Bone morphogenetic proteins promote development of fetal pancreas epithelial colonies containing insulin-positive cells

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Summary

Extracellular signals that guide pancreas cell development are not well characterized. In an in vitro culture system of dissociated pancreas cells from the E15.5 mouse fetus we show that, in the presence of the extracellular matrix protein laminin-1, bone morphogenetic proteins (BMPs-4, -5 and -6) promote the development of cystic epithelial colonies. Transforming growth factor β1 (TGF-β1) and activin A antagonise this effect of BMP-6 and inhibit colony formation. Histological analysis revealed that the colonies are composed of E-cadherin-positive epithelial cells, which in localised areas are insulin positive. The colonies also contain occasional glucagon-positive cells, but no somatostatin- or α-amylase-positive cells. These findings indicate that members of the TGF-β superfamily regulate pancreas epithelial cell development and can promote the formation of islet-like structures in vitro.

Key words: Bone morphogenetic proteins, Pancreas, Epithelial colony, Fetus, Development, β-cells

Introduction

Type 1 diabetes is a disorder in which autoimmune destruction of pancreatic islet β cells leads to insulin deficiency. Gaining the ability to generate a supply of insulin-secreting cells would be a major advance toward the cure of this disorder. Cell development is determined in part by extracellular signals from both insoluble matrix proteins and soluble growth factors. Previously, we found that the extracellular matrix (ECM) protein laminin-1, a component of the basement membrane, promotes differentiation of fetal pancreas cells into insulin-positive β cells (Jiang et al., 1999). More recently, Bonner-Weir and colleagues (Bonner-Weir et al., 2000) reported that adult human pancreas duct epithelial cells proliferate and form cysts in cultures overlayed with Matrigel, a reconstituted laminin-1-rich basement membrane gel.

Several soluble extracellular factors have been implicated in pancreatic epithelial cell development, including members of the TGF-β superfamily. Transgenic mice expressing a dominant-negative TGF-β receptor II controlled by the mouse metallothionein 1 promoter display increased proliferation and impaired differentiation of pancreatic acinar cells (Bottinger et al., 1997). Transgenic mice expressing a dominant-negative activin receptor controlled by the human insulin promoter have hypoplasia of pancreatic islets (Yamaoka et al., 1998). Hebrok et al. (Hebrok et al., 1998) found that activin B is expressed in the notochord adjacent to the domain of foregut endoderm from which the pancreatic primordia derives. Activin B represses endodermal expression of sonic hedgehog - repression is a pre-requisite for expression of the homeodomain transcription factor, Pdx-1, which is required for pancreas development (Jonsson et al., 1994; Offield et al., 1996). The bone morphogenetic proteins (BMPs), members of the TGF-β superfamily, have been shown to be important in the development of kidney tubule, lung and other organ epithelia (Hogan, 1996a; Weaver et al., 1999) and are expressed in the pancreas. BMP-7 was detected immunocytochemically in human fetal pancreas duct epithelium (Vukicevic et al., 1994) and by mRNA in situ hybridization in mouse pancreas epithelium between embryonic day (E)12.5 and E14.5 (Lyons et al., 1995). These findings prompted us to investigate the effects of TGF-β superfamily members on fetal pancreas cells in vitro.

Materials and Methods

In vitro culture of pancreatic progenitor cells

Pancreata were dissected from E15.5 CBA mouse fetuses and dissociated into single cells as described previously (Jiang et al., 1999). Briefly, dissected pancreas was digested with trypsin/EDTA for 15 minutes at 37°C in a shaking water bath. Cells were counted in a haemocytometer and the viability determined by trypan blue dye exclusion. Each fetal pancreas yielded ~50,000 viable cells (48,509±11,299; n=14). Dissociated cells were plated in 8-well chamber slides (Nunc, Naperville, USA) at 7.5×10^4 cells/well in 0.3 ml AIM V medium supplemented with N-2 (1: 100, Gibco BRL Life Technologies, Gaithersburg, USA), 500 UI/ml penicillin and 500 μg/ml streptomycin. Laminin-1 (160 μg/ml), purified from murine Engelbreth-Holm-Swarm tumour basement membrane (Becton Dickinson Labware, Bedford, USA), was overlayed on cells in the presence of various commercially available growth factors. Cultures were incubated in 10% CO_2 90% air at 37°C, for up to 6 days. Recombinant human BMP-6 and BMP-5, members of the 60A subgroup, and BMP-4, a representative of the dpp subgroup (Hogan, 1996b), recombinant human TGF-β1 and recombinant activin A were

all purchased from R&D Systems (Minneapolis, USA). These factors, which were dissolved at a concentration of 10 ng/ml in mouse tonicity phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA), were added at the start of culture. Phase contrast images of colonies were photographed with an Olympus IX70 digitized camera.

Colony quantitation

Colony formation was assessed at day 6 of culture. A colony was defined as a cellular sphere ≥30 μm in diameter that contained more than 20 cells. The number of colonies per well was counted directly under an inverted microscope at ×10. Colony counts were performed using a blind design.

Immunocytochemistry and histocytochemistry

Guinea pig anti-porcine insulin antiserum (final 1:200), rat monoclonal anti-E-cadherin IgG2a (clone ECCD-2) (1:100) and rabbit antiserum to porcine glucagon (1:100) and to human somatostatin (1:200) were purchased from Dako (Glostrup, Denmark). Fractionated rabbit antiserum to human α-amylase, a marker of acinar cells, was from Sigma. Mouse monoclonal anti-BrdU IgG2a (Clone BU-1) was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK).

After 6 days of culture, some pancreas cell colonies were washed with warm PBS, fixed in 4% paraformaldehyde (PFA) and stained for proinsulin, a marker of β cells, with 0.5 μg/ml mouse anti-human IgG1 proinsulin monoclonal antibody (clone M32337, Fitzgerald Industries International Concord, MA). Anti-proinsulin antibody was used to avoid the non-specific staining seen with guinea pig anti-insulin serum under these conditions. The specificity of this antibody was demonstrated by the complete blocking of staining in the presence of 80 μg/ml recombinant mouse proinsulin. In order to study colony cells in more detail, colonies were harvested by digestion with dispase (Becton Dickinson, Labware). Following inactivation of dispase by addition of 8% BSA, the colonies were fixed in 4% PFA, embedded into 1% low melting point agarose gel and processed for histological sections (5 μm) using standard procedures. Sections were routinely stained for hematoxylin and eosin (H&E).

For immunoperoxidase staining, endogenous peroxidase was blocked by 3% H_2O_2 in methanol for 8 minutes. Before addition of antibody, non-specific protein binding was blocked by incubation of tissues for at least 30 minutes with PBS containing 2% BSA or 2% normal rabbit serum. Negative controls were performed by replacing the first antibody with pre-immune serum from the appropriate species or by isotype-matched control monoclonal antibody. Colony sections were incubated with primary antibodies for 90 minutes at 25°C, followed by three washes with PBS. Horseradish-peroxidase-conjugated rabbit anti-guinea pig, swine anti-rabbit and rabbit anti-mouse immunoglobulins (Dako) at 1:80 were added for 30 minutes at 25°C, followed by thorough washes. Immunoperoxidase was detected with 3,3′-diaminobenzidine/H_2O_2 for 4-8 minutes. Slides were counterstained with haematoxylin and examined and photomicrographed under an Olympus microscope.

For immunofluorescence staining, fluorescein-isothyocyanate-conjugated rabbit anti-mouse immunoglobulins (Dako) were added for 30 minutes at 25°C, followed by three thorough washes. Slides were observed and photomicrographed under a Zeis Axiophot fluorescent microscope.

The periodic acid Schiff (PAS) reaction (Bancroft and Stevens, 1982) was used to stain basement membranes.

RT-PCR analysis of mRNA transcripts

Fetal pancreata were removed under a dissection microscope and snap-frozen on dry ice. Total RNA was extracted with phenol/guanidine isothiocyanate-based RNAzol B (Cinna/Biotex, Hoston, USA), treated with DNAse I and then reverse transcribed with Superscript II reverse transcriptase (GibcoBRL) in 1× transcription buffer containing 0.5 μM oligo(dT)16-18 primer (GibcoBRL) and 400 μM dNTPs. Aliquots of the cDNAs were amplified by PCR in 1× PCR buffer (Perkin, Elmer, USA) containing 200 μM dNTPs, 1 μM of each primer pair, 1.5 mM Mg^{2+} and 1 U Taq polymerase. The following primers were employed: BMP-2 (5′ GGAAAAGGACATCCGCTCCACAAACG 3′; 5′ ATTTATTCTTGCTGTGCTAACGACAC 3′, 404 bp), BMP-4 (5′ CAAACGTAGTCCCAAGCATCACCCAC 3′; 5′ TCCGCCCTCCGGACTGCCTGATCTC 3′, 453 bp), BMP-5 (5′ GAGCACAGCAAGGCTTGGGAACATG 3′; 5′ GCTGGAGATTATAATACCAGTGAAC 3′, 240bp), BMP-6 (5′ GTTCTTCAGACTACAACGGCAGTGAG 3′; 5′ GTTAGGAATCCAAGGCAGAACCATG 3′, 402bp), BMP-7 (5′ GTGTGGCAGAAAACAGCAGCAGTGAC 3′; 5′ GACATCGAAGATTTGGAAAGGTGTG 3′, 401bp), TGFß-1 (5′ ACCAACTATTGCTTCAGCTCCACAG 3′; 5′ GCAGGAGCGCACAATCATGTTGGAC 3′, 317bp), activin A (5′ CTTGGAGTGCGACGGCAAGGTCAAC 3′; 5′ CATTTTCTCTGGGACCTGGCGACTC 3′, 372 bp) and the 'housekeeping' gene *β actin* (5′ GTGGGCCGCCCTAGGCACCA 3′; 5′ CTCTTTGATGTCACGCACGATTTC 3′, 530 bp). PCR reactions were performed for 35 cycles (94°C, 30 seconds; 55°C, 30 seconds; 72°C, 30 seconds) and amplified products separated in 1.5% agarose gels.

Statistics

Multi-variable experiments were analysed by ANOVA and differences between groups by the Student's *t*-test. Data are presented as mean±s.d. of at least three experiments.

Results

TGF-β superfamily genes are expressed during pancreas development

In order to identify TGF-β superfamily genes that might be relevant to pancreas development, we performed RT-PCR analysis on mRNAs from E13.5, E15.5 and E17.5 fetal mouse pancreas. BMP-4, -6 and -7 and TGF-β1 were expressed at each age examined, whereas BMP-5, BMP-2 and activin A were detected later, at E17.5 (Fig. 1). These results were consistent with other reports (Crisera et al., 2000; Lyons et al., 1995; Vukicevic et al., 1994).

TGF-β superfamily members promote pancreatic epithelial cell colony formation

Previously, we used a low cell density culture system to demonstrate that fetal pancreas progenitor cells differentiate into insulin-positive β cells in the presence of laminin-1 (Jiang et al., 1999). When this system was modified by replacing Hybridoma medium with AIM V medium supplemented with N-2, increasing the cell density to 925 cells/mm^2 and decreasing laminin-1 concentration from 200 μg/ml to 160 μg/ml, a low frequency of cystic colonies was observed (Fig. 2). These conditions established a baseline on which the effects of other factors were studied.

In the presence of laminin-1, BMPs-4, -5 and -6 promoted the formation of cystic colonies (shown for BMP-6; Fig. 2). In the absence of laminin-1, the BMPs alone had no effect. Some colonies appeared to be initially tubular by day 2 (Fig. 2; arrow and inset) although most were cystic. By day 6, tubular colonies were hardly ever observed. Most of the

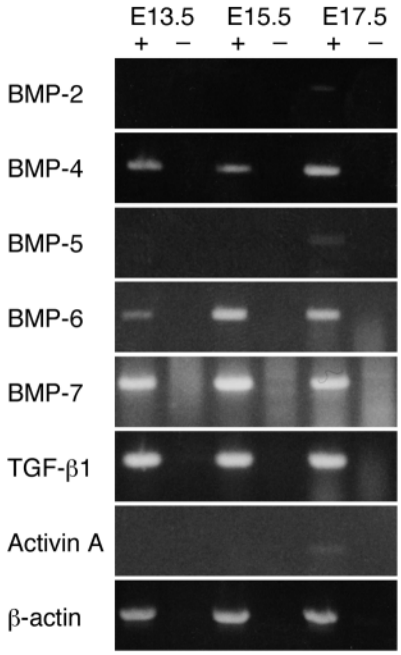

Fig. 1. RT-PCR expression profile analysis of TGF-β superfamily members. Total RNAs purified from developing pancreas between E13.5 and E17.5 are shown. mRNAs were incubated with (+) or without (–) reverse transcriptase and subjected to PCR with specific primers as described in Materials and Methods.

colonies were 50-60 μm in diameter although a few larger colonies of 100-200 μm were also observed. Maximal stimulation of colony formation occurred at concentrations of 1, 10 and 100 ng/ml for BMP-4, BMP-6 and BMP-5, respectively; at higher concentrations, fewer colonies were observed (Fig. 3).

In a number of diverse developmental settings the activity of BMPs is opposed by other members of the TGF-β superfamily, most notably by TGF-β itself and activins. TGF-β1 and activin A suppressed basal colony formation in the presence of laminin-1 alone (Fig. 4A), and antagonised BMP-6-promoted colony formation (P<0.01) (Fig. 4B). However, BMP-5 (100 ng/ml) did not antagonize BMP-6 (Fig. 4B). The dose-dependency of inhibition demonstrated that TGF-β1 was 100-fold more potent than activin A (Fig. 4C). These results suggest that an interplay between TGF-β1, activin and BMP signalling may be critical for pancreas epithelial cell development. Having established conditions that favour the formation of cystic colonies, we next examined the nature of the colonies themselves.

Colony generation involves cell proliferation

To determine whether the colonies contained proliferating cells, BrdU labelling was performed during the last 16 hours of culture. Up to 10 BrdU-positive cells per colony were detected (Fig. 5), providing evidence that cellular proliferation contributed to colony formation.

Colonies originate from epithelial cells

Histology revealed the colonies to be duct-like structures containing various forms of epithelial cells surrounding a central lumen (Fig. 6). Some colonies were composed predominantly of columnar epithelial cells (Fig. 6B), others of cuboidal cells (Fig. 6C), squamous epithelial cells (Fig. 6D) or a mixture of both columnar and squamous epithelial cells (Fig. 6E). The majority of colonies were surrounded by a PAS-positive basement membrane (Fig. 6F). Colony cells positively stained for E-cadherin, a specific epithelial cell marker involved in cell-cell interactions (Fig. 6G), indicating that the colonies most probably originated from ductal progenitor epithelial cells.

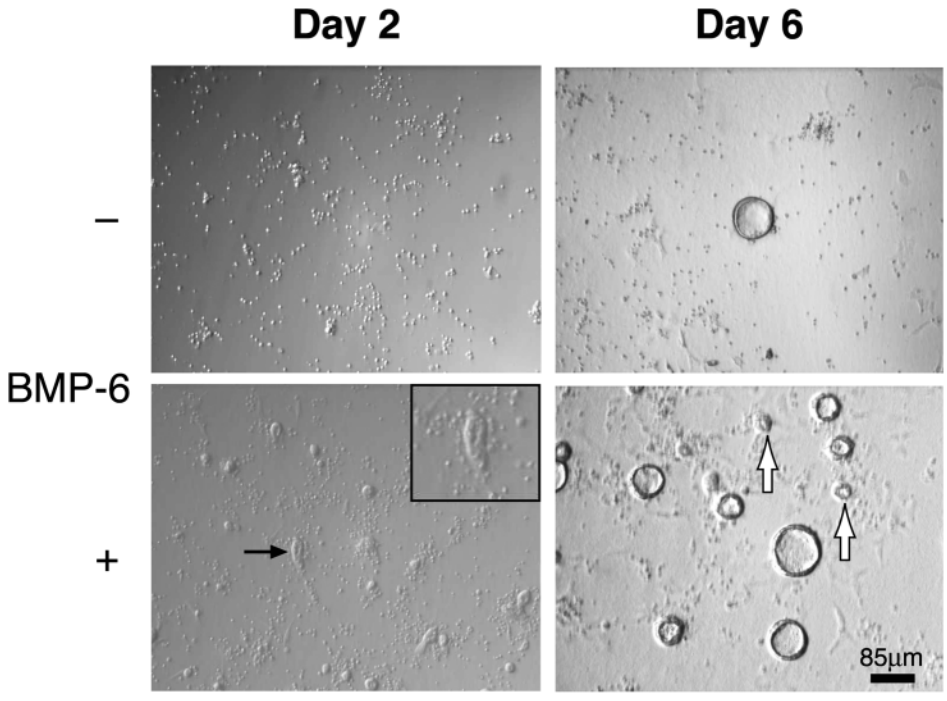

Fig. 2. BMP-6 (10 ng/ml), in the presence of laminin-1 (160 μg/ml), promotes cystic colony formation from dissociated E15.5 mouse pancreas cells. Phase contrast images show cells cultured for 2 or 6 days in laminin-1 with and without BMP-6. In the presence of BMP-6, small colonies and some tubule-like structures (closed arrow, also inset) were observed by day 2; by day 6, colonies increased in number and were of variable size. Colonies ≤30 μm (open arrow) were excluded from the quantitation of colony numbers in Fig. 3 below.

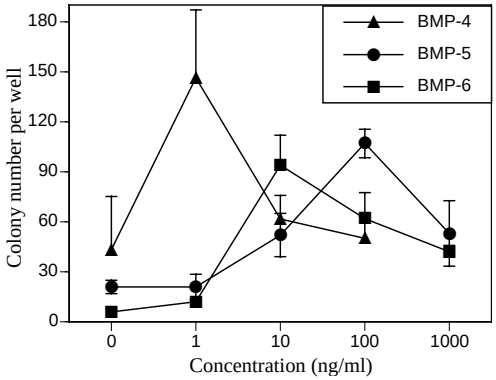

Fig. 3. Frequency of pancreatic cell colonies (mean±s.d., *n*=3) as a function of increasing concentration of BMPs. Colonies were directly counted under a phase contrast microscope (×10) 6 days after culture in the presence of 160 μg/ml laminin-1. Colonies ≤30 μm (see Fig. 2) were not counted.

Colonies contain a low number of differentiated endocrine cells

Having identified colony cells as epithelial in nature, we then sought to determine whether the colonies contained differentiated cell types or only immature ductal epithelial cells. The majority of colony cells were negative for all the differentiation markers examined. A few proinsulin-positive cells only were detected (Fig. 7A). When colony formation was suppressed by TGF-β1, activin A or a higher concentration of BMPs, more individual cells stained for proinsulin (Fig. 7B). This is consistent with our previous observation that β-cell differentiation increased when proliferation activity was inhibited (Jiang et al., 2001). In sections of harvested colonies, a few insulin-positive cells were always observed in the areas where cystic epithelial cells appeared to be delaminating or segregating from the main body of the colony (Fig. 7C-F). In addition, some individual insulin-positive cells were also observed between colonies (not shown). Glucagon-positive cells were also present in colonies, but were less frequent than insulin-positive cells (Fig. 7G), and somatostatin-positive cells were not detected. Although scattered α-amylase-positive cells were present, they were not present within colonies (Fig. 7H).

Discussion

The characterization of extracellular factors that promote islet endocrine cell development has important implications for restoring β-cell function in type 1 diabetes. We describe an in vitro laminin-1 overlay system that allows single fetal pancreas progenitor cells to proliferate, differentiate and form cystic

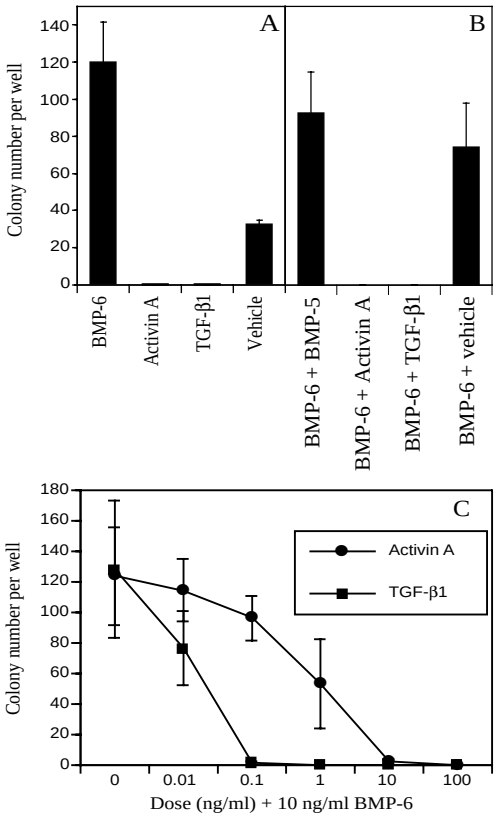

Fig. 4. Effects of TGF-β superfamily members on pancreatic cell colony formation. Colonies (mean±s.d., *n*=3 separate experiments) were counted 6 days after culture with the indicated treatment in the presence of 160 μg/ml laminin-1. (A) Effect of BMP-6, activin A, TGF-β1 (all 100 ng/ml) or vehicle alone. (B) Effect of BMP-5, activin A or TGF-β1 (each 100 ng/ml) on BMP-6 (10 ng/ml)-induced colonies. (C) Dose-dependent inhibition by TGF-β1 and activin A of BMP-6 (10 ng/ml)-induced colonies.

colonies containing hormone-positive cells in the presence of BMP-4, BMP-5 or BMP-6. This demonstrates a role for these TGF-β superfamily members in islet development.

Laminin-1 allows pancreas cell development in vitro

Pancreas duct and islet cells have previously been shown to be capable of forming cystic structures when cultured with ECM molecules. Adult human pancreas islet cells, for example, were found to ‘dedifferentiate’ into ductal epithelial cells and form cystic structures when cultured in collagen I gel (Kerr-Conte et al., 1996; Yuan et al., 1996). These cells

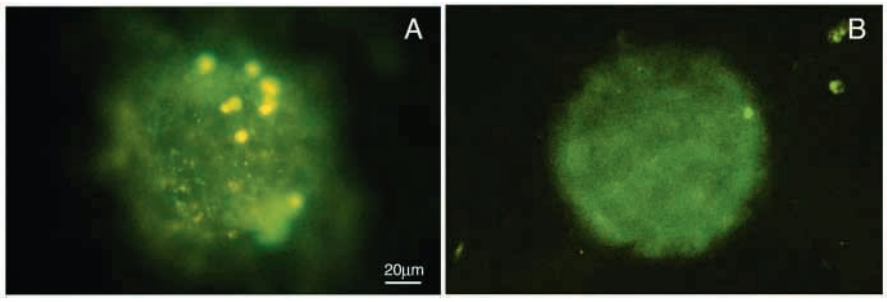

Fig. 5. Fluorescence images of proliferating cells in fetal pancreas cell colonies. BrdU (100 μM) was added during the last 16 hours of 6 day culture of E15.5 pancreas cells in the presence of laminin-1 and BMP-6. BrdU incorporated into the DNAs of proliferating cells was stained with mouse monoclonal anti-BrdU (A) and isotype control IgG2a (B) antibody and visualized with fluorescein-isothiocyanate-conjugated anti-mouse immunoglobulins.

proliferated in a 3D culture, especially in the presence of Matrigel (Kerr-Conte et al., 1996). In addition, isolated human pancreas duct cells cultured with Matrigel overlay were also shown to form ductal cysts (Bonner-Weir et al., 2000). However, because Matrigel contains a number of ECM proteins and growth factors (McGuire and Seeds, 1989) it is difficult to identify the contribution made by individual molecules. We circumvented this by using purified laminin-1 to establish a baseline from which to study the effect of specific extrinsic factors on pancreatic cell lineage development. The ability of fetal pancreas progenitor cells to proliferate, differentiate and form cystic epithelial colonies in vitro suggests that our culture system partially recapitulates development in vivo. Moreover, the endpoint of this culture system, the formation of cystic epithelial colonies containing differentiated endocrine cells, allowed us to quantify the effect of alterations in the culture parameters. It will also be of great interest to see if the nature and frequency of the various differentiated cell types present in these cultures can be influenced by other extrinsic factors.

TGF-β superfamily members regulate pancreas cell lineage development

Gene expression of some TGF-β superfamily members has been detected during pancreas development. In situ

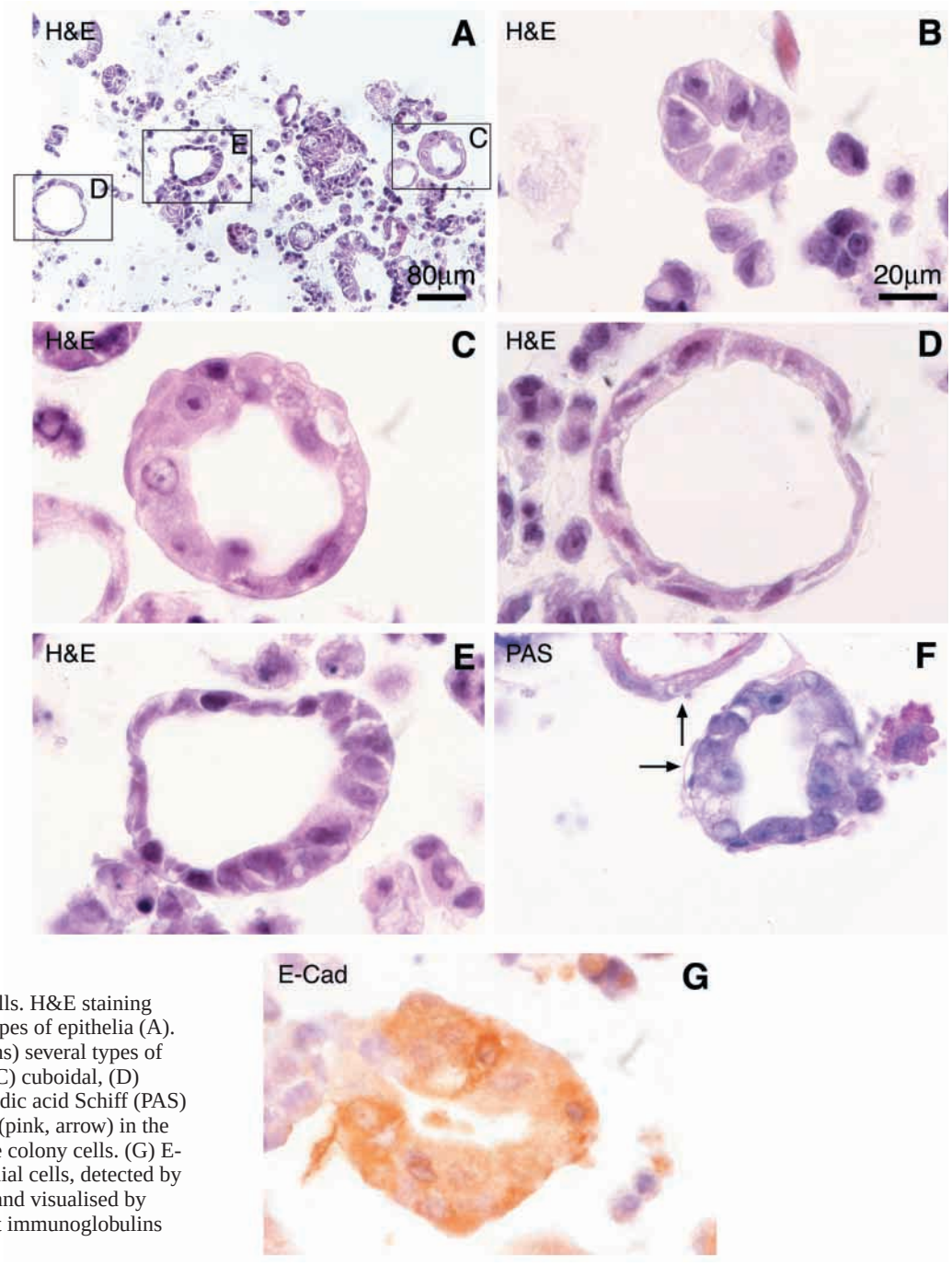

Fig. 6. Characterisation of colony cells. H&E staining showing colonies lined by various types of epithelia (A). Under a higher magnification (oil lens) several types of epithelia are shown: (B) columnar, (C) cuboidal, (D) squamous or (E) a mixture. (F) periodic acid Schiff (PAS) reaction staining of polysaccharides (pink, arrow) in the basement membrane surrounding the colony cells. (G) E-cadherin (E-cad), a marker of epithelial cells, detected by rat anti-mouse E-cadherin antibody and visualised by peroxidase-conjugated rabbit anti-rat immunoglobulins (brown).

hybridization studies showed that BMP-7, but not BMP-2, is expressed in mouse pancreas epithelium between E12.5 and E14.5 (Lyons et al., 1995). Similarly, TGF-β1 was strongly expressed in developing mouse pancreas epithelial cells and eventually in acinar cells (Crisera et al., 2000). Our RT-PCR data were broadly consistent with these results. Although activin was immunocytochemically detected in the E12.5 mouse pancreas epithelium and restricted to developing islets at E18.5 (Maldonado et al., 2000), systematic studies of the cellular localization of TGF-β superfamily members by immunocytochemistry are restricted by a lack of reliable antibodies. We showed that BMP-4, BMP-5 or BMP-6 promote and TGF-β1 or activin A inhibit colony formation, demonstrating that TGF-β superfamily members play an important role in pancreas cell lineage development.

The underlying mechanism by which these BMPs promote colony formation remains to be determined. Laminin-1 may concentrate BMPs on the cell surface for binding to their receptors, as reported for other ECMs (Taipale and Keski-Oja, 1997). Alternatively, our results might indicate some form of cross-communication between the laminin-1 and BMP receptor pathways in a similar manner to that observed for integrins and other growth factor signalling pathways in primary fibroblasts (Clark and Brugge, 1995; Moro et al., 1998). In fetal pancreas cells, we found that laminin-1 interacts with α_6 integrin to transduce a proliferation signal via the MAP

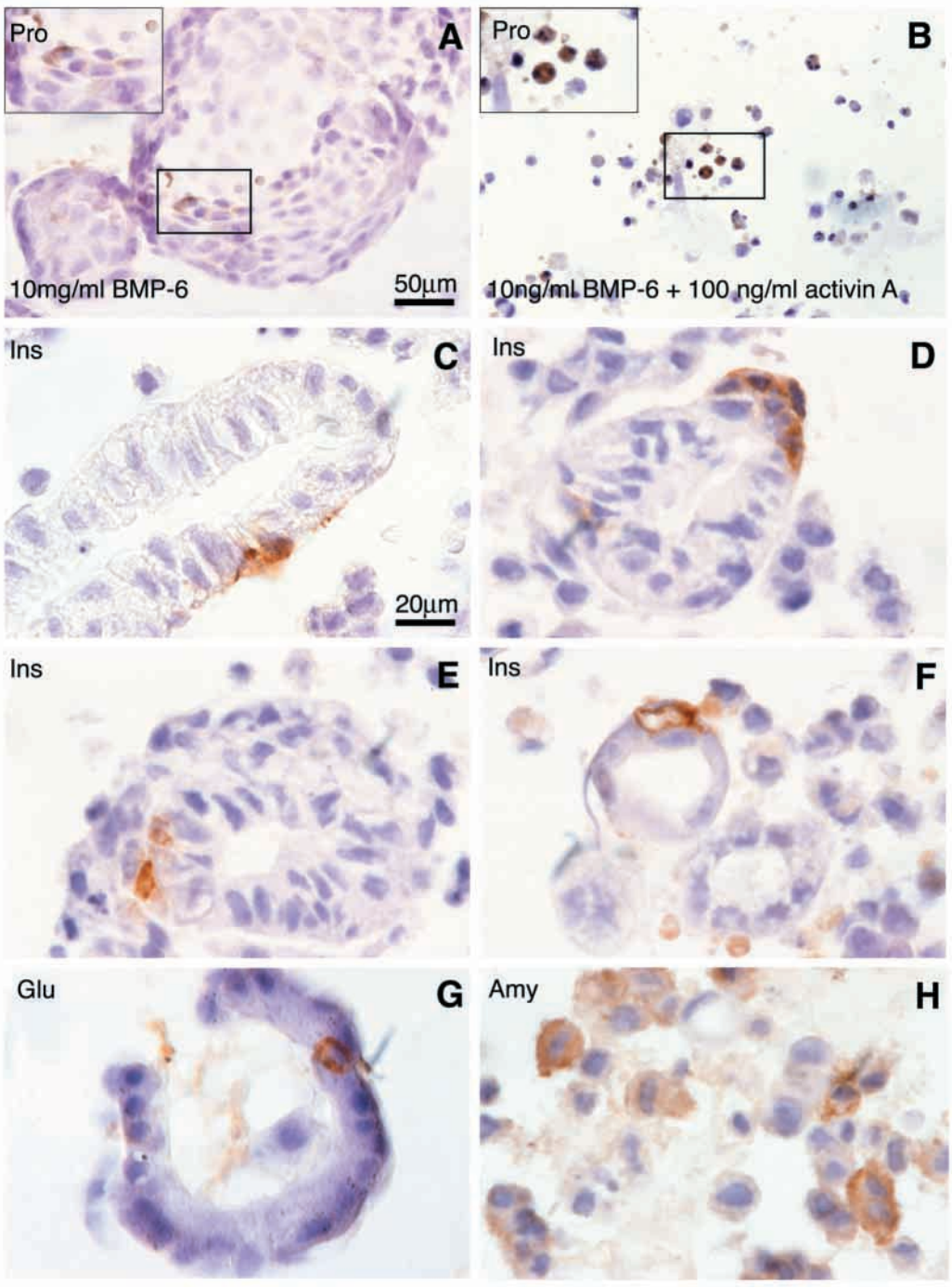

Fig. 7. Immunocytochemistry for proinsulin, insulin, glucagon and amylase in colony cells. The colony cells were directly fixed (for proinsulin only) or harvested for histological sections 6 days after culture. Proinsulin (Pro)-positive cells (also see inset) were stained with monoclonal anti-proinsulin antibody and peroxidase-conjugated rabbit anti-mouse immunoglobulins (A,B). Insulin (Ins)-positive cells were stained with guinea pig anti-insulin serum and visualised by peroxidase-conjugated rabbit anti-guinea pig immunoglobulins. Ins-positive cells constituted and in some cases appeared to bud off from colonies (C-F). Glucagon (Glu)-positive cells (G) and amylase (Amy)-positive cells (H) were stained with rabbit anti-glucagon and anti-α-amylase antibodies, respectively, and visualized with peroxidase-conjugated swine anti-rabbit immunoglobulins.

kinase pathway and with α-dystroglycan to transduce a survival/differentiation signal for insulin-positive cells (Jiang et al., 2001). It will be important to determine how signalling by members of the TGF-β superfamily intersects with these pathways. However, it can not be ruled out that another coordinating factor, secreted by non-epithelial cells, participates in promoting epithelial precursor cells to produce colonies, as reported for hepatic patterning from early foregut endoderm (Duncan and Watt, 2001; Rossi et al., 2001).

TGF-β1 and activin A antagonize BMP-induced colony formation

The ability of TGF-β1 and activin A to antagonize BMP-induced colony formation supports the notion that a balance between growth promotion and inhibition signals by TGF-β superfamily members fashions normal development of pancreas cell lineages. The ability of TGF-β1 and activin to oppose the effects of BMPs has also been noted in other cell systems. For example, BMP-7 stimulates, whereas TGF-β1 inhibits, differentiation of cultured fetal rat calvarial cells (Cheifetz et al., 1996). In the osteoblast-like cell line ROS17/2.8 and in primary rat calvarial cells, TGF-β1 stimulates, whereas BMP-7 inhibits, the expression of Smad4 (Li et al., 1998), a transduction molecule shared by the activin, BMP and TGF-β signalling pathways (Massague and Chen, 2000; Weinstein et al., 2000). An opposing effect of activin A on BMP-7-induced differentiation was also observed in human embryonal carcinoma cells (Piek et al., 1999). Our observation that activin A inhibits the formation of pancreatic cell colonies is interesting in light of the report by Yamaoka et al. that showed that pancreatic expression of a dominant-negative activin receptor type II resulted in islet hypoplasia (Yamaoka et al. 1998). This apparent discrepancy may simply reflect the complexity of the associations that occur between the various activin and BMP receptors. For example, complexes containing the activin receptor I and BMP receptor II or activin receptor II and BMP receptor I are also able to bind to BMP-2, -4 and -7 (Yamashita et al., 1995). Thus, dominant-negative activin receptors may not only block activin signalling, but also signalling by BMP-2, -4, -6 and -7 (Chang et al., 1997; Hemmati-Brivanlou and Thomsen, 1995; Schulte-Merker et al., 1994).

In summary, we demonstrate that specific BMPs promote growth and differentiation of fetal pancreas epithelial cells into cystic colonies containing insulin-positive β cells, an effect antagonised by two other members of the TGF-β superfamily, TGF-β1 and activin A. These findings may have implications for generating insulin-producing β cells in vitro for the treatment of type 1 diabetes.

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