運命決定において重要な役割を果たしているものと考えられる。様々ながん組織において、E2F1 の過剰発現（組織染色の結果）とともに、mRNA レベルでの p73 の高発現（RT-PCR の結果）が報告されている。これまでに、アポトーシス誘導能を持つ p73 の発現レベルが、何故、がん組織で高いのかについては合理的な解釈がなされていなかった。我々の研究成果は、実際のがん組織における E2F1 と p73 の発現レベルの因果関係を明らかにするものと考えられる。

E. 結論

E2F1 は p73 の転写を促進することにより、p73 依存性のアポトーシスを誘導するが、E2F1 の発現量がある閾値を越えると、p73 蛋白質の分解を促し細胞増殖を昂進させる。従って、E2F1 が持つ機能的な二面性は、その発現レベルの高低によって規定されると考えられる。

F. 健康危険情報

特記すべきこと無し。

G. 研究発表

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3．知的財産権の出願・登録状況

1． 特許取得

なし

2． 実用新案登録

なし

3． その他

なし

厚生労働科学研究費補助金（第3次対がん総合戦略研究事業）
分担研究報告書

発がんとがんの転移を制御する遺伝子の解析

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研究要旨　ミトコンドリア DNA (mtDNA) にコードされる呼吸鎖 complex I subunit である NADH dehydrogenase subunit 6 (*ND6*)遺伝子中の病因性変異が、活性酸素種（ROS）の産生を介して hypoxia-inducible factor (HIF)-1αの発現を亢進し、さらに転移能も亢進させることを報告した。そこで、ROS を介した HIF-1α発現亢進のメカニズムを検討した。その結果、ROS が phosphatidylinositol 3-kinase/Akt/protein kinase C/histone deacetylase シグナル伝達経路を介して *HIF-1α*遺伝子の転写を活性化することが明らかになった。一方、*ND1*、*ND3*、*ND4L*、*ND6* 遺伝子中の変異出現頻度が、ヒトがんの原発巣よりも転移巣の方で高いことを示した昨年度の研究を、症例数を増やすこと、及び *ND2* と *ND4* 遺伝子を解析に加えることでさらに進めた。肺がん患者の原発巣 45 例、転移巣 36 例及び大腸がん患者の原発巣 22 例、転移巣 11 例における *ND* 遺伝子のミスセンス変異を調べたところ、Grantham value が 50 以上の過激なアミノ酸置換を伴うミスセンス変異の出現頻度が、原発巣よりも転移巣で有意に高いことが判った。さらに、ミトコンドリア病に関連するミスセンス変異やナンセンス変異を考慮すると、さらに有意差が大きくなることも判った。これらの結果から、*ND* 遺伝子の変異の一部がヒトのがんの転移に何らかの影響を与える可能性が示唆された。

A.　研究目的

マウスルイス肺癌及びマウス繊維肉腫由来の低転移性および高転移性細胞株間でミトコンドリア DNA (mtDNA) を完全に交換したサイブリッド細胞を用いた先行研究により（筑波大学・林純一教授及び千葉県がんセンター研究局・越川信子博士との共同研究)、呼吸鎖 complex I subunit の１つである NADH dehydrogenase subunit 6 (*ND6*) 遺伝子中の病因性ミスセンス変異が complex I 活性の低下と活性酸素種 (ROS) の高産生を惹起し、これが原因で抗アポトーシス活性を示す Mcl-1 と hypoxia-inducible factor (HIF) -1αの高発現をもたらし、転移能を亢進させることを明らかにした。また、昨年度、ヒトがんの転移にも mtDNA 変異が関与するかどうかを検討することを目的として、肺がん患者の原発巣 25 例、転移巣 35 例及び大腸がん患者の原発巣 13 例、転移巣 9 例における mtDNA 変異を *ND1*、*ND3*、*ND4L*、*ND6* 遺伝子について調べ、転移巣においてミスセンス変異の頻度が、原発巣のそれと比べて高い傾向を示すことを示した。そこで今回は、ROS の産生により HIF-1αの発現亢進に至るシグナル伝達経路について解析を行った。また、ヒトのがんの転移における mtDNA 変異の関与の可能性を *ND2* と *ND4* 遺伝子も含めさらに解析した。

B. 研究方法

mtDNA 変異に起因する ROS 産生と HIF-1α発現との関連は、低転移性マウス肺癌細胞株 P29 細胞（*ND6* 遺伝子変異がなく、ROS 産生も HIF-1α発現も低い）と高転移性 A11 細胞（*ND6* 遺伝子変異を有し、ROS 産生と HIF-1α発現がともに高い）を用いて調べた。mRNA の発現は全 RNA を用いたノーザンブロット法で、プロモーター活性はルシフェラーゼレポーターアッセイで、SP1/SP3 の発現はウェスタンブロットで、活性はゲルシフトアッセイ及びクロマチン免疫沈降法により解析した。ヒト肺がんの原発巣 45 症例及び脳転移巣 36 症例、並びにヒト大腸がんの原発巣 22 症例及び転移巣 11 症例の試料から抽出した DNA を用いて、mtDNA 遺伝子の変異解析を行った。*ND1*、*ND2*、*ND3*、*ND4*、*ND4L* 及び *ND6* 遺伝子の塩基配列の決定はダイレクトシークエンシングで行った。

（倫理面への配慮）

ヒト腫瘍におけるミトコンドリア DNA の変異解析は、千葉県がんセンター及び島根大学医学部倫理審査委員会の承認後に、インフォームドコンセントの得られた患者の手術材料を用いて行なった。

C. 研究結果

病因性 mtDNA (*ND6*) 変異による ROS の産生亢進が HIF-1αの発現亢進に至るシグナル伝達経路を、P29 細胞と A11 細胞を用いて解析した。まず、HIF-1αの発現亢進が転写レベルであるいは翻訳レベルで起きているのかを検討したところ、転写レベルであることが判った。次に、A11 細胞に各種阻害剤を作用させたところ、*HIF-1α*mRNA の発現が ROS scavenger ebselen、PI3-kinase (PI3K)阻害剤 LY294002、pan-protein kinase C(PKC)阻害剤 Ro31-8220 で顕著に抑制されることが判った。また、A11 細胞で認められる Akt の高いリン酸化も ebselen と LY294002 で抑制されることが判った。転写因子 SP1 の阻害剤である mithramycin A でも *HIF-1α*mRNA の発現が顕著に抑制されることが明らかになったので、*HIF-1α*遺伝子のプロモーター部位中における SP1 結合部位をレポーターアッセイで特定したところ、転写開始点のすぐ上流に存在し、これが転写の活性化に必須であることが明らかになった。しかし、P29 と A11 細胞間では SP1/3 の発現量や活性に差が認められなかった。次に、A11 細胞での *HIF-1α*mRNA の発現が histone deacetylase (HDAC)阻害剤 trichostatin A (TSA) で抑制されることを見出したので、P29 と A11 細胞間で HDAC 活性を測定したところ、A11 細胞で有

意に高いことが判った。さらに、A11 細胞に ebselen、LY294002、Ro31-8220 を作用させると HDAC 活性が低下することが明らかになった。

ND1、*ND2*、*ND3*、*ND4*、*ND4L* 及び *ND6* 遺伝子中の変異の出現頻度を、肺がんと大腸がんの原発巣（合計 67 症例）と転移巣（合計 47 症例）とで比較した。mtDNA 変異（多型も含む）はミスセンス変異とナンセンス変異に絞り、さらにミスセンス変異を、①頻度が日本人一般での出現頻度よりもがん組織で２倍以上高いものとそうでないもの、②アミノ酸置換の過激度を表す Grantham value が 50 以下と 50 以上のもの、③ミトコンドリア病と何らかの関連性が示唆されているものに分けて解析した。その結果、単独では Grantham value が 50 以上のミスセンス変異の頻度が有意に原発巣よりも転移巣で高いことが判った（$p<0.04$）。また、がん組織で２倍以上出現頻度が高いもののうちで、Grantham value が 50 以上のものとミトコンドリア病に関連するものに絞るとさらに有意に転移巣で出現頻度が高いことが判った（$p<0.02$）。また、これに加えてナンセンス変異を考慮すると、顕著な有意差が認められた（$p<0.002$）。

D. 考察

病因性 *ND6* 遺伝子変異に起因する ROS の高産生を介する *HIF-1α* mRNA 発現の亢進メカニズムを調べるために、各種阻害剤の効果を検討したところ、ROS scavenger、PI3K 阻害剤、pan-PKC 阻害剤が A11 細胞での *HIF1α* mRNA の発現を顕著に抑制することが判った。また、SP1 結合が HIF-1α プロモーターの活性化に必須であるとの実験結果から SP1 の関与も考えられた。しかし、P29 細胞と A11 細胞間で SP1 の発現量や活性に差が認められないことより、SP1 は *HIF-1α* 遺伝子の発現には必須であるが、ROS の下流には位置しないことが判った。意外なことに、TSA が *HIF-1α* mRNA の発現を顕著に抑制することが判り、HDAC の関与が示された。このことは、ROS scavenger、PI3K 阻害剤、pan-PKC 阻害剤が HDAC 活性を抑制することからも支持された。以上の結果から、*ND6* 遺伝子変異による *HIF1 α* mRNA の発現亢進への ROS/PI3K/Akt/PKC/HDAC シグナル伝達経路の関与が明らかになった。

Complex I subunit をコードするいずれかの *ND* 遺伝子の病因性変異がヒトのがんの転移と関連するかどうかは興味ある点である。本研究では、ミスセンス変異のうちで、アミノ酸置換が過激であると考えられる Grantham value が 50 以上の多型あるいは変異の頻度が原発巣よりも転移巣の方で有意に高いという結果が得られた。これらが complex I 活性に何らかの影響を及ぼすのではないかと推察され、今後の研究が必要であろう。さらに、がん組織で出現頻度の高い変異や多型、ミトコンドリア病に関連することが報告されている変異及びナンセンス変異を加えるとさらに有意差が高くなることは興味深く、これらが転移と関連している可能性を示唆していると思われる。しかし、本当にこれらの多型や変異が転移と関連するのか否かを明らかにするためには、今後のさらなる研究が必要であろう。

E. 結論

Complex I subunit をコードする *ND* 遺伝子中の一部の病因性変異が ROS/PI3K/Akt/PKC/HDAC 経路を介して *HIF-1α* 遺伝子の転写を活性化することが明らかになった。また、*ND* 遺伝子の一部の変異や多型がヒトのがんの転移能の亢進に関与する可能性が示唆された。今後の注意深い解析によってこのことの信憑性が確認されれば、将来転移の予測や予防に応用できる可能性が考えられる。

F. 研究発表

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G. 知的財産権の出願・登録状況

１． 特許取得

なし

２． 実用新案登録

なし

３． その他

なし

厚生労働科学研究費補助金（第3次対がん総合戦略研究事業）
分担研究報告書

翻訳調節を介した細胞増殖と老化制御メカニズムの解明

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研究要旨
ほ乳類ポリコム群は、Ink4a/p53 経路に対して抑制的に作用して細胞老化を制御することが遺伝学的に示されている。この抑制は、ポリコム群複合体の Ink4a 遺伝子座への直接結合を介していることを今まで示してきた。昨年度までに、新規ポリコム群タンパク Pcl２はポリコム群に対し拮抗的に作用し、その作用機序は翻訳調節を介したポリコム群タンパクの発現制御メカニズムであることを新たに示し、新たながん抑制候補遺伝子であることを示した。Pcl２の機能発現機序を明らかにするために、本年度は、Pcl２が標的とする遺伝子群をゲノムワイドに解析し、他のポリコム群タンパクの標的と比較することでポリコム群抑制における Pcl2 の位置付けを明らかにした。

A．研究目的

ほ乳類ポリコム群は、Ink4a/p53 経路に対して抑制的に作用して細胞老化を制御することが遺伝学的に示されている。この抑制は、ポリコム群複合体の Ink4a 遺伝子座への直接結合を介していることを今まで示してきた。昨年度までに、新規ポリコム群タンパク Pcl２はポリコム群に対し拮抗的に作用し、その作用機序は翻訳調節を介したポリコム群タンパクの発現制御メカニズムであることを新たに示し、新たながん抑制候補遺伝子であることを示した。

　　ポリコム群による転写抑制は PRC２（ヒストンＨ３Ｋ２７をトリメチル化する酵素を含む複合体）と PRC１（トリメチル化されたＨ３Ｋ２７を認識してクロマチン結合し、その領域に H2AK119 をモノユビキチン化することができる複合体）によって媒介される。Pcl２は PRC２の一部と複合体を構成することを今まで生化学的に示してきた。今回 ES 細胞をモデルとして、Pcl２の標的を明らかにし、ポリコム抑制における Pcl２の位置付けを明らかにした。

B．研究方法

Pcl２のN端ペプチドに対する特異的な抗体を樹立し、ES細胞における標的遺伝子座をクロマチン免疫沈降-シーケンス法(ChIP-Seq法)及びそれをマイクロアレイと組み合わせた(ChIP-Chip法)により解析した。

（倫理面への配慮）

遺伝子組換え実験と動物実験については、以下に示す文部科学省及び環境省関係法令・指針に準拠して定められた理研所内規程に則って行っている。

１．遺伝子組換え実験

【関係法令・指針】

遺伝子組換え生物等の使用等の規制による生物の多様性の確保に関する法律

【理研所内規程】

横浜研究所遺伝子組換え実験実施安全管理規程

横浜研究所遺伝子組換え実験に関わる申請及び承認に関する細則

C．研究結果

H3K27 のトリメチル化が見られる遺伝子として約３２００遺伝子を明らかにし、PRC１に含まれる Ring1B の結合はその中の２３００遺伝子に見られることがわかった。PRC２と PRC１には強い相関があることが示された。H2AK119 モノユビキチン化は、その中の約３００遺伝子に見られたが、Pcl２の結合はそれを含む８３０遺伝子に見られた。

D．考察

Pcl２の標的遺伝子群は、ポリコム群による抑制が強く見られる遺伝子グループであることから、Pcl２はポリコム群による抑制をエンハンスする活性があると推察された。

E．結論

Pcl２はポリコム群が作用するにあたって、クロマチン状況を読み取って、そのポリコーム群の機能発現を制御するためのモジュールであることを昨年度までに示してきた。今回の結果は、今までの結論をサポートするものである。

G．研究発表

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H．知的財産権の出願・登録状況（予定を含む。）

なし

Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Ⅳ．研究成果の刊行物・別刷

Netrin-1 acts as a survival factor for aggressive neuroblastoma

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Neuroblastoma (NB), the most frequent solid tumor of early childhood, is diagnosed as a disseminated disease in >60% of cases, and several lines of evidence support the resistance to apoptosis as a prerequisite for NB progression. We show that autocrine production of netrin-1, a multifunctional laminin-related molecule, conveys a selective advantage in tumor growth and dissemination in aggressive NB, as it blocks the proapoptotic activity of the UNC5H netrin-1 dependence receptors. We show that such netrin-1 up-regulation is a potential marker for poor prognosis in stage 4S and, more generally, in NB stage 4 diagnosed infants. Moreover, we propose that interference with the netrin-1 autocrine loop in malignant neuroblasts could represent an alternative therapeutic strategy, as disruption of this loop triggers in vitro NB cell death and inhibits NB metastasis in avian and mouse models.

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Abbreviations used: CAM, chorioallantoic membrane; DAPK, DAP kinase; DCC, deleted in colorectal cancer; MNA, *MYCN* amplification; mRNA, messenger RNA; Myoc, myocardium; NB, neuroblastoma; PTX, primary tumor xenograft; Q-RT-PCR, quantitative RT-PCR; siRNA, small interfering RNA; TUNEL, terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end labeling.

Dependence receptors now number more than a dozen, including deleted in colorectal cancer (DCC) (1), UNC5H (2), Patched (3), some integrins (4), neogenin (5), p75^{NTR} (6), RET (7), ALK (8), and TrkC (9). Although they have no structural homology (other than possibly in a domain referred to as the DART [dependence-associated receptor transmembrane] domain) (10), they all share the functional property of inducing cell death when disengaged from their trophic ligands, whereas the presence of their trophic ligands blocks this proapoptotic activity. Such receptors thus create cellular states of dependence on their respective ligands (11, 12).

The prototype dependence receptors are the netrin-1 receptors. Netrin-1, a diffusible laminin-related protein, has been shown to play a major role in the control of neuronal navigation during the development of the nervous system by interacting with its main receptors, DCC (13, 14, 15) and UNC5H (16, 17). However, DCC and UNC5H (i.e., UNC5H1, UNC5H2, UNC5H3, and UNC5H4) have been shown to belong to the dependence receptor family (1, 2). This dependence effect upon netrin-1 has been suggested to act as a mechanism for eliminating tumor cells that would develop in settings of ligand unavailability (for reviews see references 18, 19). Along this line, disruption of the proapoptotic signaling of these netrin-1 receptors in the gastrointestinal tracts of mice, by netrin-1 overexpression or by inactivation of UNC5H3, is associated with intestinal tumor progression (20, 21).

Thus, loss of the dependence receptors' proapoptotic activity represents a selective advantage for tumor cells. In this respect, DCC was proposed in the early 1990s to function as a tumor suppressor gene, whose expression is lost in

J. Bénard, A. Bernet, and P. Mehlen contributed equally to this paper.

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the vast majority of human cancers (22, 23). This hypothesis also fits with the observation that UNC5H genes are down-regulated in most colorectal tumors, hence suggesting that loss of UNC5H genes represents a selective advantage for tumor development (21, 24, 25). We have analyzed expression of netrin-1 and its receptors in neuroblastoma (NB), the most frequent extracranial solid tumor of early childhood. The aggressive and metastatic stage 4 NB displays three distinct clinical patterns at presentation and dissemination sites based on patients' ages. Indeed, neonates and infants (<1 yr of age) with stage 4S and stage 4 without 4S features have an overall good prognosis, whereas stage 4 in children (>1 yr of age) shows a poor prognosis. We describe in this paper that, rather than the loss of netrin-1 receptor expression, a large fraction of aggressive NBs has evolved to select a gain of ligand expression that apparently represents a similar selective growth advantage. We therefore propose to use disruption of this selective advantage as an anticancer strategy in NB.

RESULTS

Netrin-1 is up-regulated in a large fraction of aggressive NB

We focused on stage 4 NB with a specific interest in comparing netrin-1 and its receptors' expression levels between the three distinct clinical patterns of stage 4, based on disease distribution and age of the patients (26). On the one hand, there are the neonates and infants (<1 yr of age) with stage 4S (2–5% of all NB) and the similarly young stage 4 without 4S features, hereafter termed [$1yr^-$] stage 4, who make up 10% of the NB population. On the other hand, there are the stage 4 children (>1 yr of age), comprising 45% of all NBs, who will hereafter be termed [$1yr^+$] stage 4. These three clinical aspects of stage 4 NB differ in their respective malignant behaviors and associated prognoses: good for stage 4S and [$1yr^-$] stage 4 (5-yr event-free survival >80%), and dismal for [$1yr^+$] stage 4 (5-yr event-free survival of ~30%) despite intensive treatment including high-dose chemotherapy and hematopoietic stem cell transplantation (27, 28).

We first analyzed the expression of netrin-1 and its dependence receptors, DCC, UNC5H1, UNC5H2, UNC5H3, and UNC5H4, by quantitative RT-PCR (Q-RT-PCR) in a panel of 102 stage 4 NB tumors including 24 stage 4S and 12 [$1yr^-$] stage 4. As shown in Fig. 1 A, netrin-1 is up-regulated in [$1yr^+$] stage 4 as compared with both stage 4S ($P < 0.05$) and [$1yr^-$] stage 4 ($P < 0.01$). Similar results were obtained when comparing netrin-1 protein level by immunohistochemistry (Fig. 1 B and quantification in Fig. S1 A). Interestingly, netrin-1 is detected mainly in tumor cells and is barely detected in stroma cells (Fig. 1 B and Fig. S1 B). Conversely, netrin-1 dependence receptor expression analysis showed that DCC was only weakly expressed in the different stage 4 NB (Fig. S1 C) as reported (29), whereas UNC5H1, UNC5H2, UNC5H3, and UNC5H4 expression showed no significant differences when comparing [$1yr^-$] versus [$1yr^+$] stage 4 (Fig. 1 C). However, we observed that the different UNC5H receptors are up-regulated specifically in stage 4S (mean increase in UNC5H expression in stage 4S vs. other stage 4 NBs: 2.98-fold, $P < 0.007$), suggesting UNC5H receptors as hallmarks of stage 4S NB. The UNC5H1 and UNC5H4, which show the highest messenger RNA (mRNA) expression, could also be seen at the protein level by immunohistochemistry (Fig. 1 D).

In an attempt to correlate netrin-1 up-regulation with the molecular signature of these tumors, we compared netrin-1 up-regulation and DCC/UNC5H1 levels to the profile of gene expression performed in a small panel of nine stage 4 NBs (30). We failed to detect any correlation between netrin-1 up-regulation or DCC/UNC5H1 levels with the molecular signature of apoptosis or invasion effectors (Fig. S1 D). Considering patients' outcomes, although 38% of [$1yr^+$] stage 4 NBs have selected up-regulation of netrin-1, this event failed to be significantly associated with poor outcome in this aggressive form of the disease (unpublished data). Moreover no association between netrin-1 up-regulation and *MYCN* amplification (MNA) was detected (unpublished data). Thus, netrin-1 up-regulation may be considered as an additional component of the genetic complexity that these tumors display.

Despite a largely favorable prognosis among infants with stage 4 NB (i.e, stage 4S and [$1yr^-$] stage 4) with no MNA, many succumb to the disease. Thus, we assessed whether netrin-1 expression may serve as a prognostic marker for the infants with stage 4 NB. As shown in Fig. 1 E, the overall survival of infants with stage 4S differed markedly based on whether the tumor displayed high levels of netrin-1 expression (netrin-1 high) or low-level expression (netrin-1 low), with the netrin-1 expression threshold being its median expression value in the 102 cases. Indeed, although 100% of the infants survived after 10 yr (including 1 MNA out of 17), when the NB 4S was netrin-1 low, the 5-yr overall survival was only 46% when the NB 4S was netrin-1 high ($P = 0.0109$). Furthermore, 43% of the non-MNA patients with high-level netrin-1 expression died. More generally, when a similar overall survival analysis was performed on all infants with stage 4 NB (i.e. stage 4S and [$1yr^-$] stage 4), a similar dichotomy was observed. Indeed, 5-yr overall survival was found to be 90% for the netrin-1–low infants yet only 48% for netrin-1–high infants ($P = 0.032$; Fig. 1 F). These data suggest that netrin-1 is a potential prognostic marker for aggressiveness in stage 4 NB diagnosed in infants. Whether or not it constitutes an independent prognostic marker of stage 4 NB in neonates and infants deserves to be tested in a larger patient cohort. Nevertheless, these data indicate that a netrin-1 threshold may turn as an alternative determinant for the biological behavior of stage 4 NB in infants, potentially suggesting its involvement in a cell death process of very early childhood neuroblasts, reminiscent of that operating during nervous system development (31).

Netrin-1 high expression is not only detected in 38% of [$1yr^+$] stage 4 and in poor outcome [$1yr^-$] stage 4 primary NB tumors but also in a fraction of NB cell lines mainly derived from stage 4 tumor material (Fig. 2 A and Fig. S2 A). Two human NB cell lines, IMR32 (netrin-1 high) and CLB-Ge2 (netrin-1 low), were evaluated further. In spite of a marked difference in netrin-1 and DCC expression, the UNC5H levels

are similar in IMR32 and CLB-Ge2 cells; UNC5H1, UNC5H3, and UNC5H4 show the highest expression (Fig. 2 B). Specifically, UNC5H1, UNC5H3, and UNC5H4 proteins could be detected at the plasma membrane by confocal analysis (Fig. 2 C). To test the hypothesis that the high netrin-1 mRNA levels detected in IMR32 cells are associated with an autocrine netrin-1 production, we next performed netrin-1 immunohistochemistry on IMR32 and CLB-Ge2 cells. As shown in Fig. 2 D, a netrin-1–specific membrane staining was detected in a homogeneous pattern in IMR32 cells, whereas no specific staining was detected for CLB-Ge2 cells. Confocal analysis further confirmed the presence of netrin-1 at the cell membrane (Fig. 2 E

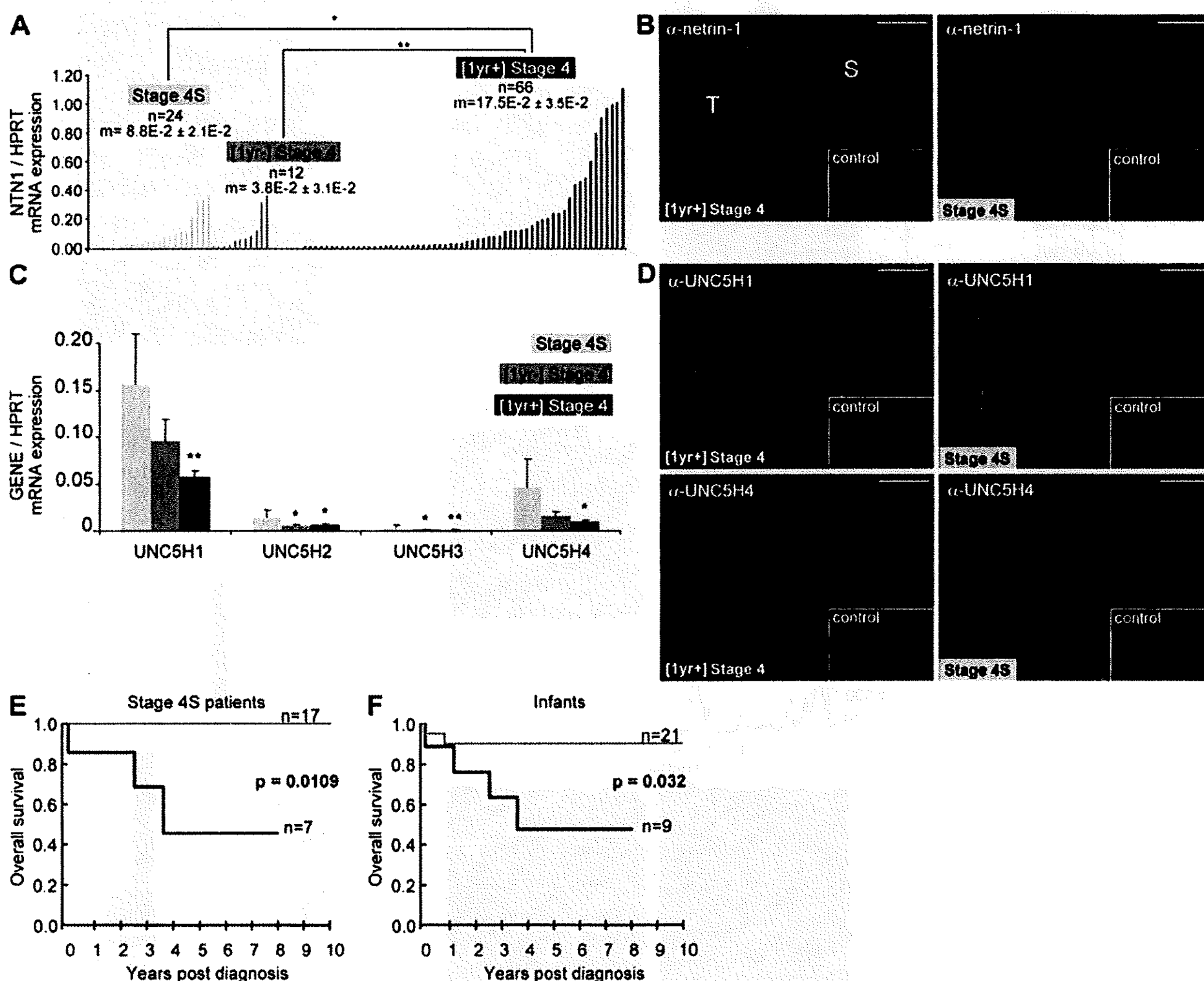

Figure 1. Netrin-1 up-regulation is detected in aggressive NB. (A) Netrin-1 mRNA levels in 102 stage 4S (n = 24), [1yr⁻] stage 4 (n = 12), and [1yr⁺] stage 4 (n = 66) NBs measured by Q-RT-PCR. HPRT housekeeping gene was used as a control. Mean netrin-1 mRNA expression value for each subgroup is indicated by an "m" value. Mean netrin-1 mRNA levels in stage 4S and [1yr⁻] stage 4 were, respectively, compared with the mean netrin-1 detected in [1yr⁺] stage 4. The data were statistically determined using Student's t test compared with levels of [1yr⁺] stage 4. *, $P < 0.05$; **, $P < 0.01$. Each sample was assessed in two independent experiments. (B) Representative netrin-1 immunohistochemistry on one [1yr⁺] stage 4 and one stage 4S tumor. Insets show control without primary antibody. Bars, 50 µm. T, tumor cells; S, stromal cells. Netrin-1 antibody specificity is further shown in Fig. 2 D and Fig. S1 B. Immunohistochemistry was performed on four [1yr⁺] stage 4 and four stage 4S tumors. (C) Mean UNC5H mRNA levels in the different stage 4 NBs. Q-RT-PCR using UNC5H1-4-specific primers was performed. Mean UNC5H1-4 mRNA levels in [1yr⁻] stage 4 and [1yr⁺] stage 4 were, respectively, compared with the mean UNC5H1-4 levels detected in stage 4S. Error bars indicate SEM. The data were statistically determined using Student's t test compared with levels of stage 4S. *, $P < 0.05$; **, $P < 0.01$. Samples were analyzed in duplicates for each gene. (D) Representative UNC5H1 and UNC5H4 immunohistochemistries on [1yr⁺] stage 4 and stage 4S tumors. Insets show control without primary antibody. Bars, 50 µm. Immunohistochemistry was performed on four stage 4 [1yr⁺] and four stage 4S tumors. (E) Netrin-1 up-regulation is a marker of poor prognosis in stage 4S NB. Overall survival in a panel of 24 infants diagnosed with stage 4S NB with primary tumors showing either netrin-1-low (gray) or netrin-1-high (black) levels. The data was statistically determined using the Kaplan-Meier method. P-value is indicated. (F) Netrin-1 up-regulation is a marker of poor prognosis in infants with NB. Data are presented as in E, with a panel of 30 infants bearing NB.

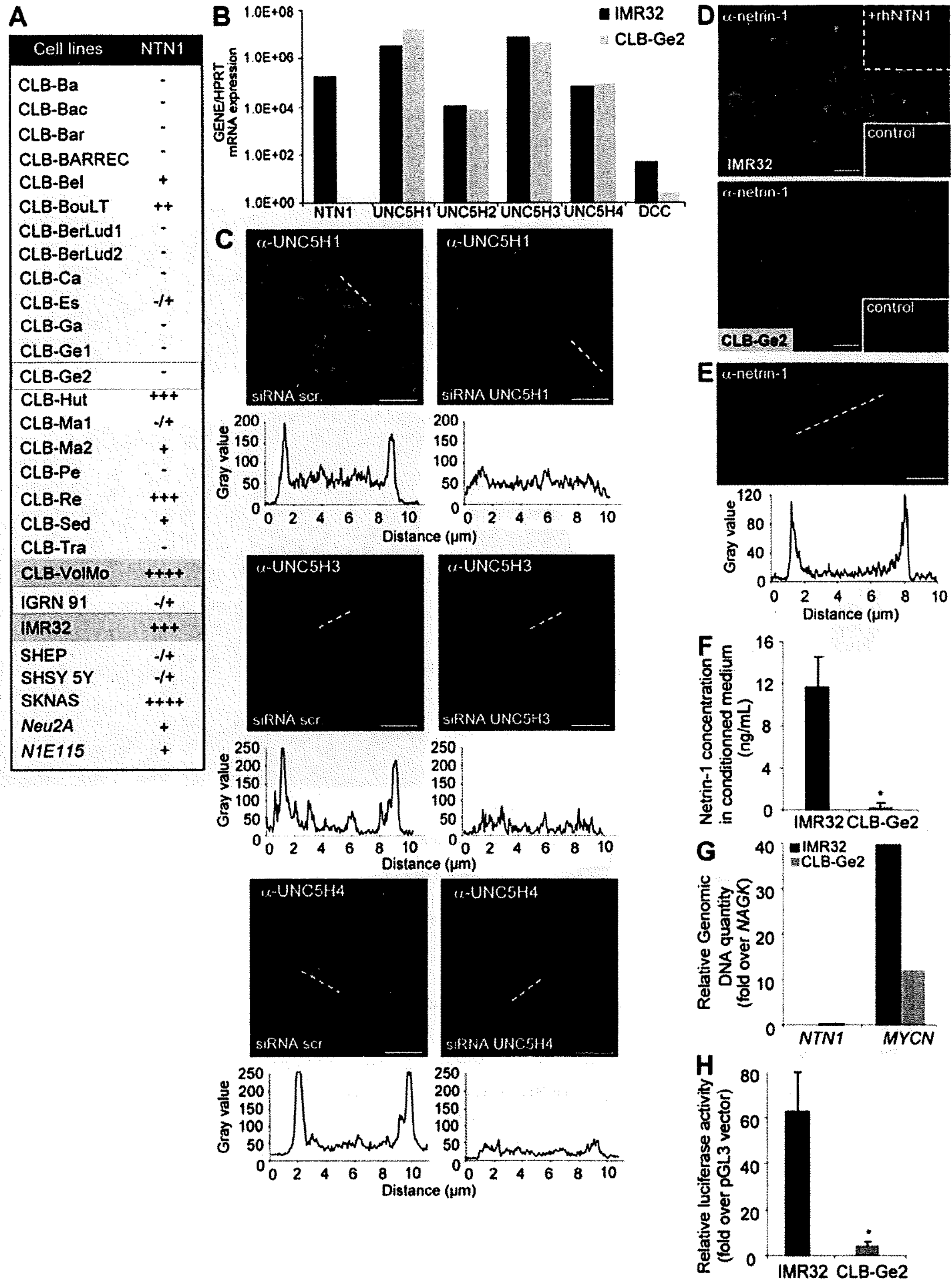

Figure 2. Netrin-1 up-regulation is detected in NB cell lines. (A) Netrin-1 expression measured by Q-RT-PCR in a panel of 28 NB cell lines. HPRT housekeeping gene was used as a control. The netrin-1 level is indicated as follows: –, not detectable; –/+, barely detectable; + to ++++, moderate to very high expression. Mouse cell lines are in italics. Cell lines outlined and highlighted in grey are, respectively, netrin-1-low and netrin-1-high cell lines further used in the experiments. (B) Netrin-1 receptor expression in IMR32 and CLB-Ge2 cell lines. DCC/UNC5H Q-RT-PCR was performed on netrin-1-expressing (IMR32) or netrin-1-low (CLB-Ge2) cells using specific primers. Ratio of netrin-1 and netrin-1 receptor expression to the HPRT housekeeping

and Fig. S2 B). To further analyze whether netrin-1 is secreted from IMR32 cells, netrin-1 ELISA assay was used to detect netrin-1 in the conditioned medium. As shown in Fig. 2 F, 11.7 ng/ml netrin-1 was recovered from the conditioned medium of IMR32 cells, whereas no netrin-1 was detected from the conditioned medium of CLB-Ge2 cells. Thus, together these data suggest that the high netrin-1 content observed in aggressive NB could result from an autocrine expression of netrin-1 in NB cells.

As a first approach to apprehend the mechanisms leading to netrin-1 up-regulation in aggressive NB, we analyzed whether netrin-1 gene (*NTN1*) is amplified in IMR32 cells. As shown in Fig. 2 G, although *MYCN* was amplified both in IMR32 and CLB-Ge2 cells compared with the *NAGK* control gene, the *NTN1* gene was not found to be amplified in these two cell lines. We then analyzed whether the increase in netrin-1 expression could be caused by a differential netrin-1 promoter activation. A luciferase reporter gene fused to netrin-1 promoter (32) was then transfected into IMR32 or CLB-Ge2 cells, and luciferase activity was reported to an internal control in each cell line. As shown in Fig. 2 H, netrin-1 promoter activity was 13.8-fold higher in IMR32 cells than in CLB-Ge2 cells, thus supporting the view that netrin-1 up-regulation in NB is related to a gain in netrin-1 promoter activation.

Netrin-1 up-regulation is a selective advantage for NB cell survival

To investigate whether the netrin-1 autocrine expression observed in IMR32 cells provides a selective advantage for survival, as would be expected from the dependence receptor theory, cell death was analyzed in response to the disruption of this autocrine loop. As a first approach, netrin-1 was down-regulated by RNA interference. As shown in Fig. 3 A, the addition of netrin-1 small interfering RNA (siRNA) to IMR32 cells was associated with a significant reduction in netrin-1 mRNA. This mRNA reduction was associated with a decrease of netrin-1 protein as observed by immunohistochemistry (Fig. 3 B). Although scramble siRNA failed to affect IMR32 cell survival, as measured by trypan blue exclusion, netrin-1 siRNA treatment was associated with IMR32 cell death (Fig. 3 C). In contrast, CLB-Ge2 cell survival was unaffected after netrin-1 siRNA treatment (Fig. 3 C). To determine whether this increase in cell death was in part caused by an increase in apoptotic cell death, caspase-3 activity was measured in response to netrin-1 siRNA treatment. As shown in Fig. 3 D, although significant apoptotic cell death was detected upon netrin-1 siRNA treatment in IMR32 cells, no such effect was observed in CLB-Ge2 cells. A similar proapoptotic effect of the netrin-1 siRNA was observed in CLB-VolMo cells, another netrin-1 high cell line (unpublished data).

Interference with netrin-1 triggers UNC5H-induced apoptosis in NB cells

As a second approach, we looked for a compound that could interfere with the netrin-1 ability to block DCC/UNC5H proapoptotic activity. We recently reported that the fifth fibronectin type III domain of DCC (DCC-5Fbn; Fig. 4 A), which is located in the DCC ectodomain, interacts with netrin-1 and blocks the ability of netrin-1 to trigger multimerization of DCC and UNC5H receptors. Because multimerization inhibits DCC or UNC5H-induced cell death (unpublished data), DCC-5Fbn antagonizes netrin-1 function, disrupting netrin-1–mediated inhibition of DCC/UNC5H proapoptotic activity. Thus, DCC-5Fbn acts as a trap for netrin-1 survival function. As shown in Fig. 4 (B–D), the addition of DCC-5Fbn, but not the unrelated protein IL3R, triggered IMR32 apoptotic cell death as measured by trypan blue exclusion (Fig. 4 B), caspase-3 activity assay (Fig. 4 C), and terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end labeling (TUNEL) staining (Fig. 4 D). This effect was specific for netrin-1 inhibition because DCC-5Fbn had no effect on CLB-Ge2 cells, and the addition of netrin-1 ultimately reversed the DCC-5Fbn–induced IMR32 apoptotic cell death (Fig. 4, B–D). Similar results were obtained with the CLB-VolMo cells (Fig. S2 D). To determine whether the ability of DCC-5Fbn to kill NB cells is restricted to established NB cell lines, a surgical biopsy from a tumor with high netrin-1 level (unpublished data) was semidissociated and further incubated with DCC-5Fbn. As shown in Fig. 4 E, DCC-5Fbn triggered cell death as measured by caspase-3 activation, demonstrating that in vitro, disruption of the netrin-1 autocrine loop is associated with NB cell death.

We next investigated whether netrin-1 autocrine expression in NB cells acts as a general cell survival factor, i.e., whether it has a trophic effect similar to that of neurotrophins, or whether it specifically inhibits death induced by

gene is presented. (C) Confocal analysis of UNC5H1, UNC5H3, and UNC5H4 receptor immunostaining in human IMR32 cells. Left and right correspond to IMR32 cells transfected with scramble siRNA and specific siRNA UNC5H, respectively. A fluorescence intensity profile corresponding to the white dashed bar is presented under each panel. Bars, 10 µm. (D) Immunostaining on human IMR32 and CLB-Ge2 cell lines using netrin-1 antibody. Bottom insets show control without primary antibody. Top inset: antibody specificity was tested by adding human recombinant netrin-1. Bars, 50 µm. (E) Confocal analysis of netrin-1 immunostaining on IMR32 cells. A fluorescence intensity profile corresponding to the white dashed bar is presented below. Bar, 5 µm. (F) Quantification of netrin-1 protein secreted in IMR32 and CLB-Ge2 cells conditioned medium by sandwich ELISA assay. Quantification in ng/ml was made according to a dose curve done with recombinant human netrin-1. Data are means of three independent experiments. Error bars indicate SEM. *, $P < 0.05$ using a two-sided Mann-Whitney test compared with level in IMR32 cells. (G) Quantification of *NTN1* and *MYCN* genomic DNA compared with control *NAGK* genomic DNA by PCR, using genomic DNA specific primers for each gene, in IMR32 and CLB-Ge2 cells. (H) Quantification of *NTN1* promoter activity in IMR32 and CLB-Ge2 cells. Both cell lines were transfected with the vector pGL3-NetP-Luc encoding luciferase under the control of *NTN1* promoter. Data presented are normalized on luciferase activity in cells transfected with pGL3 empty vector. Data are means of four independent experiments. Error bars indicate SEM. *, $P < 0.05$ using a two-sided Mann-Whitney test compared with levels in IMR32 cells.