Tag2 neuroendocrine tumor model and was associated with reduced tumor angiogenesis. Based on these results, using liposomal clodronate to target proangiogenic myeloid cells could be employed as novel therapeutic avenue in highly angiogenic tumors such as PNET.

During the last decade, the predominant role of the tumor microenvironment (TME) as modulator of tumor progression has been increasingly recognized. Nonmalignant stromal cells not only may facilitate tumor initiation, disease progression and metastasis, but also are affecting response or resistance to various therapeutic strategies. The TME comprises stromal cells such as endothelial cells, fibroblasts as well as various immune cell populations.[1] Both lymphoid and myeloid lineages are modulating tumorigenesis and tumor progression, with impact on tumor cell proliferation, invasion and survival as well as effects on the immunogenicity of the tumor.[2,3] Within the myeloid compartment, tumor-associated macrophages (TAMs) represent an important fraction. Numerous studies have demonstrated an inverse correlation between patient prognosis and TAM infiltration in various tumor entities, among them Hodgkin's lymphoma, breast cancer, glioblastoma and pancreatic cancer.[4–7] The functional impact of TAMs is still incompletely understood. TAMs release miscellaneous cytokines and proteases, among them proangiogenic

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What's new?

Tumor-associated macrophages, or TAMs, are an important component of the tumor microenvironment. These cells seem to promote tumor growth and thwart the cytotoxic effect of cancer therapy. Here, the authors investigated how the presence of TAMs affects the progression of pancreatic neuroendocrine tumors (PNETs). Using tissue samples from a variety of PNETs, they observed that more infiltrating TAMs correlate with increased angiogenesis, proliferation and metastasis. In a mouse model, treatment with liposomal clodronate depleted TAMs, which decreased angiogenesis and reduced tumorigenesis and progression. Combining clodronate with established antiangiogenic treatment provided no additional benefit, however.

factors such as VEGF as well as various cathepsins.[8–10] Further paracrine mechanisms mediating the cross-talk between TAMs and tumor cells include activation of the epidermal growth factor receptor (EGFR), colony stimulating factor 1 receptor (CSF-R1) and WNT signaling pathways which facilitate tumor cell migration and invasiveness.[11–13] In addition to their profound effects on tumor progression, TAMs equally affect the efficacy of cancer therapy. For example, TAMs influence the activity of cytidine deaminase (CDA), the key metabolizer of gemcitabine, thereby driving resistance to gemcitabine-based chemotherapy.[14] In addition, also the effects of therapeutic irradiation and targeted antiangiogenic treatment are modulated by TAMs which may acquire different functional states during tumor initiation, progression and therapeutic intervention. TAM functional plasticity has been categorized in two different opposing polarization states, namely, M1 and M2. Generally, M1 macrophages secrete proinflammatory cytokines with antineoplastic effects while M2 macrophages produce anti-inflammatory signals which facilitate tumor progression.[15,16] However, it is noteworthy that this classification is oversimplified and does not fully represent the complexity of TAM action.

Pancreatic neuroendocrine tumors (PNETs) represent a heterogeneous tumor entity within the group of neuroendocrine neoplasms. From a clinical point of view, patients with functionally active PNETs (FA-PNETs) such as insulinomas are mainly diagnosed at early stages, whereas nonfunctional PNETs (NF-PNETs) often present with symptoms when distant metastases have already occurred.[17] Several clinicopathological features have been linked to poor overall survival, including performance status, age, differentiation and proliferation capacity (Ki-67 index).[18–20] In recent years, effort has been made in deciphering the genetic basis of PNETs. Commonly mutated genes include MEN1, DAXX, ATRX and members of the mTOR pathway.[21] Recently, whole-genome sequencing results of >100 PNETs were presented by Scarpa *et al.* In this landmark paper, novel germline mutations in the genes MUTYH, CHEK2 and BRCA2 were described.[22] Moreover, distinct subgroups with altered somatic mutations were identified, including chromatin remodeling, DNA damage repair, activation of mTOR signalling (DEPDC5 and EWSR1) and telomere modification.[22]

The influence of infiltrating immune cells in neuroendocrine tumors has not been completely elucidated yet. In humans, infiltration of TAMs in primary PNETs correlates with proliferative activity, presence of liver metastases and disease recurrence after curative surgery.[23,24] Preclinical studies to decipher the role of immune cells were performed using the RIP1Tag2 genetically engineered mouse model for pancreatic neuroendocrine tumors. This model faithfully recapitulates the multistep process of insulinoma transformation, including the stages of hyperplastic islets, angiogenic islets and invasive tumors with various gradings.[25] When tissue-bound macrophages were constitutively depleted in RIP1Tag2 mice via genetic CSF-1 deficiency, the tumor burden decreased markedly.[23]

Mechanistically, TAMs modulate the tumor micromilieu via secreting multiple mediators of angiogenesis and invasiveness such as VEGF, heparanase and cathepsins.[9,26–28] To overcome TAM mediated disease progression, clinically feasible therapeutics are mandatory. To this end, we utilized liposomal clodronate as pharmacological tool to target TAMs in the RIP1Tag2 model. Moreover, in two large series of FA- and NF-PNETs and in primary and distant metastatic sites, CD68-positive macrophages were assessed to study the association between TAM infiltration and tumor progression in humans.

Material and Methods

Material and cell lines

Human neuroendocrine Bon-1 and QGP1 cells were a kind gift of R. Göke, University of Marburg, Germany[29] (passage 10–30) and cultured in DMEM/HAM's F12 medium. J774 and RAW cells were obtained by ATCC and cultured in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% FCS. All media contained 10% fetal bovine serum and 40 μg/ml gentamicin. All cells were cultured in a humidified atmosphere containing 5% CO_2 at 37°C.

Immunohistochemistry, construction of tissue arrays and evaluation

Ki-67 proliferation index was evaluated according to a standardized protocol using Leica-Bond-Max-Autostainer and the Ki-67 antibody from Dako at a dilution of 1:1000. In addition, CD31 (1:20; Dianova; Hamburg, Germany) and CD68 (Dako, 1:100) antibodies were used. Antibody binding was visualized using a biotinylated secondary antibody, avidin-conjugated peroxidase (ABC method; Vector Laboratories),

3,39 diaminobenzidine tetrachloride (DAB) as a substrate, and H&E as counterstain.

Immunohistochemical staining for CD3 (1:500; abcam), F4/80 (1:50; abDSerotec, BioRad), Caspase-3 (1:100; Cell Signaling), SV40-T-antigen V-300 (1:100; Cell Signaling), FOXP3 (1:50; Affymetrix) and Arginase I (1:50; Santa Cruz) was performed using Leica-Bond-RX. Visualization was performed using Bond Polymer Refine Detection and H&E as counterstaining.

The insulinoma tissue samples (FFPE) were obtained from the neuroendocrine tumor archives of the Departments of Pathology in Zürich (Switzerland), Düsseldorf (Germany) and Kiel (Germany) from 1975 to 2006 according to the guidelines of the local ethics committees. 1.5-mm tissue cores were taken from representative areas and inserted into four paraffin blocks. The 4 MTAs contained a total of 286 cores and additional human tonsil orientation/control cores. The construction was performed using MTA1 tissue arrayer equipment (Beecher Instruments, Sun Prairie, WI). MTAs were routinely processed for paraffin sectioning. Paraffin sections were deparaffinized, rehydrated and immunohistochemical stains were performed according to routine methods. The analysis was performed by a pathologist with expertise in endocrine and pancreatic tumors (B.S.) in a blinded fashion concerning clinical tumor parameters.

Transgenic mouse model and treatments. The RIP1Tag2 transgenic mouse model has been previously described.[25,30,31] RIP1Tag2 mice were generated and maintained in a C57BL/6 background. No glucose-enriched food or water was offered to diminish the risk of hypoglycemia. Liposomal clodronate and PBS-Liposomes were obtained from Dr N. Van Rooijen (Vrije Universiteit, Amsterdam, Netherlands). Liposomal clodronate was injected intraperitoneally twice per week at concentrations of 1.4 mg/20 mg body weight. Sunitinib malate was purchased from Cayman chemical (Ann Arbor, USA) and administered daily by oral gavage at concentrations of 40 mg/kg/d. All animal experiments were approved by the local government authorities and performed according to the guidelines of the animal welfare committee.

Statistical design and analysis. The comparisons between macrophage infiltration, tumor response and histopathological characteristics, disease stage and laboratory features were performed based on χ^2 and Fisher's exact tests, as appropriate. Recurrence-free survival was measured from surgery to recurrence, disease-related death, or last follow-up. OS was measured from the beginning of treatment to the time of last follow-up or death. Actuarial survival was measured by the method of Kaplan and Meier.[32] All data are presented as mean ± standard error deviation. Two-tailed paired Student's t test was used for statistical evaluation of the data. The one-way and nonparametric ANOVA test was used to calculate the p values for more than two groups. A p value <0.05 was considered significant. All statistical calculations were performed using SPSS (IBM SPSS Statistics) and graph pad prism (GraphPad software, La Jolla, CA).

Results

Impact of TAMs in functional-(FA) and functional nonactive (NF) PNETs

TAM infiltration was initially assessed in a cohort of PNET tissues including 21 insulinomas as well as 11 nonmetastatic NF tumors and 13 metastatic NF tumors (among them 14 metastases and 4 primary tumors) (Fig. 1*a*). Baseline characteristics of all patients within this cohort are given in Table 1. As expected due to earlier presentation with symptoms, insulinoma patients were younger than NF-PNET patients, which was independent of the presence or absence of distant metastases (median age: 48 *vs.* 54 *vs.* 58 years, Table 1). Likewise, higher grading significantly correlated with metastatic behavior (G1 1 metastatic *vs.* 9 nonmetastatic, G2 12 metastatic *vs.* 2 nonmetastatic tumors; $p = 0.006$), confirming the influence of proliferation on disease progression. In this cohort, CD68-positive macrophages were counted in at least 10 HPF. Representative immunohistochemical stainings are shown in Figure 1*b*. Mean TAM infiltration in primary tumors did not differ between insulinomas, metastatic NF-PNET and nonmetastatic NF-PNET (163, 170 and 133 CD68+ cells/10 HPF, respectively) (Fig. 1*c*). In contrast, TAM infiltration in metastases (430 CD68+ cells/10 HPF) was markedly higher compared to primary tumor tissues, with significance being reached in comparison to insulinomas and nonmetastatic NF-PNETs ($p = 0.004$ and $p = 0.021$) (Fig. 1*c*). Comparison of the FNA-PNET metastases to primary FNA-PNET tumor tissues (only 1 paired and 3 independent primary tumor samples) did not reach significance due to a small number of samples of available primary FNA-PNET tissues ($p = 0.082$; Fig. 1*c*).

In subgroup analyses, no correlations between TAM infiltration and patient age, tumor grading, Ki-67 or recurrence-free survival were detected, most likely due to small and heterogeneous sample sizes. Thus, we decided to assess TAM infiltration in an independent larger cohort. For this purpose, a multiple tissue array (MTA) was used comprising 83 tissue with benign and malignant insulinomas and normal peritumoral exocrine pancreatic tissues as internal controls. Histopathological characteristics of the patients are presented in Supporting Information, Table 1. The median age of the entire cohort was 50 years. The cohort included more males than females affected by insulinomas (68%). Twelve patients with neuroendocrine carcinomas and 48 patients with well-differentiated tumors and/or metastases were included. The mean density of TAMs was 29, 43 and 234 CD68+ cells/10 HPF in peritumoral exocrine pancreas, benign and malignant insulinomas, respectively (Fig. 2*b*, $p < 0.001$), indicating a significant correlation between increased macrophage infiltration and tumor progression (Fig. 2*a*). The group of malignant insulinomas comprised 7 primary tumors, 5 infiltrated lymph nodes and 7 liver metastases. The highest infiltration of

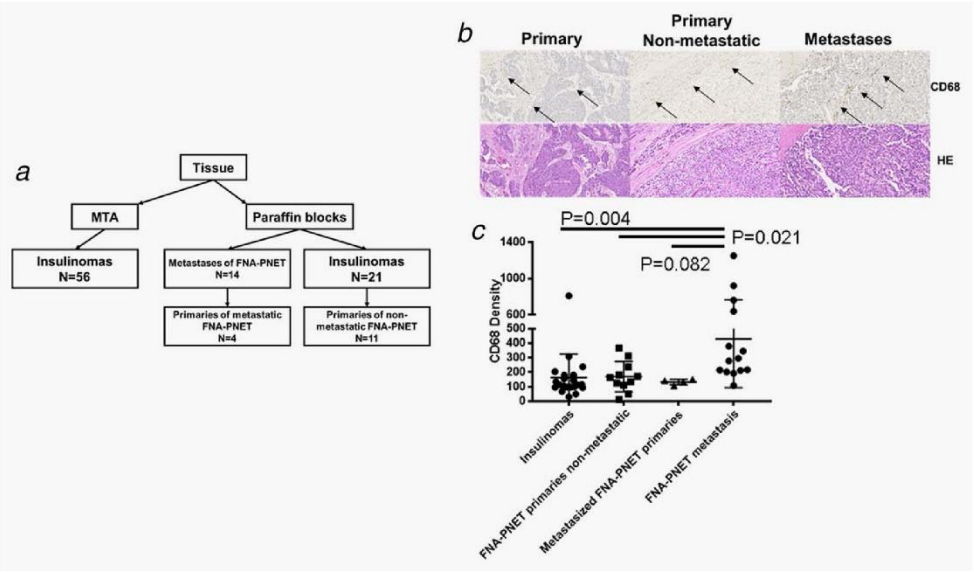

Figure 1. TAM infiltration correlates with malignant features of human PNETs. (*a*) Scheme of tissue included from patients with distinct functionality, stage and grading. Besides whole sections of paraffin blocks, an MTA carrying tissue of 56 insulinomas (including metastasis) was included. (*b*) Representative immunohistochemistry of CD68-positive cells in sections from PNETs and corresponding HE sections were shown with 40× original magnification. (*c*) Quantification of CD68-positive cells in whole paraffin sections (left) of 21 insulinomas, 11 non-metastatic NF-PNETs, 4 primaries of metastatic PNETs and 14 distant metastases. Data shown as mean ± *SD*. Symbols: dots represent data from each tissue; black horizontal line, mean percentage for each group.

TAMs has been detected in liver metastases with a mean of 315 CD68+ cells/10 HPF ($p < 0.05$ compared to primary tumors and lymph nodes with 178 and 145 CD68+ cells, respectively). Moreover, the infiltration of CD68− cells was significantly higher in malignant compared to benign insulinomas (43 *vs.* 178; $p < 0.01$, Fig. 2*b*). Similarly, poor differentiation defined as G3 neuroendocrine carcinomas (NEC) was associated to higher CD68+ macrophage infiltration compared to well-differentiated tumors (NEC *vs.* NET, 243 *vs.* 40 CD68+ cells, $p < 0.0001$). The clinical correlation of these findings showed that in 6 out of 12 patients with NEC, a disease-related death (DRD) occurred with a median overall survival of 27 months. In these patients, the CD68 score reached 303 positive cells on average. No DRD was documented in the group of patients with well-differentiated tumors.

Taken together, these results obtained from two independent cohorts demonstrate that TAMs positively correlate with tumor progression, metastatic behavior and poor outcome.

TAMs in a genetically modified mouse model

To better understand the distinct functions of TAMs during neuroendocrine tumorigenesis, we used the RIP1Tag2 transgenic mouse model, which faithfully recapitulates the stepwise progression of pancreatic neuroendocrine tumors. In this model, the p53 and RB (retinoblastoma) tumor suppressor genes are suppressed by SV40-T-antigen in the pancreatic islet cells under the control of the insulin promoter, mediating the sequential progression from hyperplastic islets via angiogenic islets to various grades of invasive tumors.[25]

To investigate a possible association between TAMs and PNET progression, we assessed TAM infiltration via F4/80 staining, the Ki-67 index and microvessel density via CD31 staining during the course of disease. Representative slides for F4/80, Ki-67 and CD31 are shown in Supporting Information, Figure 1*a*. Sequential stages of hyperplastic ($n = 17$), angiogenic ($n = 22$) and invasive tumors ($n = 18$) demonstrated increased proliferation of tumor cells (mean Ki-67 values: 9%, 28% and 67%; Supporting Information, Fig. 1*c*; $p < 0.001$). The tumor stage-dependent proliferation was paralleled by a significantly increasing number of microvessels in hyperplastic ($n = 16$), angiogenic ($n = 20$) and invasive tumors ($n = 20$) (mean values CD31-positive cells/HPF: 11, 31 and 74; Supporting Information, Fig. 1*d*; $p < 0.001$). In analogy to these findings, infiltrating TAMs have been investigated in hyperplastic ($n = 25$), angiogenic ($n = 12$) and invasive tumors ($n = 15$): the mean numbers of F4/80+ cells/10 HPF increased from 7 (hyperplastic) to 35 (angiogenic)

Table 1. Baseline characteristics of nonmetastatic and functional nonactive PNETs

	NF-PNET, $N = 11$		Insulinomas, $N = 21$		Metastatic PNETs, $N = 13$ Tissue of metastases, $N = 14$ Tissue of primaries, $N = 4$	
	Value	%	Value	%	Value	%
Age at surgery (years)						
Median	54		48		58	
Range	30–79		18–72		31–73	
Tumor differentiation						
Well-differentiated	11	100%	21	100%	18	100%
Grading						
G1	9	82%	21	100%	1	8%
G2	2	18%	0		12	92%
R-Status						
R0	11	100%	19	90.5%		
R1	0	0%	2	9.5%		
M-Status						
M0	11	100%	11	100%	0	0%
M1	0	0%	0	0%	13	100%
Recurrence-free survival in months						
Median	44		9			
Range	5–133		3–15			
CD68 positive cells						
Average	170		163			
Range	13–367		31–809			
Sites of metastasis						
Liver					13	100%
LN					9	69%
SMS-Status						
Positive					12	92%
Surgical intervention						
Primary resection					9	69%
Metastatic surgery					9	69%
No surgery					3	23%
Surgical intervention						
Primary resection					9	69%
Metastatic surgery					9	69%
No surgery					3	23%
CD68 positive cells						
Primaries					133	
Average					107–151	
Range						
Metastases					430	
Average					108–1251	
Range						

Abbreviations: NF, functional nonactive; PNET, pancreatic neuroendocrine tumor; G, grading; R, resection; M, metastases; SMS, somatostatin.

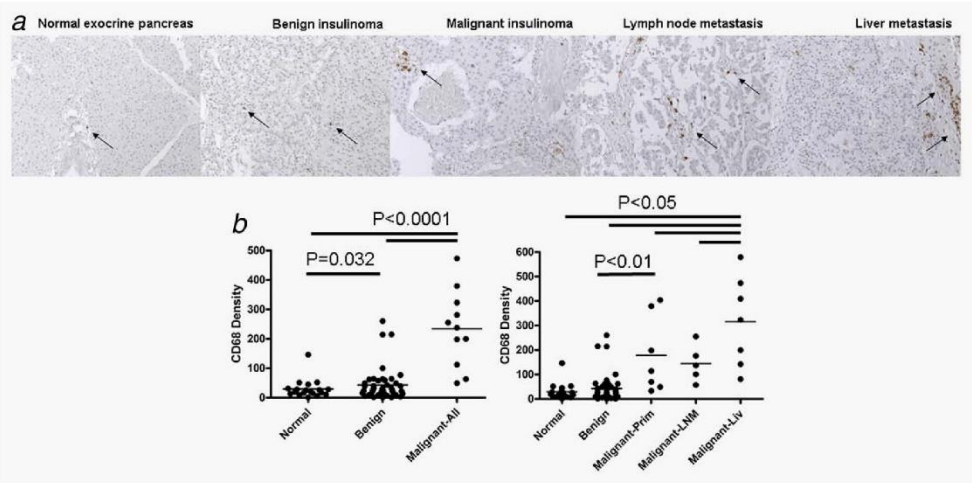

Figure 2. TAM infiltration in an MTA of human insulinomas. (*a*) 200-fold magnification of representative CD68 positive cells in normal pancreas, benign, malignant and metastasis. (*b*) Quantification of CD68 density in the insulinoma MTA (middle and right). Nineteen normal pancreata were compared to 45 benign and 11 malignant insulinomas (middle). Fractions of malignant insulinomas with their sites of metastases were added at the right figure with 7 primary malignant insulinomas, 5 lymph node and 7 liver metastases. Data shown as mean ± *SD*. Symbols: dots represent data from each tissue; black horizontal line, mean percentage for each group.

and 85 (invasive tumors) (Supporting Information, Fig. 1*b*; $p < 0.001$), indicating an increasing infiltration of TAMs during the tumor progression.

Taken together, these results suggest a pivotal role of TAMs during tumorigenesis in PNETs and underline the suitability of the RIP1Tag2 model for therapeutic studies.

Liposomal clodronate targets myeloid cells *in vitro* and *in vivo*

Based on the immunohistochemical data indicating an important role of TAMs in tumorigenesis and tumor progression of both human and murine PNET, we aimed to elucidate the impact of therapeutic TAM modulation on tumor progression. For this purpose, liposomal clodronate has been used as an effective pharmacological tool to deplete macrophages in various *in vivo* models including a mouse model of pancreatic adenocarcinomas.[33] After systemic injection, liposomes are ingested by macrophages followed by intracellular release of clodronate and accumulation of clodronate leading to subsequent apoptosis of the macrophage.[33]

To evaluate the specificity of liposomal clodronate to target TAMs, we first investigated the susceptibility of several murine myeloid cell lines and human pancreatic neuroendocrine tumor cell lines to liposomal clodronate *in vitro*. To this extent, the myeloid cell lines J774, RAW and primary murine bone marrow-derived macrophages (mBMMs) from non-transgenic C57BL/6 mice as well as the neuroendocrine tumor cell lines Bon-1 and GQP1 were utilized. While no effect on cell viability was detected in the tumor cell lines, liposomal clodronate significantly affected cell viability of all investigated myeloid cells with a reduction of cell viability ranging between 39% and 87% after 24 hr (Fig. 3).

In vivo studies to evaluate the impact of liposomal clodronate on PNET tumorigenesis and tumor progression were performed using the transgenic RIP1Tag2 model. Based on the multistage tumor progression in this model, we used two different pharmacological intervention schemes with liposomal clodronate that allowed us to assess its effects both on the angiogenic switch during tumorigenesis ("prevention trial") and on the tumor progression ("intervention trial"). In the prevention trial, mice harboring normal and hyperplastic islets were treated from Week 6 to 13, while in the intervention trial mice were treated from Week 9 to 16 (Fig. 4*a*). Liposomal clodronate effectively decreased the number F4/80+ macrophages during tumor progression as shown in Supporting Information, Figure 2. In the prevention trial, liposomal clodronate reduced the cumulative tumor burden at Week 13 significantly by 62% compared to PBS-liposomes (6.9×10^6 μm^2 *vs.* 2.6×10^6 μm^2; $p = 0.036$, Table 2). While the number of hyperplastic, angiogenic and invasive islets remained unchanged in both groups, clodronate completely prevented the development of invasive tumors in 4 of 10 mice ($p = 0.025$).

In the intervention trial, the influence of liposomal clodronate on tumor progression (number of invasive tumors: mean 4.6 *vs.* 2.2 per mice, $p = 0.069$) and tumor volume (12.7×10^6 μm^2 *vs.* 10.2×10^6 μm^2, $p = 0.47$) was less pronounced and did not reach statistical significance. Therefore,

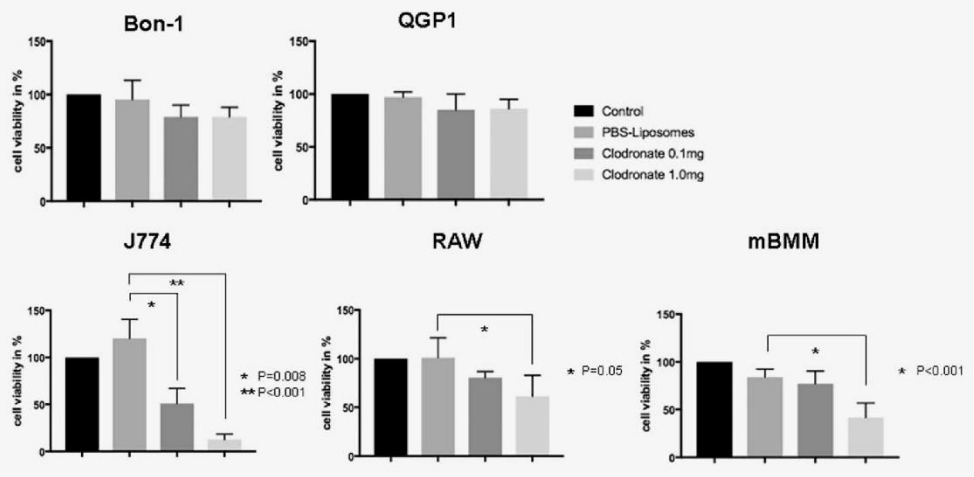

Figure 3. Liposomal clodronate decelerated tumor progression *in vitro* and *in vivo*. (*a*) Human pancreatic neuroendocrine tumor cell lines Bon-1 and QGP1 were treated with PBS-liposomes, 0.1 mg and 1 mg liposomal clodronate for 24 hr. Similarly, the myeloid cell lines J774, RAW and murine bone marrow-derived macrophages (mBMM) from nontransgenic mice were treated uniquely. Assays were performed in triplicate and cell viability was determined by ATP-based assay. Data are presented as mean ± *SD* and are representative of three independent experiments.

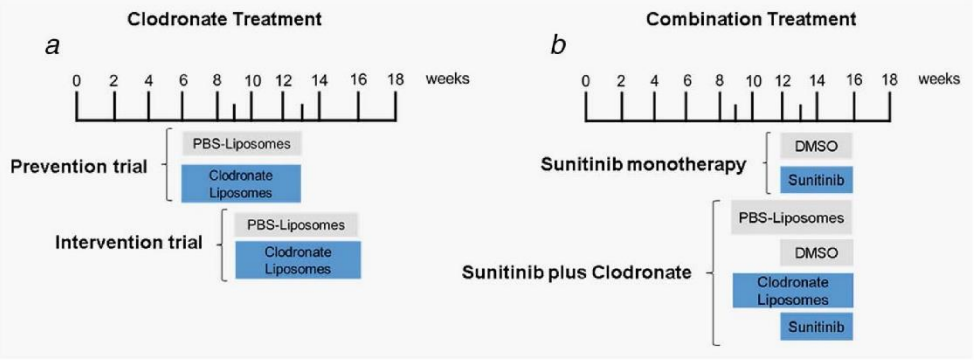

Figure 4. (*a*) Treatment scheme of the prevention (Week 6–13) and intervention (Week 9–16) trial. Liposomal clodronate or PBS-liposomes were applied intraperitoneal in RIP1Tag2 mice biweekly. (*b*) Treatment scheme of sunitnib monotherapy (Week 12–16) daily given per OS and the combination of sunitinib and liposomal clodronate (Week 9–16), which was administered biweekly intraperitoneal compared to DMSO and PBS-liposomes as control arm. [Color figure can be viewed at wileyonlinelibrary.com]

we speculated that TAMs are particularly important in early tumorigenesis during the progression from angiogenic to invasive islets. To further address this hypothesis, we assessed microvessel density (MVD) as cardinal feature of the angiogenic switch using CD31 immunhistochemistry as well as proliferation (Ki-67) and apoptosis marker (cleaved caspase-3). No differences between verum and placebo-treated animals were detected for Ki-67 and cleaved caspase-3 (Table 2). In contrast, CD31− cells in invasive tumors were dramatically diminished in the intervention trial (39 *vs.* 22 positive cells per HPF, $p = 0.006$) with a similar trend also in the prevention trial (77 *vs.* 48 positive cells per HPF, $p = 0.08$). This indicates that angiogenesis is a critical step in mediating progression to invasive tumors, which is affected by macrophage depletion using liposomal clodronate. To characterize the depletion of F4/80+ cells more thoroughly, particularly

Table 2. Results of the prevention and intervention trial in mice being treated with liposomal clodronate or PBS-liposomes

	Prevention trial					Intervention trial				
	PBS-liposomes		Clodronate liposomes			PBS-liposomes		Clodronate liposomes		
Features	Mean	*SD*	Mean	*SD*	*P* value	Mean	*SD*	Mean	*SD*	*P* value
Number of tumors	10.3	1.4	13.0	1.9	0.27	13.0	2.6	10.7	1.2	0.41
Number of invasive tumors	2.7	0.4	1.6	0.5	0.1	4.6	1.2	2.2	0.4	0.069
Prevalence of invasive tumors	**100%**		**60%**		**0.025**	100%		100%		1.0
Tumor area in μm^2	6.9×10^6	1.8×10^6	2.7×10^6	0.9×10^6	0.036	12.7×10^6	2.2×10^6	10.2×10^6	2.5×10^6	0.47
Ki-67 index in invasive tumors	58%	3%	48%	6%	0.11	60%	3%	60	5%	0.99
CD31 positive cells per HPF	76.8	7.9	47.9	13.6	0.08	**39.2**	**2.2**	**22.1**	**3.9**	**0.006**
Caspase 3 positive cells per HPF	10.6	1.3	10.6	2.4	0.99	22.1	6.7	15.5	2.7	0.36
CD3 positive cells per HPF	10.2	2.6	7.1	1.7	0.35	**9.3**	**2.2**	**15.2**	**1.2**	**0.034**
FOXP3 positive cells per HPF	3.1	1.1	3.1	0.7	0.97	4.4	0.4	10.1	2.7	0.091
Arginase positive cells per HPF	4.8	0.9	7.1	1.3	0.16	10.5	1.8	9.6	2.0	0.75

Abbreviation: SD, standard deviation.

effects on macrophage polarization, we assessed arginase expression by immunohistochemistry as established marker for the protumoral M2 polarization of macrophages. Interestingly, no change was seen between liposomal clodronate and PBS-liposomes treated mice, indicating that both M1 and M2 polarized macrophages are targeted by lipocomal clodronate.

As depletion of F4/80+ macrophages may also affect T cell populations, we also stained the T cell marker CD3 and the marker for regulatory T cells FOXP3. In late tumor stages, clodronate application fostered the infiltration of CD3-positive cells (mean: 9 *vs.* 15 positive cells per HPF, $p = 0.034$) in the intervention group. However, at earlier tumor stages, this effect was not present and FOXP3-positive T cells were not regulated in both prevention and intervention cohorts (Table 2). Supporting Information, Figure 3 depicts the representative staining of caspase 3, CD3, FOXP3 and arginase in different tumor stages and lymphnodes.

Dual antiangiogenic therapy with sunitinib in combination with liposomal clodronate

In 2011, the antiangiogenic multikinase inhibitor sunitinib has been approved for the treatment of advanced PNETs.[34] However, primary or acquired resistances frequently limit the efficacy of systemic antiangiogenic therapies in PNET patients during the course of the disease. In preclinical models including the RIP1Tag2 mouse model previous reports proposed sunitinib-induced activation of escape mechanisms such as hypoxia, epithelial–mesenchymal transition (EMT) and other hypoxia-dependent signaling pathways[35–37] as mediators of resistance, thereby accelerating tumor aggressiveness and metastasis formation. Based on these data, we aimed to examine whether these escape mechanisms to antiangiogenic drugs can be overcome by targeting macrophages (Fig. 4*b*).

First, we treated RIP1Tag2 mice with sunitinib monotherapy from Week 12 to 16. Thereby, we could confirm that sunitinib resulted in remarkable reduction of microvessel density measured using an Anti-CD31 antibody (Table 3). However, no differences could be detected in terms of tumor number and volume (Table 3). In contrast to previous reports no liver metastases could be detected by H&E staining and immunohistochemistry using an SV40 antibody (data not shown). However, in the sunitinib group, all mice ($n = 10$) survived the treatment period, whereas 2 of 10 mice in the placebo cohort died before sacrifice, potentially emphasizing a biochemical response during therapy via reducing insulin levels, although statistical significance was not reached due to small sample size.

Furthermore, we depleted macrophages via liposomal clodronate from Week 9 to 16 and simultaneously administered sunitinib (from Week 12 to 16). Combination treatment resulted in a significant reduction of both microvessel density (mean: 39 *vs.* 15 positive cells per HPF, $p < 0.001$) and infiltrating TAMs (mean: 47 *vs.* 24 positive cells per HPF, $p = 0.003$) (Table 3). However, no statistically significant

Table 3. Results of the sunitinib monotherapy arm and the combination treatment of liposomal clodronat and sunitinib in an intervention setting

	Sunitinib monotherapy					Combination treatment				
	DMSO		Sunitinib			DMSO + PBS-liposomes		Sunitinib + clodronate liposomes		
Features	Mean	SD	Mean	SD	P value	Mean	SD	Mean	SD	P value
Number of tumors	8.9	1.9	12.6	2.3	0.25	16.7	3.1	17.3	3.6	0.89
Number of invasive tumors	3.4	0.7	4.4	0.9	0.43	4.8	0.5	4.0	0.9	0.5
Prevalence of invasive tumors	100%		100%		1.0	100%		100%		1.0
Tumor area in μm^2	10.6×10^6	2.3×10^6	10.3×10^6	2.6×10^6	0.92	16.1×10^6	2.4×10^6	11.6×10^6	3.2×10^6	0.32
Ki-67 index in invasive tumors	70%	3%	65%	5%	0.42	72%	4%	77%	3%	0.32
CD31 positive cells per HPF	**33.1**	**2.9**	**16.3**	**2.4**	**0.004**	**39.2**	**2.7**	**14.6**	**2.7**	**0.001**
F4/80 positive cells per HPF	30.7	4.7	23.3	3.8	0.24	**43.9**	**4.2**	**23.4**	**1.7**	**0.003**
Caspase 3 positive cells per HPF	15.6	3.1	16.7	3.6	0.82	18.9	5.3	24.1	3.8	0.43
CD3 positive cells per HPF	19.5	3.3	14.1	1.8	0.14	22.8	4.5	19.6	3.5	0.59
FOXP3 positive cells per HPF	7.1	1.5	7.3	1.7	0.92	7.6	2.1	4.6	1.1	0.19
Arginase positive cells per HPF	5.4	1.4	4.2	0.9	0.49	**6.4**	**1.1**	**11.6**	**1.4**	**0.018**

Abbreviation: SD, standard deviation.

synergies of dual antiangiogenic treatment regarding number of invasive islets or tumor volumes compared to monotherapy could be detected. Similarly, no differences in proliferation and apoptosis markers or infiltrating CD3−, FOXP3+ or Arginase+ cells have been observed between combination and monotherapy strategies (Table 3). Similar to sunitinib monotherapy more mice reached the study endpoint of 16 weeks in the combination arm (9 *vs.* 6) compared to placebo therapy; however, this study has not been designed to evaluate overall survival.

Discussion

In this study, we utilized two independent cohorts of PNET tissue samples to investigate the infiltration of TAMs and its impact on tumor progression and metastasis. Our data clearly show that infiltrating TAMs are significantly correlated to tumor hallmarks such as angiogenesis, proliferation and metastasis. In addition, this study introduces liposomal clodronate as new therapeutic tool to target macrophages in pancreatic neuroendocrine tumors. Liposomal clodronate by depleting TAMs significantly impaired angiogenesis in the RipTag2 genetic mouse model of PNET. Our data suggest that pharmacological depletion of TAMs could serve as a suitable new therapeutic avenue for patients with unresectable disease. However, dual targeting of angiogenesis by simultaneous use of the clinically established multi-tyrosine kinase inhibitor sunitinib and liposomal clodronate in RIP1-Tag2 mice failed to show synergistic antitumor or antiangiogenic efficacy.

Recently, TAMs were assessed in human PNETs samples with the degree of infiltration strongly correlating to proliferative activity, tumor grade and stage.[23] However, this study included only a small heterogeneous patient cohort with 22 functional nonactive (NF) and 5 functionally active (FA) PNETs without detailed characterization. TAM infiltration was only investigated in primary tumor samples. In our study, we used two independent cohorts of PNET including both primary tumors and metastases. In the first cohort, we could detect a remarkable difference between TAMs in primary tumors and distant metastases. However, this cohort also included heterogeneous tumor entities, both functionally active and nonactive. Therefore, we aimed to confirm our data in a more homogeneous PNET cohort and used a TMA comprising benign and malignant insulinoma tissues including lymph node and liver metastases. Hereby, we could confirm the association between TAM infiltration, tumor progression and grading. In analogy to our findings, Wei *et al.* in 2014 reported a positive association between TAM infiltration and recurrence in a small cohort of 38 patients after curative resection of NF-PNETs.[24] In this report, TAM infiltration was only compared semiquantitatively. In contrast, in our series comprising >100 human samples, TAM infiltration was quantified in absolute numbers per HPF which to our knowledge represents the largest study on TAMs in human PNET tissues.

In various cancer models, TAMs have been shown to affect angiogenesis.[38] TAMs secrete a variety of proangiogenic growth factors, most prominently the vascular endothelial growth factor (VEGF), a cardinal mediator of tumor angiogenesis.[39,40] TAM-induced upregulation of VEGF results in the so-called angiogenic switch that leads to enhanced microvessel formation and tumor progression. Our study clearly corroborates these findings showing a significantly reduced microvessel density following macrophage depletion.

Several trials are currently ongoing to evaluate antibody- or small-molecule-based strategies targeting macrophage-derived proteins such as CCR2 or CSF1 that, however, might be associated with a considerably toxicity. In contrast, systemic application of macrophage-modulating bisphosphonates represents a promising therapeutic avenue with a favorable toxicity profile. This "drug repurposing" approach makes use of a compound which has been used in clinical practice of osteoporosis treatment for many years.

Clodronate coated in liposomes has been widely used as experimental tool for macrophage depletion in several disease models including autoimmune disorders, atherosclerosis and cancer.[41] The use of liposomal clodronate has been reported in several xenograft tumor models.[42,43] Recently, we depleted TAMs by liposomal clodronate in a mouse model of pancreatic adenocarcinoma (PDAC).[33] Interestingly, in this model liposomal clodronate induced a significant decrease in metastasis formation. The size of the primary tumor, however, was only reduced to a minor extent.[33] We speculate that different angiogenesis patterns in PDAC versus PNET might be responsible for this difference: PDAC primary tumors are largely hypovascular which limit the antiangiogenic effect of liposomal clodronate on the primary tumor. In these tumors, the effect of premetastatic niches in target organs liver and lung might be more important. In contrast, PNET tissues are highly vascularized resulting in an increased efficacy of antiangiogenic strategies in PNET tumors including therapeutic targeting of TAMs.

The depletory effect on macrophages appears to be a group effect and not to be restricted to liposomal-encapsulated clodronate. It has also been observed with other bisphosphonates such as zoledronate which has been shown to reduce viability of macrophages without affecting cancer cells *in vitro* and to diminish intratumoral macrophages in xenograft models *in vivo*.[44,45] However, liposomal coating of drugs such as clodronate enhances their phagocytosis by cells such as macrophages, thereby increasing therapeutic efficacy. Generally, nanoparticle-coating of cytotoxic drugs such as doxorubicin or irinotecan with liposomes is increasingly used to decrease toxicity and improve tolerability.[46,47]

Despite of the pronounced effects of liposomal clodronate action on microvessel density and tumor progression, we failed to detect a significant synergism of liposomal clodronate with the antiangiogenic multikinase inhibitor sunitinib, which has been shown to target several VEGF-dependent signaling pathways *in vitro* and to significantly improve progression-free survival in PNET patients. This is in contrast to a report using xenograft HCC mouse models in which zoledronate was shown to overcome sunitinib- and sorafenib-induced IL12-dependent prometastatic resistance mechanisms.[48] The reasons for the lack of synergism in our *in vivo* model have yet to be elucidated. Previously, we have shown that liposomal clodronate leads to a pronounced downregulation of VEGF as main secretion product of TAM.[33] It may be speculated that both macrophage depletion and sunitinib application predominantly affect VEGF-dependent angiogenic pathways which could explain in part the lack of synergism in the highly VEGF-dependent PNET model.

Taken together, our data indicate that TAM plays a major role in tumor progression of pancreatic neuroendocrine tumors. Pharmacological macrophage depletion by liposomal clodronate leads to a pronounced antiangiogenic effect decreasing tumorigenesis and tumor progression, which could represent a new therapeutic avenue for this highly angiogenic tumor entity. Based on our data, further studies are warranted to evaluate the role of bisphosphonates alone or in combination with established therapies such as somatostatin analogs or cytotoxic drugs in pancreatic neuroendocrine tumors.

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Tumor-assoziierte Makrophagen (TAM) spielen in vielen Entitäten eine wichtige Rolle als Mediator der Tumorprogression. In pankreatischen neuroendokrinen Neoplasien sind TAM bislang nur unzureichend charakterisiert worden. Wir haben daher TAM an verschiedenen humanen Kohorten mittels CD68 Immunhistochemie untersucht und mit Patientencharakteristika und der Tumorpathologie verglichen. Dabei zeigte sich in Primärtumoren zwischen nicht funktionellen und funktionellen sowie nicht metastasierten und metastasierten Stadien kein Unterschied der Makrophageninfiltration. Die TAM Dichte in Metastasen zeigte sich allerdings signifikant erhöht im Vergleich zu denen in Primärtumoren. In einer großen und homogenen Kohorte an Insulinomen (n=60) korrelierte die TAM Infiltration mit der Malignität, der Metastasierung und auch mit dem Grading. Daraufhin wurde in in vitro und in vivo Ansätzen der therapeutische Effekt auf Makrophagen disseziert. Dazu verwendeten wir ein in Liposomen verpacktes Bisphosphonat (Clodronat), welches nur von Makrophagen phagozytiert wird und bei Kumulation zur Apoptose dieser Zellen führt. Dabei zeigte sich ein solitärer antiproliferativer Effekt von liposomalen Clodronat auf myeloische Zelllinien (J774, RAW) und isolierte mBMM (murine bone marrow macrophages) im Vergleich zu den neuroendokrinen Tumorzelllinien Bon1 und QGP1. Danach wurde der Effekt von liposomalen Clodronat im RIP1Tag2 Mausmodell in einem präventiven und einem interventionellen Ansatz untersucht. Im präventiven Ansatz konnte die maligne Transformation in dem Tumorprogressionsmodell signifikant gesenkt werden, was begleitet war von einer Reduktion F4/80 positiver Makrophagen und der Gefäßdichte (Untersuchung auf CD31). Im späteren Stadium (Interventionsansatz) zeigten sich die Effekt nicht mehr signifikant, was darauf hindeutet, dass die Angiogenese als entscheidender Schritt vom angiogenetischen

Tumor zum invasiven Tumor durch TAM mediiert wird. Ein synergistscher Effekt des liposomalen Clodronat in Kombination mit dem Tyrosinkinaseinhibitor Sunitinib bestätigte sich nicht. Dabei sollten durch TAM induzierte Resistenzmechanismen, nach deren Depletion mit Clodronat, überwunden und die Wirkung von Sunitinib verstärkt werden. Zusammenfassend spielen TAM eine essentielle Rolle in der Tumorprogression und Therapieresistenz von PanNEN. Neue therapeutische Ansätze sollten neben einer klassischen zytotoxischen oder zielgerichteten Therapie auch eine Therapie des Tumormicroenvironment einschließen.

4 Diskussion

In neuroendokrinen Neoplasien (NEN) inklusive pankreatischen neuroendokrinen Neoplasien (PanNEN) sind das TNM Staging System sowie das von der WHO etablierte Grading System unter Angabe des Ki-67 Index die bislang robustesten prognostischen Marker [5, 6].

Neben diesen etablierten Biomarkern wurden weitere Marker auf genetischer, epigenetischer und RNA-Ebene untersucht. Veränderungen im PI3K/Akt/mTOR finden sich häufig und korrelieren mit dem Verlust von PTEN, TSC2 und MEN1 in PanNET. In sporadischen PanNET korreliert dabei eine geringe TSC2 und PTEN Expression mit einem schlechten Gesamtüberleben und krankheits-freien Überleben nach kurativer Resektion [22]. Zusätzlich zeigen sich epigenetische Veränderungen als Treiber der Tumorprogression und mit prognostischer Relevanz. Ein DAXX oder ATRX Verlust war mit einer schlechten Prognose in PanNET und einer chromosomalen Instabilität (CIN) vergesellschaftet [27, 28]. Das Vorliegen einer CIN korrelierte wiederum mit einem aggressiveren biologischen Verhalten in PanNEN [20]. Der DAXX/ATRX Komplex reguliert zudem Histone (z.B. H3K9me3). Veränderungen in Histon H3 und H4 waren in einer Subgruppe von PanNET mit dem Ki-67 und der Differenzierung assoziiert [74]. Weitere häufig epigenetisch regulierte und methylierte Gene sind MGMT (O6-Methylguanin-DNA-Methyltransferase), THSB1, P14 und P16. Der MGMT Status ist seit längerer Zeit in kritischer Diskussion, weil für ihn auch prädiktive Eigenschaften postuliert werden. Mehrere retrospektive Studien validierten den prognostischen Wert von MGMT und kamen zu unterschiedlichen Ergebnissen. Die Arbeitsgruppe von Perren zeigte, dass der Expressionsverlust von MGMT auf Proteinebene (immunohistochemische Darstellung) mit einem geringem Überlegen assoziiert war [75].

In der vorliegenden Habilitation wurden zwei auf Proteinebene dysregulierte Zielgene, der Tumorsuppressor Pdcd4 sowie der Transkriptionsfaktor CUX1, in verschiedenen pankreatischen neuroendokrinen Tumorzelllinien als Mediatoren der Tumorprogression charakterisiert. Die Funktionen von Pdcd4 sind Zelltyp-spezifisch [76]. In neuroendokrinen Tumorzellen inhibierte der Tumorsuppressor über die Regulation von eIF4E die Proteinsynthese und steuerte die Carboanhydrase II, ein essentielles Enzym multipler intrazellulärer Signalwege, u.a. involviert in die Pyrimidinsynthese, Glukoneogenese und Aminosäurensynthese [76]. Weitere Untersuchungen in Bon1 Zellen zeigten einen Einfluss auf die dUTPase, ein wichtiges Substrat für die Synthese von Thyminnukleotiden über die Thymidylat-Synthase. Eine hohe dUTPase Expression stellt einen Resistenzmechanismus von Tumorzellen dar, vor allem gegen Antimetaboliten wie 5-FU und deren Derivate [77]. Unsere Ergebnisse reihen sich in die beschriebenen tumorsuppressiven Eigenschaften von Pdcd4 ein. Neu ist die Erkenntnis, dass Pdcd4 seine Effekte über Ang-2 vermittelt und direkt Endothelzellen und damit die Angiogenese beeinflussen kann. Die Angiogenese ist ein entscheidender Faktor im Rahmen der Tumorprogression und der „angiogenic switch“ ist daran maßgeblich beteiligt [78]. Bekannt ist, dass das Zytokin VEGF in diesem Prozess eine zentrale Rolle einnimmt. Obwohl zirkulierendes VEGF in unterschiedlichen Konzentrationen abhängig von der Primärtumorlokalisation vorhanden ist, zeigten sich höhere VEGF Spiegel in NET Patienten mit nachgewiesenen Tumorprogress im Vergleich zur Kontrollgruppe mit stabiler Erkrankung [79]. Dabei zeigen gut-differenzierte neuroendokrine Tumore eine hohe Gefäßdichte und starke VEGFA Expression, was mit einer besseren Prognose assoziiert ist, als die wenig stark vaskularisierten und aggressiveren neuroendokrinen Karzinome. Dieser Effekt wird als neuroendokrines Paradoxon bezeichnet [80]. Im Verlauf konnten translationale Arbeiten eine ebenso prägende Funktion von Ang-2 als

Prädiktor für eine starke Angiogenese in NET darlegen. Ang-2 und deren Rezeptor TIE2 fanden sich signifikant erhöht in NET Patienten und zeigten eine Korrelation zur Metastasierung und Prognose [81]. Deutlich erhöhte Werte an zirkulierendem Ang-2 korrelierten außerdem mit einem schnelleren Tumorprogress [82]. Antiangiogen wirkende Substanzen wie Sunitinib und Everolimus konnten in großen klinischen Phase III Studien einen signifikanten Effekt auf das progressions-freie Überleben präsentieren und beide Therapieansätze sind zur Therapie von pankreatischen NET zugelassen worden [62, 83]. Allerdings zeigen einige Patienten ein primäres oder sequentielles Therapieversagen auf antiangiogene Therapien. Gründe dafür liegen in primären oder sekundären Resistenzmechanismen. Die primär vorhandene Resistenz basiert auf dem Ausgleich der Angiogenesehemmung durch weitere Zytokine und proangiogene Faktoren. Neben dem VEGF-Signalweg gibt es VEGF unabhängige Mechanismen z.B. die Freisetzung von Zytokinen über proinflammatorischen Zellen (IL-6, TNF-a, IL-1) [84]. Die VEGF Blockade über die etablierten Therapie mit Sunitinib und Everolimus kann damit kompensiert und überwunden werden. Adaptive Mechanismen der erworbenen Resistenz sind vielfältig. Ein zentraler Mediator der Resistenz ist HIF-1a (Hypoxia-inducible factor-1 alpha). HIF-1a ist ein Transkriptionsfaktor, der durch hypoxische Zustände in seiner Expression induziert wird und Einfluss auf die Angiogenese in PanNET besitzt [85]. Dabei führt die Induktion des Transkriptionsfaktor zur Regulationen von diversen Genen, welche in die Sauerstoffversorgung und –Freisetzung und anaerobe Stoffwechselvorgänge involviert sind [85]. Neben hypoxischen Zuständen durch ein rasantes Tumorwachstum oder antiangiogene Therapie ist ein weiterer Mechanismen der HIF-1a Regulation die Inaktivierung des VHL Gens über somatische oder epigenetische Veränderungen. Bis zu 25% der spontanen PanNET weisen Veränderungen des VHL Gens auf [86]. Die Induktion von HIF-1a führt dann zur Induktion von alternativen

Signalwegen über Angiopoietine, den FGF-Rezeptor, ggf. über EMT (Epithelial-mesenchymale Transition) zur Resistenz. In zwei humanen Kohorten von GEP-NET war die immunhistochemische Expression von HIF-1a ein unabhängiger Prädiktor für das Gesamtüberleben [87]. Dabei korrelierte HIF-1a mit einer schlechten Differenzierung und höherer Proliferation [88]. Wir konnten zeigen, dass der Transkriptionsfaktor CUX1 durch die Regulation von HIF-1a und MMP-9, einem weiteren proangiogenem Zytokin, die Angiogenese signifikant moduliert. CUX1 (Cut homeobox 1) ist auf der Chromosomenbande 7q22.1 lokalisiert und existiert in verschiedenen Isoformen. CUX1 wurden in verschiedenen Malignomen als Mediator der Tumorigenese und Tumorprogression charakterisiert, in PanNEN gab es bis dato keine Untersuchungen von CUX1 [89, 90]. Spätere Arbeiten charakterisierten CUX1 als Repressor NF-kB regulierter Chemokine in inflammatorischen Zellen und zeigten auch einen Einfluss auf die Angiogenese im Adenokarzinom des Pankreas [91]. Die besondere Bedeutung von CUX1 in PanNEN wurde außerdem unterstrichen durch die Validierung der in vitro Arbeiten im Xenograft Modell und an einer humanen Kohorte von funktionell aktiven PanNEN. CUX1 agiert als Modulator der Tumoraggressivität in PanNEN, insbesondere weil CUX1 auf verschiedenen Ebenen (Proliferation, Apoptose und Angiogenese) die Tumorprogression beeinflussen kann.

Einen weiteren Schwerpunkt der Habilitation bildete die Untersuchung von Tumor-assoziierten Makrophagen in PanNEN. Neben der Tumorzelle selbst ist der zunehmende Einfluss der Tumormikroumgebung, insbesondere von infiltrierenden Immunzellen wie den TAM, auf Tumorinitiierung, -progression und Metastasierung in den letzten Jahren in den Fokus gerückt. Während in einigen Entitäten TAM schon länger erforscht wurden, war die Rolle von TAM in PanNEN nur in wenigen Arbeiten untersucht worden. TAM können durch die Expression und Sezernierung von proangiogenen Faktoren wie VEGF, Heparanase und Cathepsinen indirekt und direkt

auf das Tumorwachstum und das Mikromilieu Einfluss nehmen [92]. In klinischen Studien korrelierte die TAM Infiltration in Primärtumoren mit dem Proliferationsindex Ki-67, dem Auftreten von Lebermetastasen sowie dem Rezidiv-freien Überleben nach kurativer Resektion [93, 94]. Unsere humanen Ergebnisse von TAM in zwei großen Kohorten von funktionell aktiven und inaktiven unterstützen die Hypothese, dass die TAM Infiltration rein deskriptiv an der Tumorprogression beteiligt ist. Um den Mechanismus von TAM auf die Tumorprogression zu erörtern haben wir das genetische neuroendokrine Mausmodell RIP1Tag2 benutzt. Die Gruppe von D. Hanahan hat das international vielfach verwendete transgenes Mausmodell entwickelt, das die humane Erkrankung von PanNEN gut rekapituliert. In diesem murinen Modell werden spezifisch in endokrinen pankreatischen Inselzellen über das Onkogen SV40-T-Antigen die Tumorsuppressoren p53 und RB herunterreguliert und dadurch die Entwicklung von funktionell aktiven und inaktiven PanNEN induziert. In kurzer Zeit entwickeln sich in diesem Modell aus normalen Inselzellen invasive Tumore [29]. Durch die gute Charakterisierung des Modells zeigte sich, dass aus den ursprünglich etwa 400 murinen Langhans-Inseln regelhaft ab der 5. Lebenswoche hyperplastische und dysplastische Vorstufen entstehen. Nach der 5. Lebenswoche kommt es zu dem sog. „angiogenen switch“, welcher maßgeblich an der Progression von gut vaskularisierten Inselzellhyperplasien zu Inselzelladenomen beteiligt ist. Nach der Entwicklung von Inselzelladenomen bis zur 10. Lebenswoche führt die weitere neoplastische Transformation zur Entstehung von invasiven Tumoren. Zusätzlich konnte im RIP1Tag2 Modell, ähnlich wie in humanen PanNEN bewiesen werden, dass die Angiogenese, gesteuert durch mehrere gut definierte Signalwege (IGF-II/IGF-1R, MMP-9 und MMP-2, VEGF-A/VEGFR2, mTOR, EGFR und PDGF-B/PDGFRβ), eine essentielle Rolle im Rahmen der Tumorprogression spielt [95, 96]. In einer Arbeit von Pyonteck wurden ortsständige Makrophagen durch eine genetische Modulation von

CSF-1 (colony-stimulating factor-1) im RIP1Tag2 Modell depletiert [93]. Während die Makrophageninfiltration um 50% in allen Tumorstadien sank, zeigte sich innerhalb der verschiedenen Tumore kein Unterschied auf Proliferation, Apoptose, Invasion und Angiogenese. Die insgesamt reduzierte kumulative Tumorlast in diesem Versuchsansatz wurde durch das Auftreten von weniger Tumoren und die Einflussnahme auf den „angiogenic switch“ erklärt [93]. Unser Ansatz konzentrierte sich auf die pharmakologische Depletion von TAM, sowohl von ortsständigen als auch potentiell zirkulierenden TAM. Damit bietet sich ein selektiverer Ansatz an eine bestimmte Makrophagenpopulation anzugreifen. Unsere Untersuchungen unterstützen allerdings die Hypothese, dass Makrophagen/TAM in der frühen Phase in präinvasiven und angiogenetischen Stadien essentiell für die Tumorprogression sind. Allerdings sahen wir unabhängig vom Tumorstadien im Gegensatz zu Pyonteck et al. durchgängig einen antiangiogenen Effekt von TAM. Zudem korrelierte die Makrophageninfiltration mit dem Proliferationsindex Ki-67 und dem MVD (micro-vessel density) Marker CD31. In späteren Tumorstadien scheint die alleinige Modulation von TAM nicht auszureichen, um signifikante Effekte zu erzielen. Dabei hatte die duale sequentielle Therapie von liposomalem Clodronat und Sunitinib folgende Rationale. Durch die Depletion von Makrophagen sollten vorhandene primäre Resistenzmechanismen ausgeschaltet werden, damit eine klassische antiangiogene Therapie mit Sunitinib eine effektivere Wirkung zeigt. Dieses Vorgehen konnte im RIP1Tag2 Modell nicht bestätigt. Während andere in vivo Studien im RIP1Tag2 Modell mit einer dualen Therapie z.B. mit VEGFR/FGFR Inhibitoren vielversprechende Ergebnisse erzielten [97] zeigten klinische Studien der Kombinationstherapie keinen Zusatznutzen [98, 99].

Die ersten drei Arbeiten der kumulativen Habilitation haben neue proangiogene Marker charakterisiert, welche zusätzlich, durch Modulation der Proliferation und Apoptose in vitro und in vivo, einen prognostischen Charakter aufwiesen. Neben der präklinischen und translationalen Evaluation von Biomarkern haben wir eine monozentrische Kohorte am ENETs Zentrum Marburg evaluiert und entsprechend einer therapeutischen Stratifizierung nach prädiktiven und prognostischen Effektoren gesucht. Unter den GEP-NEN zeigen hauptsächlich PanNET eine Sensitivität auf zytotoxischen Therapien. Die aktuellen europäischen und deutschen Leitlinien empfehlen daher als Erstlinientherapie eine Streptozocin basierte Chemotherapie [48, 100]. Allerdings sind die randomisierten Studien zu Streptozocin (STZ) aus den 80-iger Jahren sehr heterogen und entsprechen, was die radiologische Auswertung und supportive Therapie betrifft, nicht dem heutigen Standard [101]. Neuere Studien mit RECIST Auswertung beweisen weiterhin einen Benefit von STZ Kombinationen mit Ansprechraten von 30-40%.[102]. Der Nutzen der STZ basierten Therapie wurde von uns und zwei weiteren großen ENETs Zentren (ebenso retrospektive monozentrische Arbeiten) publiziert [103, 104]. Im Marburger Kollektiv zeigten sich 91% der Patienten im metastasierten Stadium, 78% gut differenzierte Tumore, 88% mit Lebermetastasen (und fast 40% Knochenmetastasen), wobei nur 20% der Patienten Therapie-naiv waren und eine leitlinienempfohlene Erstlinienchemotherapie erhielten. Während sich die Ansprechraten und die Krankheitsstabilisierung (70-90%) in allen Studien ähnelten, waren im mPFS und mOS deutliche Unterschiede erkennbar. Die mPFS und mOS Ergebnisse aus Marburg waren mit 16 und 28 Monaten niedriger als die der Vergleichsgruppen aus Berlin und Uppsala. Gründe dafür waren der geringe Einsatz von STZ als erste Therapie und 15% der eingeschlossenen Patienten hatten keinen pankreatischen Ursprung, was einen zusätzlichen Bias präsentierte. Alle Studien untersuchten prädiktive und prognostische Marker innerhalb der Kohorte. Dabei

ergaben sich robuste Ergebnisse für Ki-67 und Grading als prognostische Faktoren. In unserer Kohorte zeigte sich zudem neben dem biochemischen Ansprechen (Chromogranin A Abfall mehr als 30% zum Basalwert) auch eine positive SSTR Bildgebung als prädiktiv. Da die SSTR Expression ebenso korreliert mit der Differenzierung ist diese auch als Surrogatparameter für gut differenzierte NET anzusehen, unabhängig davon, ob es sich hierbei um einen G1,G2 oder G3 Tumor handelt. Weitere prädiktive Marker für eine STZ basierte Chemotherapie in PanNET Patienten wurden im Verlauf veröffentlich mit dem Fokus auf das DNA-Reparatursystem. Dazu wurde der MGMT-Status immunhistochemisch und der MGMT Promotermethylierungsstatus erhoben. Vielversprechende Daten publizierten Schmitt [75], Walter [105] und Kulke [106]. Die aktuellste Arbeit von Cives et al. konnte diese Daten an ihrer Kohorte allerdings nicht bestätigen [107]. Ein neues Werkzeug zur Stratifizierung von GEP-NET Patienten unter Therapie könnte der NETest sein. Der NETest beinhaltet eine Gen-basierte Signatur von 51 Genen [108]. Diese werden nach verschiedenen Algorithmen ausgewertet und nach Tumoraktivität bewertet. In mehreren Studien zeigte der NETest seine Robustheit mit guten inter- und intra-Variationskoeffizienten. Dabei war der NETest in der Lage einen Tumorprogress früher zu detektieren als die Bildgebung. Eine geringe Aktivität des NETest korrelierte mit einer langen Krankheitsstabilität (~5 Jahre), während hohe Werte einen zügigen Progress innerhalb von 2 Jahren vorhersagten [109, 110]. Ziel ist es prädiktive Marker in großen prospektiven randomisierten Studien zu validieren, um deren Implikation in der klinischen Routine zu evaluieren.

5 Schlussfolgerung

Der Tumorsuppressor Pdcd4 und der transkriptionelle Repressor CUX1 modulieren über Ang-2, HIF-1a und MMP-9 die Angiogenese und die Prognose von PanNEN. Neben ihren proangiogenen Effekten in vitro sind beide Gene an einem aggressiven Phänotyp durch Aktivierung anti-apoptotischer und pro-proliferativer Signalkaskaden involviert. Für CUX1 wurden diese Effekte auf Proliferation und Malignität im Xenograft- und im transgenen Mausmodell (RIP1Tag2) in vivo sowie an einer humanen Kohorte funktionell aktiver PanNEN korroboriert. Neben direkten Mediatoren der Tumorprogression innerhalb der Tumorzelle, modellierten wir den Einfluss von Tumor-assoziierten Makrophagen (TAM) auf PanNEN. Deren prognostische Bedeutung wurde in 2 humane Kohorten und deren Einfluss auf die Tumorprogression in frühen Stadien im genetischen RIP1Tag2 Modell validiert. Die TAM Infiltration zeigte dabei eine starke Korrelation zur Proliferation und Angiogenese. Außer präklinischen Untersuchungen wurden Biomarker an einer retrospektiven Kohorte von PanNET Patienten evaluiert. Unter zytotoxischer Chemotherapie wurden die etablierten prognostischen Biomarker Ki-67 und das WHO Klassifikationssystem bestätigt. Prädiktive Marker für die Chemotherapie waren biochemisches Ansprechen und ein positiver Somatostatinrezeptorstatus.

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7 Thesen

1. Pdcd4 und CUX1 modulieren über Ang-2, HIF-1a und MMP-9 die Angiogenese und die Prognose von PanNEN.
2. Pdcd4 inhibiert und CUX1 induziert einen aggressiven Phänotyp durch Modulation anti-apoptotischer und pro-proliferativer Signalkaskaden.
3. Die Effekte von CUX1 auf Proliferation und Malignität sind im Xenograft-, im transgenen Mausmodell (RIP1Tag2) sowie in humanen funktionell aktiven PanNEN präsent.
4. Tumor-assoziierte Makrophagen (TAM) haben einen Einfluss auf die Tumorprogression in humanen PanNEN.
5. Die TAM Infiltration korreliert im RIP1Tag2 Mausmodell mit der Proliferation und Angiogenese.
6. Eine pharmakologische Depletion von TAM verhindert die Ausbildung invasiver PanNEN.
7. Bei Patienten mit PanNEN unter zytotoxischer Chemotherapie sind der Biomarker Ki-67 und das WHO Klassifikationssystem etablierte prognostische Faktoren.
8. Prädiktive Marker für das Ansprechen unter Chemotherapie sind ein biochemisches Ansprechen und ein positiver Somatostatinrezeptorstatus.

8 Selbstständigkeitserklärungen

Halle (Saale), den 12.7.2018

Sehr geehrte Damen und Herren,

hiermit erkläre ich, dass ich diese Habilitationsleistung selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Sebastian Krug

9 Erklärung über frühere Habilitationsversuche

Halle (Saale), den 12.7.2018

Sehr geehrte Damen und Herren,

hiermit erkläre ich, dass an keiner anderen Fakultät oder Universität ein Habilitationsverfahren anhängig ist. Bislang gab es keine etwaigen früheren Habilitationsverfahren oder abgelehnte Habilitationsgesuche an anderen Universitäten.

Sebastian Krug

10 Lebenslauf

Angaben zur Person:

Name:	Dr. med. Sebastian Krug
Geburtsdatum/-ort:	20.07.1985 in Bad Salzungen, Thüringen
Familienstand:	verheiratet, 2 Kinder

Schulausbildung :

1992 – 96	Grundschule Unterbreizbach
1996 – 2004	Johann Gottfried Seume Gymnasium Vacha
2004	Abitur (Durchschnittsnote 1,7)

Studium:

2004 – 2010	Studium der Humanmedizin an der Universität Marburg
8/2006	I. Abschnitt der Ärztlichen Prüfung
10/2010	II. Abschnitt der Ärztlichen Prüfung (Gesamtnote 2,0 „gut“)
07/2009 – 06/2010	Praktisches Jahr: VTG-Chirurgie, Innere Medizin und Radiologie am Universitätsklinikum Marburg

Beruflicher Werdegang:

12/2010	Approbation
01/2011 – 03/2015	Wissenschaftlicher Angestellter in der Klinik für Innere Medizin, Schwerpunkt Gastroenterologie (Direktor: Prof. Dr. T.M. Gress) der Philipps-Universität Marburg
Seit 04/2015	Wissenschaftlicher Angestellter in der Klinik für Innere Medizin I, Schwerpunkt Gastroenterologie und Pulmologie (Direktor: Prof. Dr. P. Michl) der Martin Luther Universität Halle/Wittenberg
07/2016	Facharzt für Innere Medizin

Wissenschaftlicher Werdegang:

5/2008 – 05/2011	Experimentelle Promotionsarbeit Universitätklinikum Marburg, Bereich Gastroenterologie, Note: „cum laude“ (Direktor: Prof. Dr. T.M. Gress)
01/2011 – 03/2015	Mitglied des molekulargenetischen Forschungslabors von Prof. Dr. Thomas Gress Klinik für Innere Medizin, SP Gastroenterologie, Forschungsbereich 3, AG Prof. Dr. Michl
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01/2018	Clinical Scientist Programm MLU Halle/Wittenberg

Wissenschaftliche Schwerpunkte:

1. Charakterisierung der Angiogenese und der Therapie-induzierten Resistenzmechanismen in Neuroendokrinen Neoplasien
2. Klinisch translationale Ansätze zur Detektion prognostischer und prädiktiver Marker in NEN
3. Immunmodulation und neue zielgerichtete Therapieansätze in NEN

Fortbildungen/Zusatzqualifikationen:

03/2012	Durchführung klinischer Prüfungen gemäß Arzneimittelgesetz (AMG) und GCP-Verordnung (GCP-V)
12/2012	FELASA B Zertifikat für versuchstierkundliche Grundlagen und Methoden
02/2013	Durchführung klinischer Prüfungen gemäß Arzneimittelgesetz (AMG) und GCP-Verordnung (GCP-V), Update Gesetzesänderungen
10/2013	Theoretischer Teil für die Zusatzbezeichnung Notfallmedizin Marburger Kompaktkurs
03/2016	DGVS Zertifikat Onkologische Gastroenterologie
04/2016	GCP-Refresher-Kurs
09/2017	Hochschullehrerlehrgang Martin-Luther Universität Halle/Wittenberg
09/2017	Prüfarztschulung für klinische Prüfung nach MPG/ISO 14155

Drittmittelförderungen:

2012 Pharmakologische Immunmodulation im transgenen RIP-Tag-Modell (THERANOSTIC RESEARCH NETWORK 10.000€)
2013 Biomarker in neuroendokrinen Neoplasien (80.000€)
2013 Charakterisierung der molekularen Mechanismen der CUX1-induzierten Angiogenese bei Neuroendokrinen Neoplasien (Röntgen-Behring-Stiftung 180.000€)
2014 Interactions between SSTR modulation via lanreotide and TKIs in sequential and combination approaches in vitro and in vivo (100.000€)
2017 Everolimus-Resistenz in NEN (Novartis Pharma) Kooperationsprojekt (110.000€)
2018 The impact of epigenetic changes on long non-coding RNAs as a mediator of tumor progression in pancreatic neuroendocrine tumors (160.000€)

Stipendien, Preise und Auszeichnungen:

10/2013	Travel scholarship recipient, UEGW Berlin, Dotation: 1000€
04/2014	Gewähltes Mitglied zum 7. Kurs Pancreas 2000
06/2015	ESDO fellowship, NCI Amsterdam, Dotation: 1000€
04/2017	Graduiertenstipendium der Novartis-Stiftung für therapeutische Forschung: 8000€
07/2017	Clinical Scientist Programm MLU Halle/Wittenberg

Mitgliedschaften in wissenschaftlichen Fachgesellschaften/Organisationen:

Seit 2011	Deutsche Gesellschaft für Verdauung und Stoffwechsel (DGVS)
Seit 2011	Deutsche Gesellschaft für Innere Medizin (DGIM)
Seit 2013	European Neuroendocrine Tumor Society (ENETS)
Seit 2015	European Society of Digestive Oncology
Seit 2015	Deutsche Krebsgesellschaft
Seit 2015	AIO Arbeitsgemeinschaft Internistische Onkologie
Seit 2017	NET-Z Werk Mitglied für grundlagenwissenschaftliche Kooperationen

Reviewer- und Gutachter-Tätigkeiten:

PLOS ONE, British Journal of Cancer, Journal of Zhejiang University, Zeitschrift für Gastroenterologie, World Journal Gastroenterology, Histochemistry and Cell Biology, BMC Cancer, Pancreas, Journal of the Pancreas, Journal of Experimental & Clinical Cancer Research, Oncotarget, Pancreatology, Scientific Reports, Digestion

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