表1　中空バイオナノ粒子と従来型遺伝子治療用ベクターの比較

	レトロウイルス	アデノウイルス	リポソーム	中空バイオナノ粒子
遺伝子導入効率	高い	高い	低い	高い
ベクター自身の病原性	低い	高い	低い	低い
ウイルスゲノム導入の危険性	高い	低い	無し	無し
生体内細胞及び組織標的化能	無し	無し	無し	有り
任意の細胞および組織への再標的化	困難	困難	困難	容易
ベクターの生産性	低い	低い	高い	高い
ベクター生産における感染危険性	高い	高い	無し	無し
DDSへの応用	不可能	不可能	可能	可能

となった．また，1999年にフランスで開始された重症複合型免疫不全症に対する遺伝子治療では，レトロウイルスベクターを用いて遺伝子導入を行ったものであり，長期の症状改善がみられていたが，2002年にベクターを投与された2人の患者に白血病が発症した．これは，ベクター由来の遺伝子が患者の染色体ゲノム中の癌抑制遺伝子近傍に挿入されたことが引き金になったと考えられている．また，ウイルスベクターは非常に強い感染力を有するが，細胞および組織特異性がないので，投与に際しては患部を外科的に露出する必要がある．これは繰り返し投与を困難にし，患者のQOL(Quality of Life)を損ねるので，遺伝子治療を一般的な医療技術として普及できない主要因になっている(表1)．そこで，今後の遺伝子治療では，DDS技術を用いて(ウイルス由来遺伝子を排除して)治療用遺伝子のみを患部に正確に導入することが必須である．

以上のように，DDS技術に対する医薬品領域における要求は，これまでで最高に高まっている．しかし，DDS技術が提唱されてから約30年が経過するが，今までのDDS技術は，薬剤の細胞膜透過性を向上させてBioavailabilityを向上させたり，血液中の薬剤の濃度を一定に長期間保つようにしたりするアプローチが主流であった．最近では，癌組織は増殖能が旺盛なので細胞間隙が他の通常組織よりも大きい(約100nm)こと(EPR効果：Enhanced Permeability and Retention Effect)を利用して，ブロックコポリマーに代表されるような一定のサイズのナノ粒子に薬剤を包含して患部に送達させる方法(受動的薬剤送達法)が盛んになりはじめているが，血液中の薬剤量は依然として高濃度で維持する必要があるのが欠点である．そこで，血液中の薬剤量は低濃度でも積極的に薬剤を患部に集中投与できる方法(能動的薬剤送達法)の開発が期待されているが，まだ抗体医薬品以外に決定打がないのが現状であった．2003年夏，われわれは生体内でヒト肝臓特異的に，薬物(遺伝子・タンパク質・薬剤)をピンポイントで送達できる担体「中空バイオナノ粒子」を開発した(図1)[1]．この粒子は従来のDDSおよび遺伝子治療用担体が抱える問題点をほぼ全て克服するので(表1)，Nature Biotechnology誌(2003年8月号)，The Lancet誌(2003年7月5日号)，Nature Materials誌(2003年8月号)等に，「強い感染力と高い細胞特異性を持つウイルスゲノムフリーな画期的な生体内物質送達法」として紹介された．本稿では「中空バイオナノ粒子」の概要とその技術により実現されるであろう医療技術の一例を紹介する．

2．中空バイオナノ粒子とは

われわれのグループでは，従来ベクターの欠点を克服するための新規遺伝子治療用ベクターの開発を進めてきた．この新規ベクターはB型肝炎ウイルス表面抗原(hepatitis B virus surface antigen，以下HBsAg)粒子を基としており，酵母を用いて生産されるナノサイズの中空粒子であることから“中空バイオナノ粒子”と呼んでいる．この中空バイオナノ粒子は，今までとは大きく異

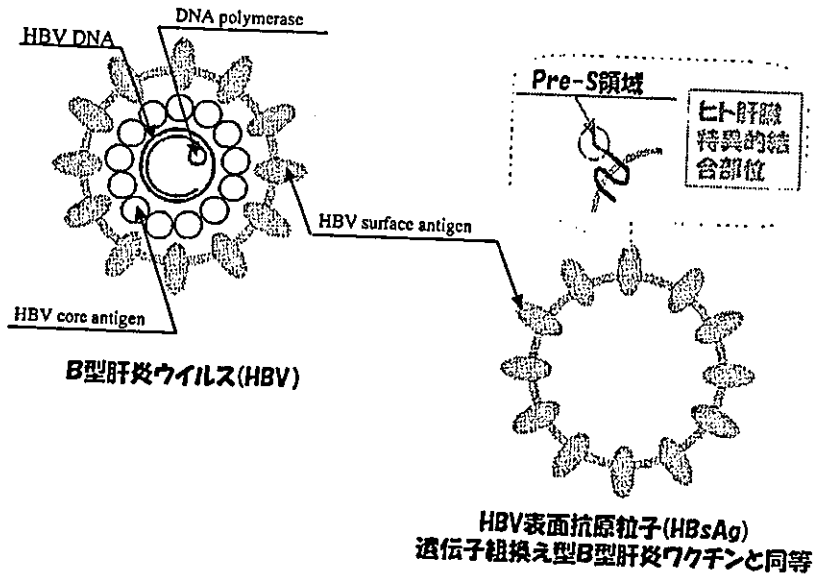

図1　B型肝炎ウイルスとB型肝炎ウイルス表面抗原

なるベクターであり，想定される短所をほぼ完全に克服しており，遺伝子治療のみならず種々のアプリケーションに応用できると期待される．

中空バイオナノ粒子の骨格となるHBsAg粒子は，B型肝炎ウイルス(hepatitis B virus，以下HBV)の外殻に相当するもので，外被タンパク質(HBsAg分子)と宿主由来の脂質二重膜から成る中空状粒子である[2,3]．HBsAg粒子は，HBVのようにウイルス本体の遺伝子や遺伝子を複製するのに必要となる酵素などを含む核となるタンパク質群を一切もたない(図1)．ウイルスには外殻をもつタイプは他にも知られているが，HBsAg粒子のように外被タンパク質が単独で脂質二重膜を含む形で中空状粒子を形成するものは他にない．HBsAg分子はHBVゲノム上にコードされ，翻訳開始位置の違いによって3種類のタンパク質に分かれ，それぞれS(small)，M(middle)，L(large)と呼ばれる．Sタンパク質は226アミノ酸から成り，MタンパクはSタンパク質のN末端側に55アミノ酸から成るpre-S2領域が付加された形になっている．このpre-S2領域にヒト血清由来重合アルブミンを介してヒト肝細胞に結合する活性があるが，われわれの研究からHBVがヒト肝臓に感染するには必ずしも必要ではないことが判明している．さらに，LタンパクはMタンパクのN末端側に108(血清型y)または119アミノ酸(血清型d)から成るpre-S1領域が付加している．このpre-S1領域のN末端側約70アミノ酸残基は肝細胞と直接結合する部位を含んでおり，ヒト肝細胞へ特異的に感染する際に中心的な役割を担っている．また，pre-S1領域の作用により，HBVのトロピズムはチンパンジーとヒトの肝臓に厳格に限定されている[4~7]．

HBsAg粒子はHBVの抗原として免疫原性をもち，HBVに対する抗体を誘導することから，HBVに対するワクチン用抗原として有効であることが早期から知られている．B型肝炎感染者は世界的に多いので，1980年代初めはHBsAg粒子をB型肝炎患者血漿から精製し，B型肝炎用ワクチンとして使用した．その後，血液製剤であるので組換え酵母により生産したSタンパク質からなる粒子(S粒子)が第2世代ワクチンとして実用化された．1980年代後半には，抗pre-S2抗体が早期の感染防御に有効で，M粒子が一層効果的であることが判明したので，武田薬品工業㈱のTGP-943等の第3世代ワクチンが実用化された．さらに，抗pre-S1抗体が抗pre-S2抗体と同様に有効と判明したが，pre-S1領域の生合成阻害活性によりL粒子の生産は困難であった．1992年，われわれはpre-S1領域のN末端にシグナルペプチドを付加して，該阻害活性を回避することに成功した[8]．酵母におけるLタンパク質の発現量は全可溶性タンパク質の40%以上に達し，1L培養液から約20mgのL粒子を精製できた．現時点において，これは組換え酵母を用いたタンパク質生産の中でもっとも生産量の高い例である．これ

は高価な動物細胞を用いるウイルスベクター生産量の1,000倍以上であり，対費用効果は極めて高い．また，このL粒子はS粒子あるいはM粒子に比べ，HBVに対する抗体の誘導能が優れていることも判った．L粒子の物理的性質を調べたところ，HBVと同様に熱に対して非常に安定であった．また，原子間力顕微鏡による観察から，その平均直径は100nmであることが判明した[9]．さらに，酵母由来SおよびM粒子は各種毒性試験で陰性であることからL粒子も同様と期待された．以上のようなL粒子の性質を受けて，われわれはL粒子をワクチンとしてでなく，ヒト肝臓特異的なピンポイント物質送達法に応用することを発案した．

3．中空バイオナノ粒子による遺伝子導入

中空バイオナノ粒子による遺伝子導入を検証するために，モデル遺伝子として緑色蛍光タンパク質(green fluorescence protein：GFP)の発現遺伝子を用いた．L粒子内部に遺伝子を封入するために種々の手法を検討した結果，エレクトロポレーション法を用いることで簡便に遺伝子を封入できることがわかった．GFP発現遺伝子を封入したL粒子を培養ヒト肝細胞株および数種のヒト肝癌由来細胞株の培養上清に血清存在下で添加すると，いずれの細胞においてもGFPに由来する蛍光を観察することができた(図2)．また，研究用遺伝子導入試薬(リポソームの一種)を用いた場合より遺伝子の使用量が1％未満で充分であった．一方，対照として数種の非肝臓系の細胞株にも同様にGFP発現遺伝子の導入を試みたが，GFPに由来する蛍光は観察されなかった．以上の結果から，本中空バイオナノ粒子は，ヒト肝細胞に対してのみウイルスベクターに匹敵する遺伝子導入効率を有していることが判明した．

中空バイオナノ粒子による生体内遺伝子導入を検討するために，ヒト肝癌由来細胞株とヒト大腸癌由来細胞株を固形癌として同じマウスの背部へ移植した担癌マウスを作製した．同様の手順で粒子内部にGFP発現遺伝子を封入したL粒子を用いて，マウス尾静脈より血流へ投与した．GFP発現遺伝子含有L粒子は，移植したヒト肝癌由来固形癌のみに到達し，その結果，組織内にてGFPの発現を観察することができた．また，対照組織並びにマウス各臓器においてはGFPに由来する蛍光は観察されなかった．以上のことより，生体内でも中空バイオナノ粒子は内包する遺伝子をヒト肝細胞・組織特異的に送達することが可能であることが示された[1]．

続いて，遺伝子治療で用いられる治療用遺伝子を用いた遺伝子治療モデルの検証実験を行った．対象疾患には血友病Bを，治療用遺伝子には血友病Bの遺伝子治療で利用されている血液凝固第9因子(blood clotting factor Ⅸ：FⅨ)発現遺伝子を用いた．血友病の治療用遺伝子は非常に大きく，ウイルスベクターに乗らないことが多い．われわれの中空バイオナノ粒子は大きさもフレキ

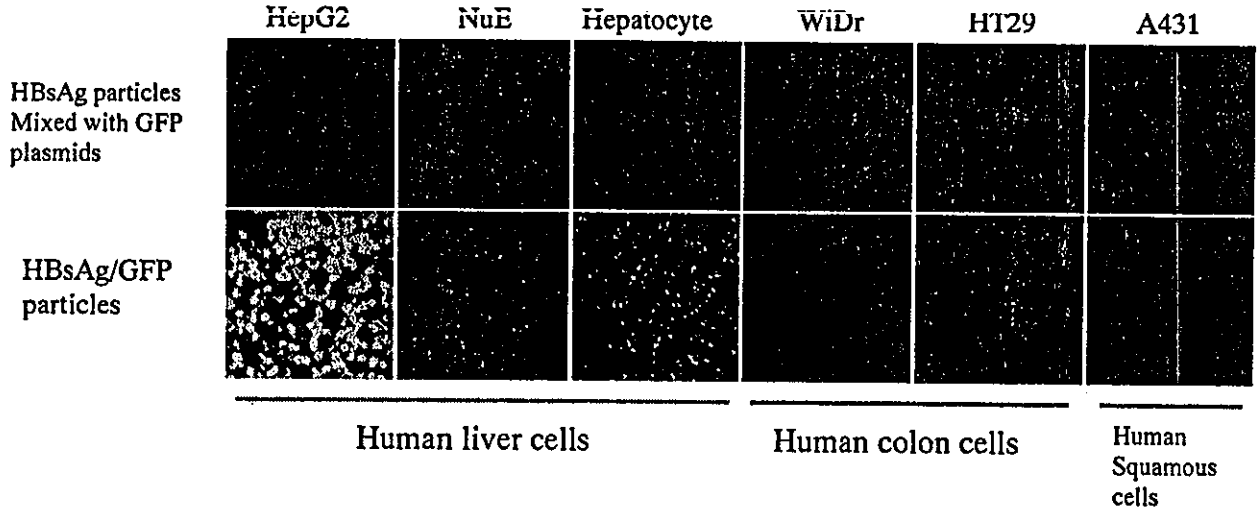

図2　培養細胞レベルでのGFP発現遺伝子のヒト肝細胞特異的導入

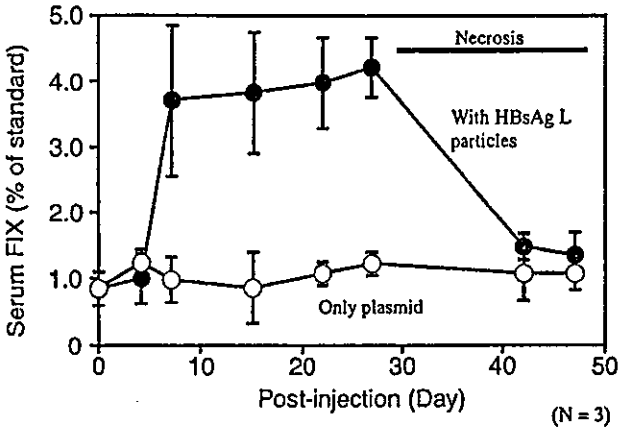

図3　Xenograft モデルマウスを用いた血友病 B 治療用遺伝子導入

シブルなので，封入できる遺伝子長も従来のウイルスベクターと比べて遥かに大きいので，中空バイオナノ粒子は本遺伝子治療に最も適していると考えられた．FIX 発現遺伝子の封入には，GFP 発現遺伝子の場合と同様に，エレクトロポレーション法にて行った．FIX 発現遺伝子含有 L 粒子を担癌マウスの尾静脈より投与した後に経時的に採血を行い，ELISA 法にて血中 FIX 量を求めた．その結果，投与後 4 日目にマウス血中に FIX が発現し，3 週間にわたり血友病 B の重症患者に対する治療効果が期待できる発現レベルを維持した(図 3)[1]．このように 1 回の静脈注射で 1 ヵ月近い治療効果が現れる血友病治療薬剤は他には存在しないことからも，本技術が極めて有効であることが分かる．

4．中空バイオナノ粒子による薬剤導入

中空バイオナノ粒子は，薬剤などの生理活性物質も内部に包含し，標的細胞へ送達させることが期待できる．われわれは，モデル化合物として蛍光物質カルセインを用いて薬剤送達の検証を試みた．GFP 発現遺伝子の場合と同様に，エレクトロポレーション法を用いることで簡便にカルセインを L 粒子内部に封入できることが判明した．カルセインを封入した L 粒子をヒト肝癌由来細胞株の培養上清に添加すると，カルセインに由来する蛍光を観察することができたが，対照として数種の非肝細胞系の細胞株では観察されなかった[1]．また，ヒト肝癌由来固形癌とヒト大腸癌由来固形癌を移植した担癌マウスの尾静脈よりカルセイン含有 L 粒子を血流へ投与した結果，移植したヒト肝癌由来固形癌の組織内にてカルセインに由来する蛍光を観察することができた．また，対照組織並びにマウス各臓器においては蛍光を観察することができなかった．以上のことより，低分子化合物の場合でも培養細胞・生体内にかかわらず中空バイオナノ粒子はヒト肝細胞・組織特異的に送達することが可能であることが示された[1]．

5．中空バイオナノ粒子の再標的化

中空バイオナノ粒子の骨格となっている L 粒子表面には肝細胞結合部位(約 70 アミノ酸)が提示されている．この部位が中空バイオナノ粒子に肝細胞への特異性を付与していることから，任意の生体認識分子に置換することで肝細胞以外の細胞へ標的化することが期待される．モデル生体認識分子として上皮成長因子(epidermal growth factor : EGF)を選択し，上記肝細胞結合部位とカセット式に置換した EGF 提示型 L 粒子を創製した．EGF 提示型 L 粒子の生産は，L 粒子と同様に組換え酵母を用いた生産系にて行うことが可能である．生産した EGF 提示型 L 粒子を用いてカルセインの薬剤送達を行ったところ，EGF と結合する EGF 受容体を細胞表面に発現している細胞にのみカルセインを導入することができた[1]．また，われわれは，さまざまなサイトカインや抗体を粒子表面に提示して，それらの特異性により物質送達することにも成功した．最近では，生体内で高度な組織認識が可能なペプチド(Homing Peptide)の研究が進んでおり，われわれは中空バイオナノ粒子と融合して再標的化の幅を大きく広げている．

以上のことより，中空バイオナノ粒子には遺伝子治療用ベクター並びに DDS に適した以下のような特長をもっている．

①中空バイオナノ粒子は，その表面に肝細胞結合部位を提示しており，HBV と同様にヒト肝細胞への高い親和性と高い導入効率(感染能力)を有する．

②内部が中空状であるため，遺伝子・薬剤などの生理活性物質を封入することが可能．

③副作用の原因となるウイルス由来の遺伝子を一切含まないため病原性はない．

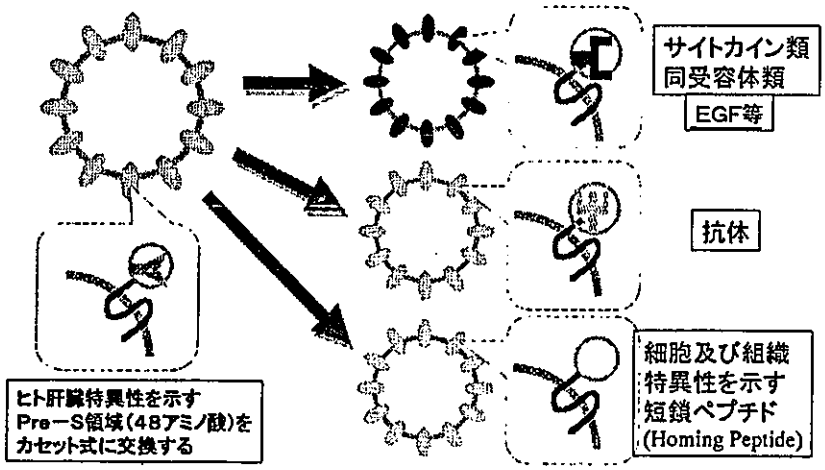

図4　中空バイオナノ粒子の再標的化ストラテジー

④組換え酵母にて大量生産することが可能.
⑤粒子表面に提示されている肝細胞結合部位を，他の生体認識分子へ置換することで分子に応じた標的細胞へピンポイントで送達可能(図4).

6．中空バイオナノ粒子の今後

中空バイオナノ粒子は，内部に封入した物質(遺伝子のみならず薬剤やタンパク質)を提示する生体認識分子に応じた細胞に，生体内でピンポイントに送達することができる画期的な遺伝子治療法でありDDS法でもある．本技術の用途は医薬品以外にも多数あるが，ここでは医薬品用途に限って言及する．以下に示す応用例は，2002年にわれわれが本技術の実用化のために設立した大学発ベンチャー・㈱ビークル(本社・岡山市)の当面の課題であり，一部は3～5年以内の臨床試験開始を目標にしている．

①ウイルス性肝炎治療にはインターフェロンが投与されるが，重篤な全身性の副作用と中和抗体産生が問題となっている．そこで，われわれはL粒子によりインターフェロン(遺伝子，タンパク質)を肝臓特異的に投与を行い安全なウイルス性肝炎治療を目指している．
②従来の抗癌剤は全身性の副作用を引き起こすので，われわれはL粒子内部に抗癌剤を高濃度で封入して肝臓特異的に投与を行い安全な肝癌治療を目指している．
③癌組織表面に提示されるさまざまな糖鎖や抗原を認識する改変型L粒子を作製し，種々の癌のピンポイント遺伝子治療もしくは抗癌剤治療を目指している．
④遺伝性疾患において単一タンパク質の発現が欠損していることが多い(血友病，フェニルケトン尿症等)ので，われわれはL粒子を用いて欠損タンパク質遺伝子を肝臓に導入する遺伝子補充療法を目指している．
⑤さまざまな感染症が世界的に問題となっているが，これらの治療に当たっては感染細胞と非感染細胞の生体内での識別が非常に大事である．われわれは，幾つかのウイルス性疾患において感染細胞を特異的に認識する改変型L粒子を作製して，その効果を検討中である．
⑥将来的には個人の遺伝子情報に基づくテーラーメイド医療が普及するので，各患者にパーソナライズされた遺伝子治療やDDSのプラットホーム技術として本粒子を活用できる．

7．中空バイオナノ粒子の課題

本技術は完成したばかりであるので，上述するような医薬品として実用化するには解決すべき課題も多い．特に，免疫原性および抗原性を下げて連続投与を可能にしなければならない．そのためには，エレクトロポレーションよりも極めて効率の高い封入法を考案して必要投与量を著しく下げること，ヒト免疫系から認識されにくいHBVエスケープ変異株のアミノ酸変異を模倣してステルス化L粒子を完成すること，更に静脈注射以外の効率的な投与法を見出すことが課題と考えている．さらに，L粒子よりも優れた性質を有する他の分子を探索することも必要であるが，現在まで

にL粒子を凌ぐものは見出されていない．この非常に高いポテンシャルを有する中空バイオナノ粒子は，現状のDDS技術が解決すべき問題点を大きく改善しうる可能性を秘めている技術であり，生物医学研究，先進医療を前進させることが可能であると考える．

謝　辞

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Epithelial Mesenchymal Transition Is a Characteristic of Hyperplasias and Tumors in Mammary Gland From MMTV-Cripto-1 Transgenic Mice

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Epithelial-mesenchymal transition (EMT) facilitates migration and invasion of epithelial tumor cells. Cripto-1 (CR-1), a member of the epidermal growth factor-CFC protein family increases migration of cells in vitro. Here the expression of molecular markers and signaling molecules characteristic of EMT were assessed in mammary gland hyperplasias and tumors from mice expressing the human CR-1 transgene by the MMTV promoter (MMTV-CR-1) and in mouse mammary epithelial cell line HC-11 overexpressing CR-1 (HC-11/CR-1). Western blot analysis showed decreased expression of E-cadherin in MMTV-CR-1 tumors and in HC-11/CR-1 cells. The expression of N-cadherin, vimentin, cyclin-D1, and of the zinc-finger transcription factor, snail, was increased in MMTV-CR-1 tumors. Increased snail mRNA was also found in HC-11/CR-1 cells. Expression of phosphorylated (P)-c-Src, P-focal adhesion kinase (FAK), P-Akt, P-glycogen synthease kinase 3β (GSK-3β), dephosphorylated (DP)-β-catenin, and various integrins such as, alpha 3, alpha v, beta 1, beta 3, and beta 4 was also increased in MMTV-CR-1 tumors. Immunohistochemistry showed positive staining for vimentin, N-cadherin, cyclin-D1, smooth muscle actin, fibronectin, snail, and β-catenin in MMTV-CR-1 tumor sections. HC-11/CR-1 cells treated with the c-Src inhibitor PP2 reduced the expression of P-c-Src and of P-FAK, P-Akt, P-GSK-3β, DP-β-catenin all known to be activated by c-Src. Migration of HC-11/CR-1 cells was also reduced by PP2 treatment. These results suggest that CR-1 may play a significant role in promoting the increased expression of markers and signaling molecules associated with EMT. J. Cell. Physiol. 201: 266–276, 2004. © 2004 Wiley-Liss, Inc.

One aspect of tumor biology is the capacity for primary malignant epithelial cells to exit their site of origin, degrade the surrounding extracellular matrix (ECM), migrate into blood vessel, and invade secondary organs (Liotta, 1986). In order for tumor cells to carry out this invasive process, they must first modify their phenotype by altering the expression of a new repertoire of cell surface receptors, growth factors, proteases, and adhesion molecules that participate in the activation of particular signaling pathways needed to facilitate cell proliferation and migration (Price et al., 1997). Tumor cells that migrate must first undergo an important process known as epithelial-mesenchymal transition (EMT). EMT is a normal physiologic process by which epithelial cells loose their relatively strong adhesiveness, become more motile and assume a spindle-shaped morphology resembling cells of mesenchyme origin (Boyer et al., 2000; Savagner, 2001). This process normally occurs during embryonic development and organogensis, tissue growth and wound healing and repair (Perez-Pomares and Munoz-Chapuli, 2002). EMT

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facilitates migration and invasion of epithelial tumor cells and has recently been suggested as an index of aggressiveness and increased metastatic potential in different types of malignant tumors (Nishihara and Tsuneyoshi, 1993; Fuchs et al., 2002; Gilchrist et al., 2002; Xue et al., 2003).

A well-characterized series of biochemical modifications occurs during EMT. For instance, degradation or loss of expression of the epithelial adhesion molecule E-cadherin and a concomitant increase in expression of N-cadherin is a common finding in cells undergoing EMT (Hatta and Takeichi, 1986; Islam et al., 1996; Kim et al., 2000). In fact, N-cadherin was shown to induce increased motility, invasion, and metastasis of breast cancer cells (Nieman et al., 1999; Hazan et al., 2000). Loss of E-cadherin expression that occurs during EMT is coupled to the intracellular accumulation of β-catenin, which normally participates in adherens junctions with E-cadherin (Nieset et al., 1997; Chitaev and Troyanovsky, 1998; Boyer et al., 2000; Savagner, 2001). In most cases intracellular accumulation of β-catenin is prevented by glycogen synthease kinase 3β (GSK-3β)-dependent phosphorylation of β-catenin and subsequent ubiquitination and degradation through the proteosome (Henderson and Fagotto, 2002). Interference with β-catenin degradation such as occurs during activation of the canonical Wnt signaling pathway leads to accumulation of dephosphorylated (DP)-β-catenin that translocates to the nucleus and functions as a transcription factor in a complex with Tcf/Lef-1 to activate genes such as *c-myc*, *cyclin-D1*, and *snail*, that are involved in cell survival, proliferation, and migration (Polakis, 2000). Members of the snail family zinc-finger transcription factors are important for mesoderm development and are expressed in adult tissues and organs including cardiac and skeletal muscle (Paznekas et al., 1999). Snail can bind to E-boxes in the promoter regions and regulate the expression of genes such as, E-cadherin, N-cadherin, fibronectin (FN), vimentin, and smooth muscle actin (SMA) (Nieto, 2002). Since cells undergoing EMT acquire a more mesenchymal phenotype, cytoskeletal components undergo changes often characterized by an increased expression of vimentin and/or SMA, which are cytoskeleton molecules normally expressed in mesenchymal cells (Steinert and Roop, 1988; Masszi et al., 2003). Increased expression of certain growth factors such as fibroblast growth factor (FGF), transforming growth factor-beta 1 (TGF-β1), and scatter factor/hepatocyte growth factor (SF/HGF), and activation of signaling molecules such as c-Src, Akt, focal adhesion kinase (FAK), and integrin linked kinase (ILK) have also been shown to occur during EMT and probably contribute to the increased migration and invasive capacity of tumor cells (Birchmeier et al., 1996; Thiery and Chopin, 1999).

Cripto-1 (CR-1) is a signaling protein and member of the epidermal growth factor (EGF)-CFC protein family, which plays an important role during early embryonic development (Minchiotti et al., 2002). Increased expression of CR-1 has been observed in a variety of human carcinomas including gastrointestinal tumors, lung cancer, breast cancer, cervical, ovarian, and testicular cancers (Salomon et al., 2000). Overexpression of mouse CR-1 in mouse mammary epithelial cells in vitro increases proliferation, chemotaxsis, and migration when these cells are cultured on plastic or extracellular matrix (ECM)-coated porous filters (Wechselberger et al., 2001). CR-1 can function as a co-receptor for the TGF-β1-related protein Nodal and activate an Alk4 and Smad-dependent signaling pathway (Yeo and Whitman, 2001; Bianco et al., 2002a). Recently, the EGF-like domain of CR-1 was found to bind to mouse mammary epithelial cells and mouse mammary gland tissue sections suggesting the presence of a specific CR-1-receptor interaction (Bianco et al., 2002b). In fact, CR-1 was later shown to bind glypican-1, a glycosylphosphatidylinositol (GPI)-linked membrane associated heparan-sulphate proteoglycan, and to specifically activate the tyrosine kinase c-Src which was required for cell proliferation and migration of mammary epithelial cells (Bianco et al., 2003). These effects of CR-1 are coincident with EMT in vitro and were blocked by the specific Src inhibitor, PP2, suggesting that glypican-1 and c-Src activity may be important components of CR-1-dependent EMT.

To better understand the potential role of CR-1 in EMT, we have assessed the expression of molecular markers and signaling molecules characteristic of EMT in hyperplasias and tumors that develop in the mammary gland of transgenic mice that overexpress the human CR-1 transgene under the transcriptional control of the MMTV promoter (MMTV-CR-1) (Wechselberger, submitted).

MATERIALS AND METHODS

Antibodies

The following antibodies were used for both immunohistochemistry and Western blotting: rabbit anti-E-cadherin (H108), rabbit anti-N-cadherin (H-63), goat anti-vimentin (C-20), rabbit anti-integrin alpha v (H-75), goat anti-integrin alpha 3 (N-19), rabbit anti-integrin beta 1 (M-106), rabbit anti-integrin beta 3 (H-96), rabbit anti-integrin beta 4, rabbit anti-cyclin-D1 (C-20), rabbit anti-FN (H-300), and goat anti-snail (T-18) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); rabbit anti-Akt, anti-phosphorylated (P)-Akt, anti-GSK-3β and anti-P(Ser9)-GSK-3β were purchased from Cell Signaling (Beverly, MA); mouse anti-c-Src (GD11) and rabbit anti-β-catenin were purchased from Upstate (Charlottesville, VA); mouse anti-FAK and anti-P-FAK were purchased from Chemicon (Temecula, CA); mouse anti-DP-β-catenin from Calbiochem (San Diego, CA); mouse anti-SMA from Zymed (San Francisco, CA); rabbit anti-P-c-Src from Biosource (Camarillo, CA); rabbit anti-CR-1 (1579) from Biogen, Inc. (Cambridge, MA) and mouse anti-actin (JLA20) from Oncogene (Boston, MA).

Cells and transgenic mammary tissue samples

Normal mouse mammary epithelial cells HC-11 wild-type (HC-11/WT) and HC-11 cells that are overexpressing CR-1 (HC-11/CR-1) were established and grown as previously reported (Wechselberger et al., 2001; Bianco et al., 2002a,b, 2003). Mammary tissue lesions consisting of four hyperplasias and five adenocarcinomas were obtained each from separate, multiparous MMTV-CR-1 G4 female mice. Mammary tissues from three FVB/N strain normal multiparous mice were used as controls.

All samples had been previously (Wechselberger, submitted) categorized and catalogued according to pathology as determined by an independent veterinary pathologist. In brief, multiparous MMTV-CR-1 female mice developed ductal hyperplasias and multifocal lobules of hyperplastic epithelium. At 14–18 months, approximately 35% of the G4 MMTV-CR-1 mice that had undergone at least five pregnancies developed tumor lesions. These lesions are papillary adenocarcinomas containing multiple layers of relatively differentiated tumor cells, which in some areas line solid undifferentiated foci containing large anaplastic and elongated mesenchyme-like tumor cells.

Western blotting

HC-11/WT and HC-11/CR-1 cells were grown until approximately 80% confluent and then serum starved for 24 hr before being harvested for Western blot analysis. Protein extracts in lysis buffer were obtained from these cells and Western blotting was performed as previously described (Bianco et al., 2002a). For inhibition experiments, approximately 2×10^5 HC-11/CR-1 cells were seeded and grown until 80% confluent, serum starved for 24 hr then treated for 15 min with 1 μM of the c-Src inhibitor PP2 (Calbiochem) before being harvested for Western blotting as above. The specificity of this inhibitor and ideal concentration was determined in our previous study (Bianco et al., 2003). Mammary tissue from normal FVB multiparous, MMTV-CR-1 hyperplasias and MMTV-CR-1 tumors, previously stored in liquid nitrogen, were crushed while still frozen and rapidly homogenized in lysis buffer using a polytron (Powergen 35, Fisher Scientific, Pittsburgh, PA). Cellular debris was spun down by centrifugation at 14,000 rpm for 10 min at 4°C. Protein concentration was determined for each supernatant and equal amounts were used for Western blotting. Equal loading of proteins was determined by Western blotting for actin. A 1:1,000 dilution was used for all antibodies except for anti-CR-1 and anti-β-catenin, which were used at 1:3,000. To determine the fold difference in expression of the molecules examined, blots were scanned and densitometric readings of the bands was obtained by using NIH Image 1.62f software (Research Services Branch, WIH). All densitometric readings were normalized against actin expression.

Immunohistochemistry

For immunohistochemistry, 5 μm thick sections of paraffin-embedded, formalin-fixed mammary tissue were deparafinized in xylene, rehydrated in a series of graded ethanols, and pre-digested with ready to use pepsin solution (Digest-All3, Zymed) for 10 min at 37°C. Endogenous peroxidase activity was blocked with 3% H_2O_2. The sections were then incubated for 30 min at RT with the primary antibodies listed above all used at 1:100 dilution. Negative controls were obtained by replacing the primary antibodies with irrelevant control isotype IgG. Immunostaining was carried out using the Vectastain ABC kit appropriate for the type of primary antibody used (Vector, Burlingame, CA) and following the manufacture's instruction. Color was developed with either DAB (brown) or AEC (red) peroxidase substrates (Vector) and sections counterstained with haematoxylin.

RT-PCR for snail mRNA expression

Mouse mammary gland samples were subjected to RNA preparation with RNeasy Mini kit (Qiagen, Valencia, CA) and treated with DNase I (Invitrogen, Carlsbad, CA). The resultant RNA was spectrophotometrically qualified and quantified at 260, 280, and 320 nm. The RNA was transcribed to cDNA with Superscript II (Invitrogen) and random hexamer primers (Invitrogen). To obtain cDNAs, 100 ng of RNA were amplified with PCR Master (Roche Diagnostics, Indianapolis, IN) for 35 cycles (94°C for 1 min, 60°C for 1 min, and 72°C for 1 min) using primers for mouse snail: forward-5′-AGCTGGCCAGGCTCTCGG-3′ and reverse-5′-TAGCTGGGTCAGCGAGGG-3′.

Cell migration assay

Cell migration across FN-coated, modified Boyden chambers was evaluated using the QCM™-FN Migration Assay (Chemicon) following manufacture's instructions. Briefly, approximately 2×10^5 cells (HC-11/WT, HC-11/CR-1 and HC-11/CR-1 + PP2, 1 μM) were seeded onto the FN-coated migration membranes and incubated for 3 hr. Non-migrating cells were carefully wiped off the inner surface of the chambers with a swab. Migrated cells on the outer surface were stained with crystal violet, rinsed, and solubilized with extraction buffer. The extraction buffer was then transferred into a microtiter plate and optical density (OD) read at 540 nm.

Statistics

Student's *t*-test was used to determine significance of the mean densitometric values of the Western blots and of the mean OD values obtained from the cell migration assay. Calculations and analysis was performed using the SPSS 7.5 for Windows statistical package. A P value of <0.05 was considered statistically significant.

RESULTS

Expression of EMT markers in MMTV-CR-1 lesions

Western blotting of mammary gland tissue extracts showed a reduction in expression of E-cadherin in tumor lesions from MMTV/CR-1 transgenic mice compared to mammary tissue that was obtained from normal FVB multiparous mice (Figs. 1 and 2). E-cadherin expression was actually increased in the majority of the hyperplastic lesions studied (Figs. 1 and 2). The adhesion molecule N-cadherin was overexpressed in hyperplastic and tumor lesions in the MMTV/CR-1 mammary gland (Figs. 1 and 2). The expression of the mesenchyme cytoskeleton molecule vimentin was also enhanced in MMTV/CR-1 mammary tumor lesions (Figs. 1 and 2). Levels of the cell-cycle regulator cyclin-D1 increased in the hyperplasias with highest expression found in extracts from the MMTV/CR-1 tumor lesions (Figs. 1 and 2).

Increased expression of the integrins alpha 3, alpha v, beta 1, beta 3, and beta 4 was also observed in most of the MMTV/CR-1 mammary gland hyperplasias with highest expression found in the MMTV/CR-1 mammary

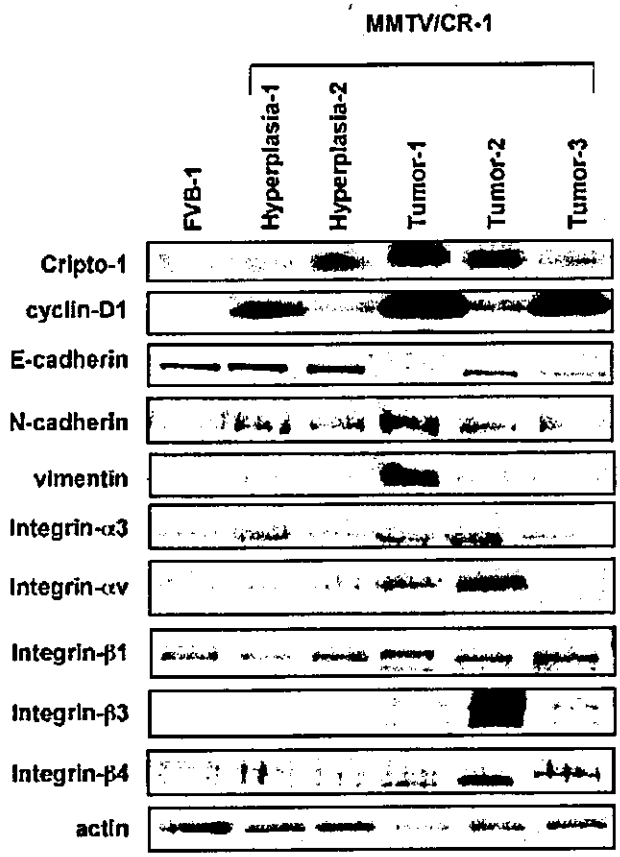

Fig. 1. Western blots representative of the change in expression of epithelial-mesenchymal transition (EMT) markers and integrins found in extracts from mammary gland tissues obtained from normal FVB strain multiparous mice (FVB) and from mammary glands with hyperplasias and tumors in MMTV transgenic mice overexpressing human Cripto-1 (MMTV/CR-1).

tumor samples (Figs. 1 and 2). Immunostaining of the MMTV/CR-1 tumor sections showed positive staining for CR-1, vimentin, N-cadherin, cyclin-D1, SMA, and FN especially in areas of the tumors containing large, poorly differentiated, tumor cells of which some also exhibited an elongated, mesenchyme cell-like morphology (Fig. 3).

Expression of snail in MMTV-CR-1 transgenic mammary lesions and in HC-11 CR-1 transfected cells

Like the previous EMT markers, snail was expressed in the poorly differentiated areas of the MMTV/CR-1 mammary tumor lesions (Fig. 4A). Very little staining for snail was observed in the epithelial cells or stroma of normal FVB mammary tissue sections (Fig. 4A, inset). Positive staining of muscle tissue in FVB mammary tissue sections was obtained (Fig. 4A, inset). Focal staining of sparse epithelial cells lining the hyperplastic acinar lesions was observed (Fig. 4D). Western blotting confirmed the increased expression for snail in the MMTV-CR-1 mammary tumor tissue (Fig. 4B,C). To investigate whether CR-1 expression is associated with increased snail expression, we assessed the expression of snail mRNA in HC-11 mouse mammary epithelial cells that were overexpressing CR-1. RT-PCR showed increased expression of snail RNA in the HC-11/CR-1 cells compared to HC-11/WT cells (Fig. 4E).

Expression of signaling molecules associated with EMT in MMTV/CR-1 transgenic mammary lesions

Compared to mammary tissue obtained from wild-type FVB multiparous animals, increased expression of the phosphorylated and activated forms of c-Src, FAK, and Akt were found by Western blotting in mammary tissue extracts from the mammary hyperplastic and tumor lesions in the MMTV/CR-1 transgenic mice after normalizing for expression of the non-phosphorylated forms of the same molecules (Fig. 5A,B). Since P-Akt was expressed in both the hyperplastic and tumor MMTV/CR-1 lesions, we also investigated whether there was expression of one of its substrates, GSK-3β, in these lesions. In fact, increased P-GSK-3β was found in the MMTV/CR-1 mammary gland lesions compared to FVB control animals (Fig. 5A,B). Western blotting showed a gradual increase in the expression of DP-β-catenin being lowest in the FVB normal multiparous control tissue, intermediate in the MMTV/CR-1 hyperplastic lesions and highest in the MMTV/CR-1 tumors (Fig. 4F,G). Immunohistochemisrty showed positive staining for DP-β-catenin in the cytoplasm of large tumor cells found in the stroma of the MMTV/CR-1 mammary tumors (Fig. 4H).

Expression of EMT markers and signaling molecules in HC-11 cells expressing CR-1

Compared to HC-11/WT there was a decrease in expression of E-cadherin in HC-11/CR-1 cells and an increase in vimentin expression as observed by Western blotting (Fig. 6A). No differences in the expression of N-cadherin or SMA were observed between HC11/WT and HC11/CR-1 (data not shown). Beta 1 integrin expression was increased in HC11/CR-1 cells compared to HC11/WT cells (Fig. 6A) but there was no significant difference observed in the expression of the integrins alpha v and alpha 3 between the two types of cells (data not shown). Western blotting of protein extracts also showed increased expression of P-c-Src, P-Akt and P-GSK-3β in HC-11/CR-1 cells as compared to wild-type HC-11 cells (Fig. 6A). In addition, there was no difference in expression in total β-catenin between these two cell types. However, there was increased expression of DP-β-catenin in HC-11/CR-1 cells compared to HC-11/WT cells (Fig. 6A).

Effect of PP2 on expression of EMT signaling molecules and cell migration in HC11 cells overexpressing CR-1

CR-1 is known to form a complex with glypican-1 on the cell surface and promote activation of c-Src suggesting that c-Src may play a pivotal role in CR-1-dependent signaling (Bianco et al., 2003). When HC-11/CR-1 cells were treated with the c-Src specific inhibitor PP2, Western blotting of lysates obtained from treated cells showed a decrease in expression of

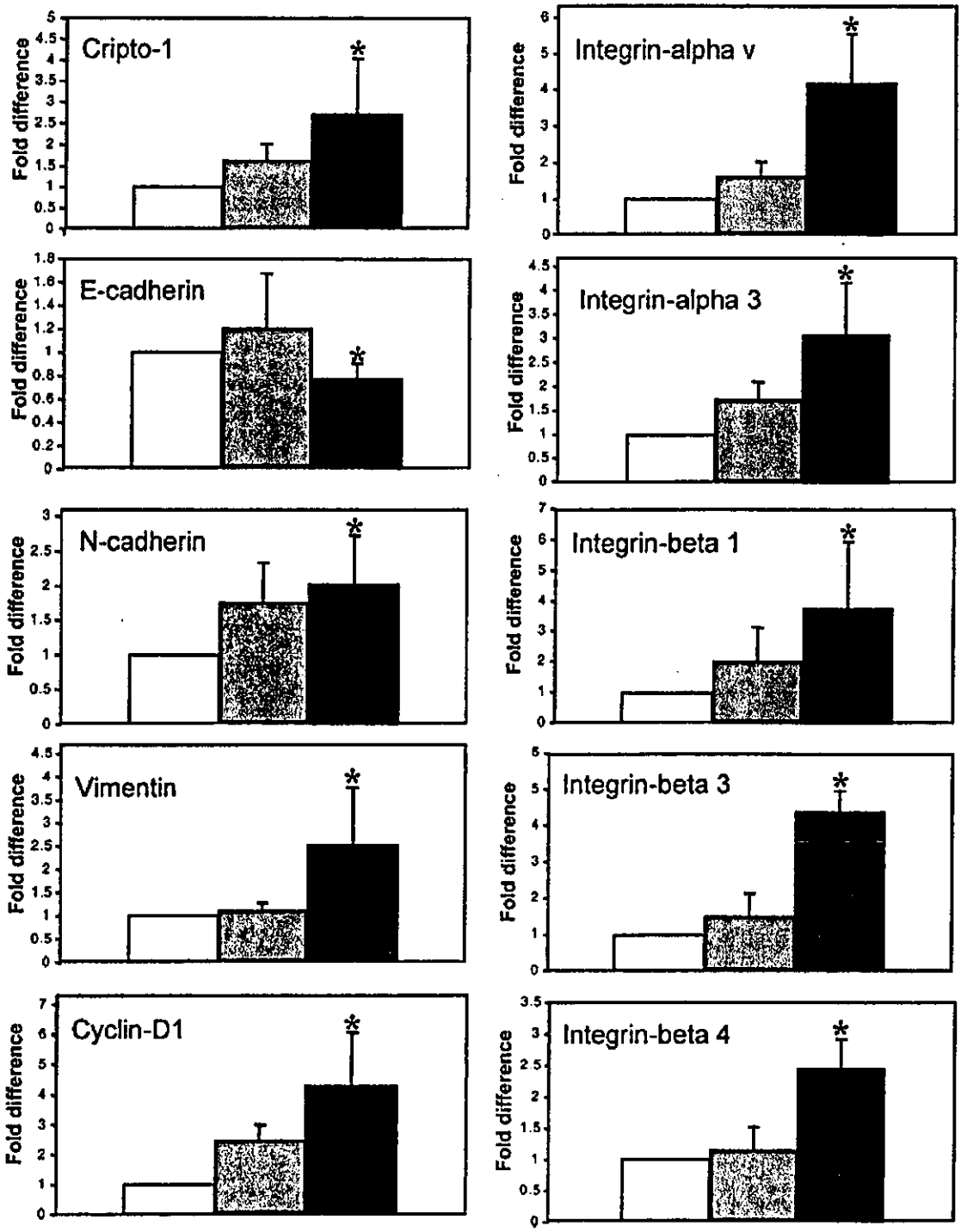

Fig. 2. Histograms showing the fold difference in expression of EMT markers and integrins determined by densitometric reading of Western blots. Each reading was normalized relative to actin expression. White bars, mean fold difference in expression among three FVB strain multiparous mice; gray bars, mean fold difference in expression in hyperplastic mammary lesions from four MMTV/CR-1 transgenic mice; black bars, mean fold difference in expression in mammary tumors from five MMTV/CR-1 transgenic mice (*, $P < 0.05$).

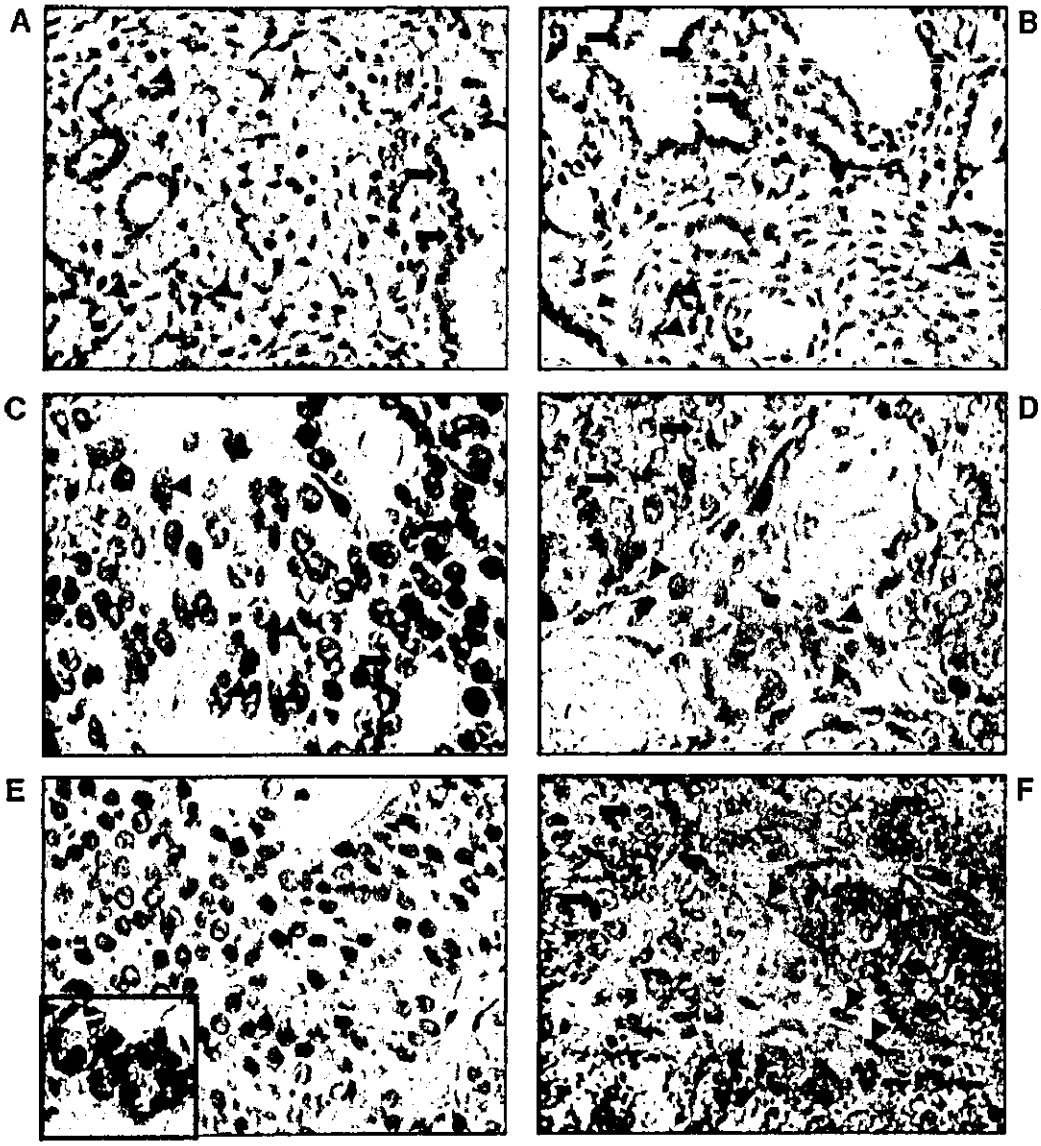

Fig. 3. Immunohistochemistry of sections of mammary gland tumors from MMTV/CR-1 mice shows expression of: (A) human Cripto-1 (40×); (B) vimentin (40×); (C) N-cadherin (100×); (D) cyclin-D1 (100×); (E) smooth muscle actin (SMA) (100×); (F) fibronectin (100×). Inset in center panel in box (E) shows SMA expressed in myoepithelial cells (yellow arrow) surrounding glandular structures in a section of normal mammary gland from multiparous FVB mouse. Solid arrows point to relatively differentiated epithelial tumor cells with respect to the larger more elongated, anaplastic tumor cells (arrow heads).

P-FAK, P-Akt, P-GSK-3β and also a reduction in the expression of DP-β-catenin (Fig. 6B,C). This was correlated with a significant reduction in the migration across FN-coated migration membranes of PP2 treated HC-11/CR-1 cells compared to untreated HC-11/CR-1 cells (Fig. 6D).

DISCUSSION

We have recently shown that when a human CR-1 transgene in an MMTV-CR-1 expression vector was used to generate several different heterozygous transgenic mouse lines, these mice exhibited a spectrum of transgene expression in the mammary gland (Wechselberger, submitted). In that study, multiparous G4 MMTV-CR-1 females exhibited multifocal ductal hyperplastic lesions and approximately 35% of these animals after four to five pregnancies developed papillary adenocarcinomas and solid undifferentiated tumors that probably originate from the hyperplasias after 15–18 months. This study demonstrates that these hyperplasias and tumors in the MMTV-CR-1 transgenic mammary gland exhibit a phenotype that suggests that epithelial cells within these lesions are undergoing EMT.

In normal tissues, epithelial cells are bound to each other through tight intercellular junctions. Adherens

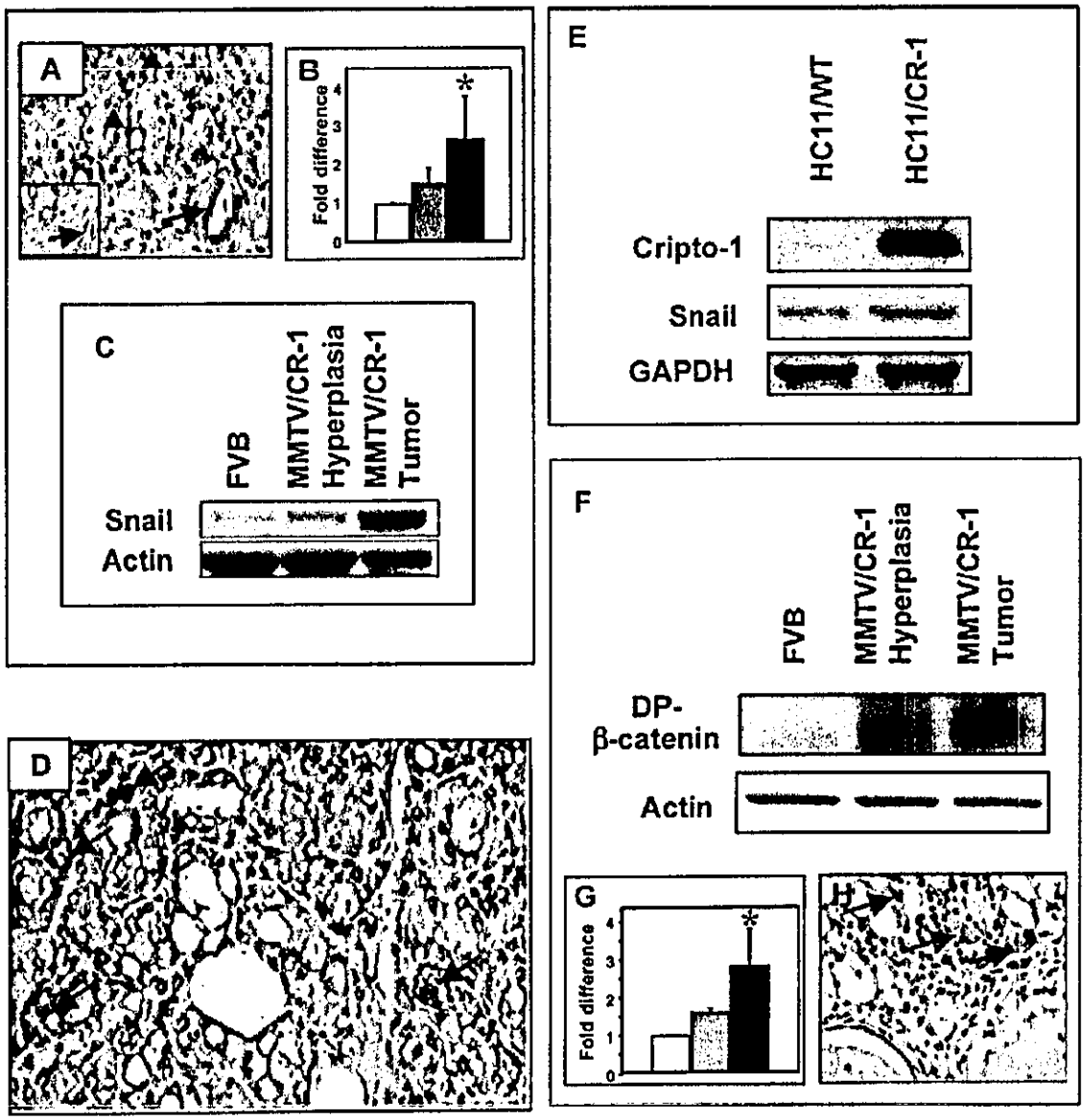

Fig. 4. Immunohistochemistry in part (A) shows diffuse staining for snail in solid tumor areas (arrow heads) of tissue section from MMTV/CR-1 mammary gland. Inset in part (A) shows no staining for snail in normal mammary glands from FVB multiparous mouse but positive staining in adjacent muscle tissue (arrow). Part (B) shows mean fold difference in snail expression measured by densitometric readings of Western blots of mammary tissue obtained from: three normal FVB strain multiparous mice (white bar); hyperplastic mammary lesions from four MMTV/CR-1 transgenic mice (gray bar); mammary tumors from five MMTV/CR-1 transgenic mice (black bar). Representative Western blot in part (C) shows increased snail expression in mammary tumors from MMTV/CR-1 transgenic mice compared to mammary tissue from normal FVB strain multiparous mice. Focal staining for snail in hyperplastic lesions (arrows) is shown in part (D). In part (E), result from RT-PCR shows higher levels of snail mRNA in normal mouse mammary epithelial cells transfected with Cripto-1 (HC11/CR-1) compared to non-transfected control cells (HC11/WT). A representative Western blot in part (F) shows increased expression of dephosphorylated-β-catenin (DP-β-catenin) in mammary gland tissue extracts from MMTV/CR-1 transgenic mice. Part (G) shows the fold difference in expression determined by densitometry of Western blots of DP-β-catenin expression in all mammary tissue samples studied. Immunohistochemisrty in part (H) shows positive staining for DP-β-catenin of tumor cells (arrows) in the stroma of MMTV/CR-1 mammary gland tumor section (*, $P < 0.05$).

junctions constitute a major component of these intercellular junctions and are composed of the transmembrane molecule E-cadherin, which binds to β- and γ-catenin through its cytoplasmic tail. This complex then binds to α-catenin, which forms a bridge for connecting these to actin (Tsukita et al., 1992). EMT is characterized by an increase in cell motility as a consequence of the loss of intercellular contact, which is evidenced by a decrease in E-cadherin expression and disassembly of the adherens junction complex. This leads to a change in the subcellular localization of β-catenin while the morphology of the epithelial cells becomes more

elongated and mesenchyme-like as the cells exhibit an increase in motility and invasiveness (Nieset et al., 1997; Chitaev and Troyanovsky, 1998; Boyer et al., 2000; Savagner, 2001).

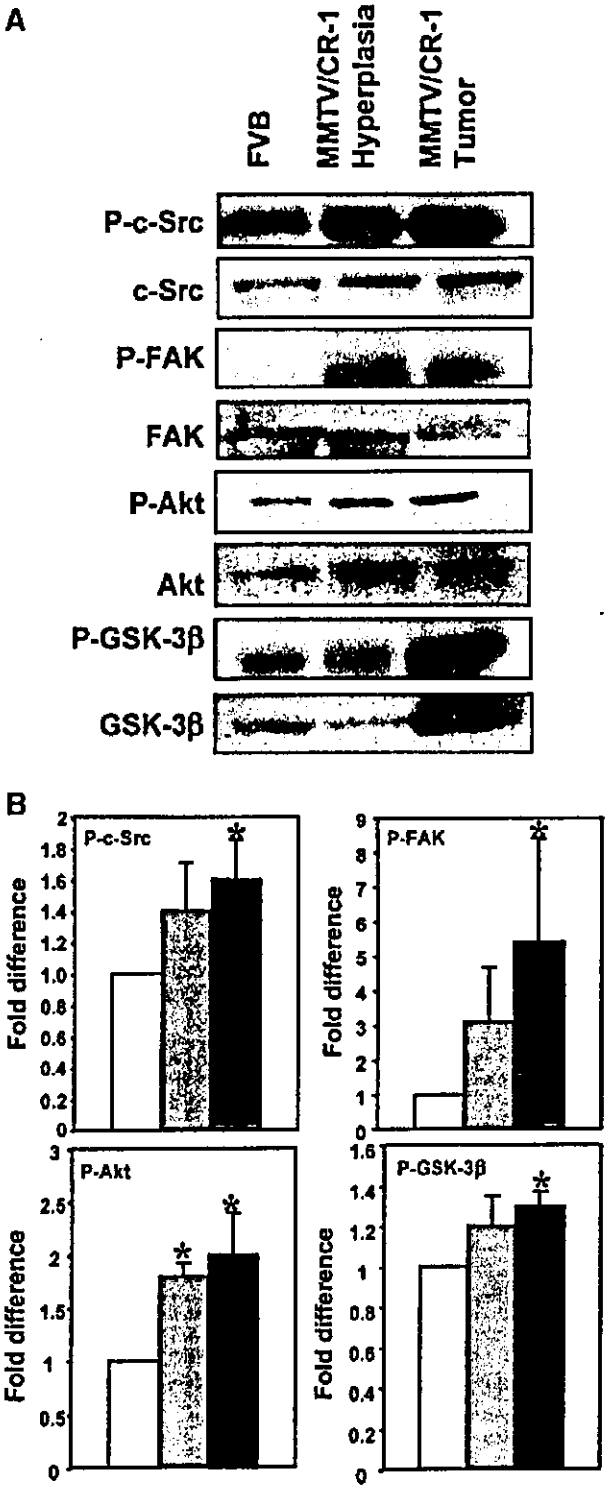

Fig. 5. Representative Western blots in part (A) shows increased expression of activated signaling molecules c-Src, FAK, Akt, and GSK-3β in mammary lesions from MMTV/CR-1 transgenic mice compared to mammary tissue extracts from FVB strain normal multiparous mice. Part B shows mean fold difference in expression of P-c-Src, P-Akt, P-FAK, and P-GSK-3β determined by densitometry of Western blots of mammary tissue extracts from three FVB strain normal multiparous mice (white bar), hyperplastic mammary lesions from four MMTV/CR-1 transgenic mice (gray bar) and mammary tumor lesions from five MMTV/CR-1 transgenic mice (black bar) (*, $P < 0.05$).

Previous experiments have demonstrated that overexpression of CR-1 in mammalian epithelial cell lines is associated with morphological, functional, and molecular alterations that are suggestive of EMT (Ebert et al., 2000; Wechselberger et al., 2001; Bianco et al., 2003; Normanno et al., 2004). The present study demonstrates that hyperplasias and tumors from the mammary gland of transgenic mice that overexpress the human CR-1 transgene also show morphological and biochemical characteristics of EMT. The decreased expression of E-cadherin in tissue extracts from the MMTV/CR-1 tumors compared to extracts from mammary gland tissue of multiparous normal FVB/N mice and a corresponding increase in the expression of N-cadherin in the transgenic mammary lesions suggests that CR-1 may play a role in promoting EMT in these lesions. In fact, positive immunostaining for N-cadherin was observed in tumor cells with a more elongated mesenchymal-like morphology compared to adjacent relatively more differentiated, round epithelial tumor cells forming rudimentary glandular structures. These same elongated tumor cells also showed increased cell-cycle activity as evidenced by positive staining for cyclin-D1. Interestingly, the expression of E-cadherin was actually increased in the MMTV/CR-1 hyperplastic lesions. This is most likely due to the preponderance of epithelial cells still tightly adherent to each other in the hyperplastic lesions compared to the epithelial structures present in the mammary tissue of FVB normal multiparous mice and of MMTV/CR-1 transgenic mice with adenocarcinomas.

Another characteristic of EMT is the increase in expression of vimentin, an intermediate filament protein normally expressed in mesenchyme tissues, and in motile cells during embryonic development (Lane et al., 1983) and during disease (Fuchs and Weber, 1994; Gilles et al., 1996). Recombinant mouse or human CR-1 was shown to enhance the motility of epithelial cells and these effects were associated with an increase in vimentin expression (Ebert et al., 2000; Wechselberger et al., 2001; Bianco et al., 2003). Expression of vimentin was highest in tissue extracts of the MMTV/CR-1 tumor lesions and, as observed with N-cadherin expression, vimentin staining by immunochemistry showed increased expression in the undifferentiated, elongated tumor cells adjacent the papillary tumor structures.

Integrins are heterodimers composed of non-covalently associated, transmembrane alpha and beta subunits that interact with an array of ECM molecules, such as type IV collagen, laminin and fibronectin, and orchestrate fundamental cell processes such as adhesion, proliferation, and migration (Coppolino and Dedhar, 2000; Danen and Sonnenberg, 2003). Studies have shown that increased expression of specific integrins is

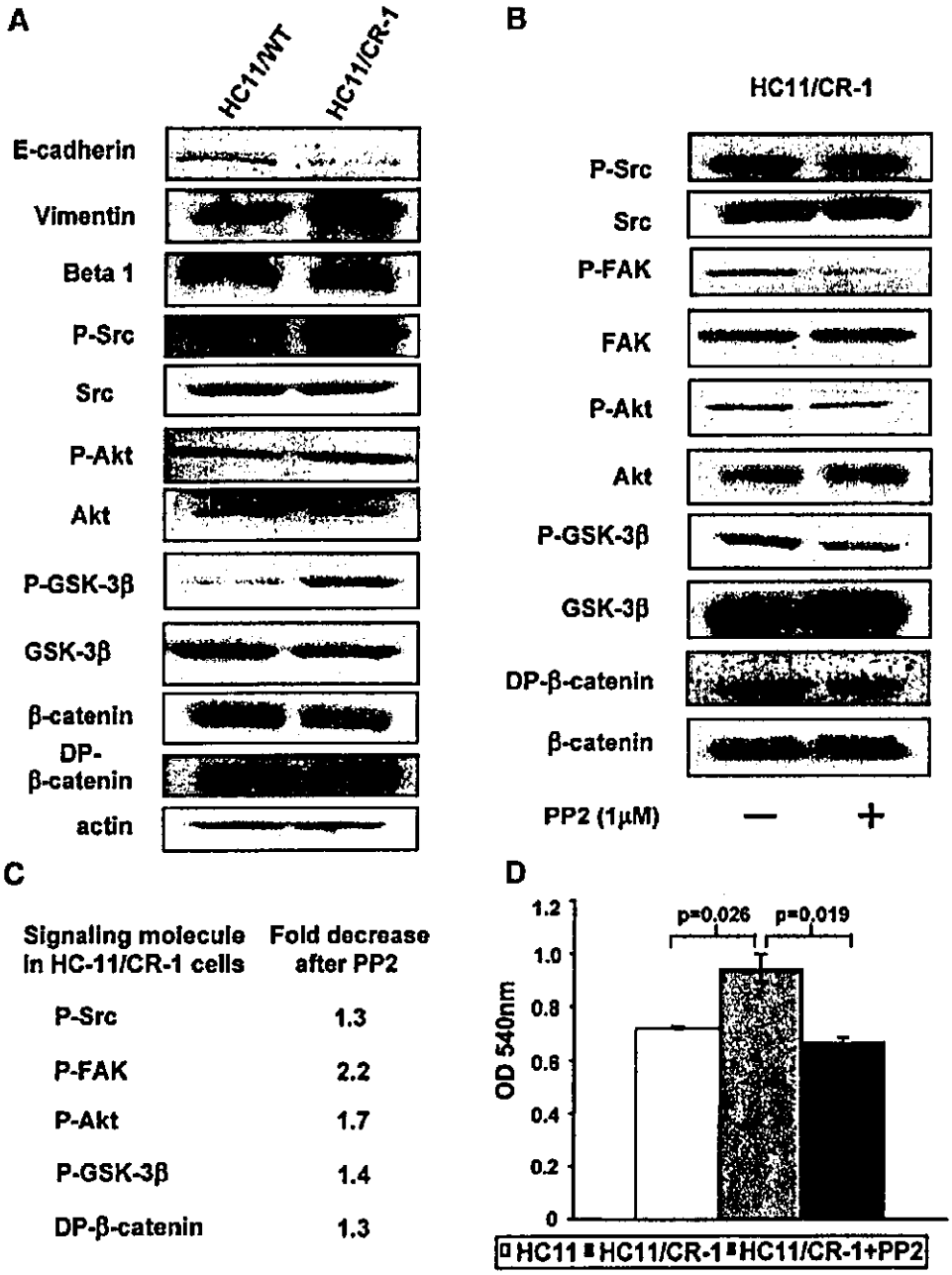

Fig. 6. Part A: Western blots of lysates from normal mouse mammary epithelial cells overexpressing Cripto-1 (HC11/CR-1) shows decreased expression of E-cadherin and increased expression of vimentin and beta 1 integrin as well as increased expression of active phosphorylated (P) signaling molecules important for EMT like c-Src and Akt. Increased expression of inactive P-GSK-3β and dephosphorylated-β-catenin (DP-β-catenin) was associated with overexpression of Cripto-1 in HC11 cells. Western blotting of lysates from HC11/CR-1 treated with the specific c-Src inhibitor PP2 in part (B) shows decreased expression of P-c-Src, P-FAK, P-Akt, P-GSK-3β, and DP-β-catenin. In part (C), results from readings obtained by densitometry shows the fold decrease in expression of P-c-Src, P-FAK, P-Akt, P-GSK-3β, and DP-β-catenin in HC-11/CR-1 cells after PP2 treatment. PP2 also significantly reduced migration of HC11/CR-1 as shown in part (D).

associated with enhanced tumor aggressiveness. For example, TGF-β1-mediated EMT of SiHa cells and Syndecan-1-mediated cell spreading of human breast carcinoma cells were associated with increased expression of integrins alpha v and beta 3 (Yi et al., 2002; Beauvais and Rapraeger, 2003). Also, EGF-induced EMT of colon carcinoma cells was associated with an increase in expression of the alpha 2 integrin and EMT of human breast carcinoma cells and rat bladder carcinoma cells was associated with an increase in

expression of alpha 2 and beta 1 integrins (Maemura et al., 1995; Valles et al., 1996; Solic and Davies, 1997). Our study suggests that one possible mechanism by which CR-1 may facilitate EMT is through the induction of expression of integrins. We found increased expression of alpha v, alpha 3, beta 1, beta 3, and beta 4 integrins in the MMTV/CR-1 mammary tumor lesions and beta 1 integrin, which was overexpressed in HC-11 mouse mammary epithelial cells expressing CR-1. Integrins cluster on the cytoplasmic membrane when binding to specific ECM molecules and facilitate the formation of focal adhesions thereby promoting tyrosine phosphorylation of FAK, which initiates intracellular signaling pathways leading to cell spreading (Burridge et al., 1992). Akt is also involved in integrin directed cell signaling and in turn phosphorylates and inactivates GSK-3β activity (Lynch et al., 1999). Unphosphorylated or active GSK-3β phosphorylates and targets β-catenin for subsequent ubiquitination and proteosome degradation (Lynch et al., 1999). When GSK-3β is inactivated by phosphorylation, β-catenin is free to accumulate in the cell where it is capable of entering the nucleus to form a complex with the transcription factor Tcf/Lef and activate target genes such as cyclin-D1 which are important for cell survival, proliferation, and migration (Henderson and Fagotto, 2002). The presence of increased P-FAK, P-Akt, and P-GSK-3β in the MMTV/CR-1 mammary tumor tissue extracts correlates with the increased expression of non-phosphorylated β-catenin in these lesions. This suggests that CR-1 may be involved in regulating certain aspects of the Wnt signaling pathway. In fact, inactive GSK-3β and accumulation of β-catenin are characteristics of the activated canonical Wnt signaling pathway (Huelsken and Behrens, 2002; Pandur et al., 2002). In this respect, increased expression of Wnt target gene products such as cyclin-D1 and the zinc-finger transcription factor snail was also found in MMTV/CR-1 mammary lesions. By immunohistochemistry, we found most of the snail expression in the undifferentiated areas of the MMTV/CR-1 transgenic tumors. HC-11 cells transfected with CR-1 also showed increased snail mRNA expression compared to control cells. Since snail has been shown to down-regulate E-cadherin expression (Cano et al., 2000; Poser et al., 2001; Jiao et al., 2002), snail overexpression in MMTV/CR-1 mammary tumor lesions may be responsible for the low level of E-cadherin expression found in these samples and in HC11 cells expressing CR-1.

We also found increased expression of active P-c-Src in both MMTV/CR-1 tumor tissue extracts and in HC-11 cells expressing CR-1 suggesting that c-Src may also play a role in CR-1-dependent signaling. Studies have shown that c-Src can directly or indirectly activate the same signaling molecules that are also involved in the integrin-signaling cascade such as FAK and Akt (Cary et al., 1999; Jones et al., 2000; Jiang and Qiu, 2003). In addition, activated c-Src has been shown to stabilize cytoplasmic β-catenin through tyrosine phosphorylation (Hinck et al., 1994; Gottardi and Gumbiner, 2001). It is possible that either CR-1 dependent EMT is a result of c-Src activation, which in turn activates downstream signaling molecules such as FAK and Akt, or that CR-1 is capable of binding to one of the specific types of integrins thereby directly activating FAK, which is also capable of activating c-Src. A previous study demonstrated that active c-Src kinase is necessary for CR-1 dependent transformation and migration of mouse mammary epithelial cells (Bianco et al., 2003). The results from the present study demonstrating that PP2 treated HC-11 cells transfected with CR-1, and not blocking antibodies against either integrin beta 1 or beta 3 (data not shown), exhibited a decreased activation of phosphorylated FAK, Akt, GSK-3β, and β-catenin as well as a significant decrease in migration across FN-coated membranes supports a c-Src-dependent pathway that is activated by CR-1. However, activation of signaling by CR-1 through the interaction with integrins like beta 4 needs to be assessed for potential CR-1-dependent integrin signaling. It may also be possible that CR-1 can activate downstream integrin signaling indirectly by stimulating the cells to synthesize and deposit specific ECM proteins that are important for cell spreading. In fact, FN, which is an ECM molecule known to bind beta integrins and regulate diverse cellular activities (Danen and Yamada, 2001), was expressed in the MMTV/CR-1 mammary tumors.

Our data further supports the oncogenic potential of CR-1. We show that CR-1 expression is associated with the turnover of intercellular adhesion proteins, the expression of mesenchyme markers associated with increased tumor aggressiveness and the activation of signaling molecules important for cell survival, proliferation, and migration. All these characteristics are highlights of EMT, an important mechanism for tumor cell migration and metastasis. Through activation of c-Src and other signaling molecules like FAK, Akt, and GSK-3β important for EMT or interaction with cell surface molecules like integrins, CR-1 may be capable of promoting cellular migration. The increased expression of snail and of DP-β-catenin also suggests that other signaling pathways such as Wnt may cross-talk with CR-1 signaling and regulate cell adhesion and migration.

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RAPID COMMUNICATION

Reversal of Streptozotocin-induced Hyperglycemia by Transplantation of Pseudoislets Consisting of β Cells Derived from Ductal Cells

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Abstract. The present study was conducted in an attempt to treat streptozotocin (STZ)-induced hyperglycemia by transplanting β cells derived from pancreatic ductal cells. Ductal cells obtained from neonatal rats were cultured *in vitro*. Approximately 70% of the cells were converted to insulin-secreting cells by incubating with betacellulin and activin A. Differentiated cells responded to a depolarizing concentration of potassium, tolbutamide and a high concentration of glucose, and insulin secretion increased by 2.5-, 2.3- and 1.6-fold, respectively. We then prepared pseudoislets using the differentiated cells, which exhibited greatly improved glucose-responsiveness, with a high concentration of glucose inducing a 3-fold increase in insulin secretion. We transplanted these pseudoislets into the portal vein of STZ-treated nude mice. Before transplantation, the plasma glucose concentration was above 400 mg/dl, and after transplantation it was markedly reduced, the effect of which persisted for two weeks. These results indicate that STZ-induced hyperglycemia can be treated by transplanting pseudoislets consisting of β cells derived from ductal cells.

Key words: β Cell, Insulin, Presudoislet, Transplantation, Ductal cell

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PANCREATIC β cells are highly differentiated endocrine cells that secrete insulin, a principal hormone regulating glucose metabolism in the body. Reduction in the mass of functioning β cells impairs glucose metabolism and causes diabetes. Recent advances in cell biology have enabled researchers to induce differentiation of pancreatic precursor cells to β cells in a culture system [1–4]. Insulin-producing cells have been obtained *in vitro* from precursor cells located in the pancreatic islets [2] and from epithelial cells in the pancreatic ducts [3, 4]. If a large number of β cells are obtained from those precursors in the culture system, diabetes mellitus can be treated by transplanting these functioning β cells. To achieve this therapeutic approach, several issues still remain to be resolved. First of all, a large number of functioning β cells are necessary to control glucose metabolism. Secondly, those β cells should have reasonably good insulin secretory response to ambient glucose and incretins. Thirdly, a method for effective transplantation should be established. In this regard, islets have been successfully transplanted into the portal vein in human islet transplantation [5]. This approach has an advantage in that transplanted islets are located in the upstream of the liver, a major target organ of insulin.

The present study was conducted in an attempt to establish a method for β cell transplantation using cells derived from precursor cells located in the pancreatic duct. Our strategy was to induce differentiation of precursor cells *in vitro* and to prepare pseudoislets using insulin-secreting cells differentiated from the pancreatic ductal cells. The pseudoislets were then transplanted into the portal vein of the diabetic animals. The present results indicated that transplanted pseudoislets effectively improved hyperglycemia in streptozotocin (STZ)-treated mice.

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Experimental Procedure

Materials

Recombinant human activin A was generously provided by Dr. Y. Eto of the Central Research Laboratory, Ajinomoto Inc. (Kawasaki, Japan). Recombinant human BTC was prepared as described previously [6]. Fetal calf serum (FCS) was obtained from Gibco (Grand Island, NY, USA).

Cell culture

Pancreatic ducts were obtained from seven-day old male Wistar rats after collagenase digestion of the pancreas [7]. Ductal cells were dispersed by using Dispase (Godo Shuzo, Tokyo, Japan). Cells were then cultured in Dulbecco's modified Eagle's medium (DMEM) containing 2.7 mM glucose and 0.5% FCS, for two weeks. The medium was then changed to DMEM containing 25 mM glucose and 0.5% FCS and cultured for three weeks. To induce differentiation, duct epithelial cells were incubated with a combination of 2 nM activin A and 1 nM BTC for various periods.

Formation of pseudoislets

Ductal cells were incubated for five to seven days with 2 nM activin A and 1 nM BTC. Differentiated cells were then dispersed by trypsin digestion. To obtain pseudoislets, cell suspension (5×10^5 cells/ml) was incubated for 36 to 48 hrs in a gelatin-coated plastic dish with DMEM containing 1 nM BTC under constant stirring (30 rotations per min).

Measurement of mRNA expression

Total RNA was extracted by using TRIzol reagent (Invitrogen Japan, Tokyo, Japan). Messenger RNA was extracted using the Quick Prep Kit (Pharmacia LKB Biotechnology, Piscataway, NJ). Messenger RNA samples were pretreated with DNase to remove contamination of genomic DNA. First-strand cDNA was synthesized using a Preamplification System for SUPERSCRIPT First-Strand Synthesis System (Invitrogen Japan). To confirm no contamination of genomic DNA, samples without reverse transcription treatment were prepared. Primers used in this study were described elsewhere [8]. The reactions were conducted in a DNA Thermal Cycler (Perkin Elmer, Norwalk, CT) under the following conditions: denaturation at 94°C for 1 min, and annealing and extension at 65°C for 2 min. The number of cycles was 30, with the exception of 17 cycles for GAPDH. No PCR products were detected in the real-time PCR prodedure without reverse transcription.

Immunohistochemistry

For immunohistochemistry, cells were cultured on non-coated glass coverslips. Cells were fixed and immunostained with anti-insulin, anti-glucagon, anti-somatostatin or anti-pancreatic polypeptide antibody as described previously [9, 10]. Images were obtained using a Zeiss microscope equipped with fluorescein and rhodamine filter sets (Axiophoto, Carl Zeiss, Thornwood, NY, USA).

Measurement of insulin

For measurement of insulin secretion, differentiated cells or pseudoislets were incubated for 60 min in Krebs-Ringer bicarbonate (KRB) buffer containing 0.1% bovine serum albumin. Samples were kept in –20°C until the assay. Insulin was measured by time-resolved immunofluorometric assay as described previously [9].

Transplantation of pseudoislets

Four-week old nude mice were injected peritoneally with 150 μg/g STZ. On the next day (designated as day 0) pseudoislets were injected into the portal vein via the cecal vein and the morning plasma glucose concentration was measured daily by using Accu-Check Active (Roche Diagnostics GmbH, Germany). Statistical significance was determined by one-way ANOVA. Difference between the two groups was assessed by Student's t-test.

Results

Differentiation of ductal cells

Fig. 1A depicts the morphology of ductal cells in culture. To induce differentiation of ductal cells, we examined various factors including FGF, VEGF, EGF,

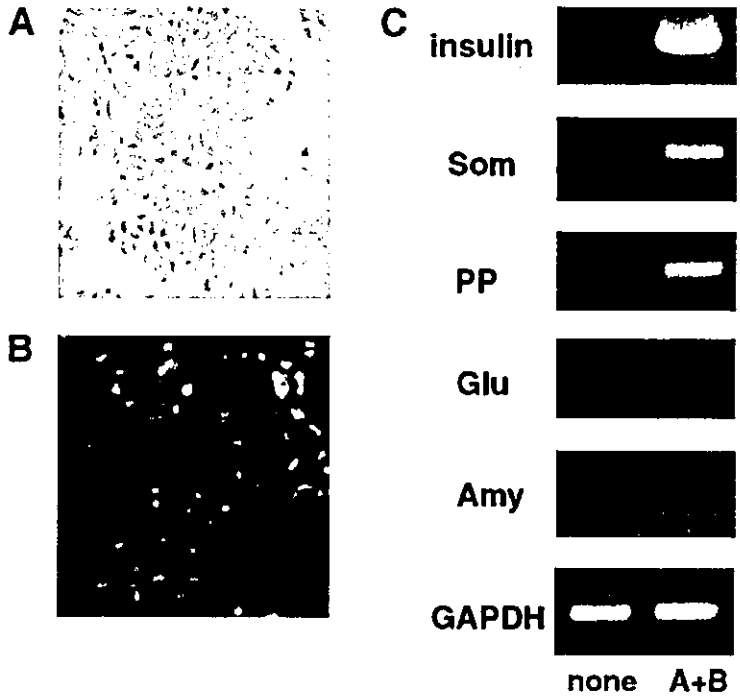

Fig. 1. Differentiation of Ductal cells
A: Morphology of ductal cells in culture. B: Immunostaining of insulin in differentiated ductal cells. Ductal cells were incubated with activin A and BTC for 5 days. Immunohistochemistry was performed using anti-insulin antibody. C: Changes in the mRNA expression of various pancreatic markers. Ductal cells were incubated in the absence (none) or presence (A + B) of 1 nM activin A and 1 nM BTC for 5 days. mRNA was extracted and the mRNA expression of insulin, somatostatin, glucagon and amylase was measured by RT-PCR.

NGF, BTC, TGF-α, TGF-β and activin A. Among them, a combination of activin A and BTC provided the best results. Fig. 1B shows the insulin-staining of the cells treated with activin A and BTC. Immunoreactive insulin was positive in approximately 70% of the cells treated with activin A and BTC. Somatostatin was detected in approximately 10% of the cells, but somatostatin/insulin double-positive cells were not observed. As shown in Fig. 1C, cells incubated with activin A and BTC expressed mRNA for insulin, somatostatin and pancreatic polypeptide, but mRNA for glucagon was not detected. Similarly, mRNA for amylase was not expressed. Fig. 2 depicts the insulin secretory response of differentiated cells. These cells responded to a depoparizing concentration of potassium and tolbutamide, an inhibitor of the ATP-sensitive potassium (K_{ATP}) channel. A high concentration of glucose also induced a significant increase in insulin secretion although the response was modest (Fig. 2).

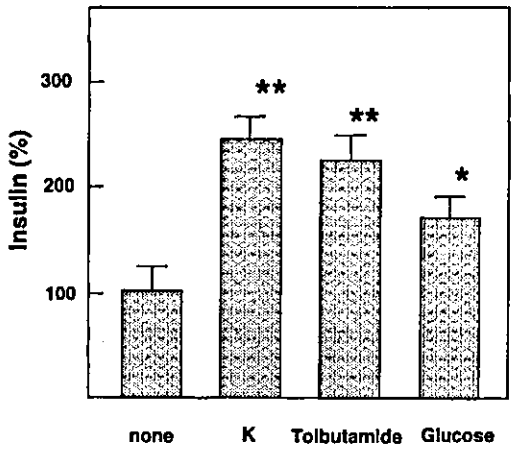

Fig. 2. Insulin Secretion From Differentiated Ductal Cells
Ductal cells were incubated with 1 nM activin A and 1 nM BTC for 5 days. Cells were then incubated with KRB buffer containing 50 μM tolbutamide and 16.7 mM glucose for 60 min and insulin released was measured. Values are the mean ± S. E. for four experiments. *: $p<0.01$ vs none.