Interplay of Glucagon-Like Peptide-1 and Transforming Growth Factor-β Signaling in Insulin-Positive Differentiation of AR42J Cells

Kok-Hooi Yew, Krishna L. Prasadan, Barry L. Preuett, Mark J. Hembree, Christopher R. McFall, Christina L. Benjes, Amanda R. Crowley, Susan L. Sharp, Zhixing Li, Sidhartha S. Tulachan, Sheilendra S. Mehta, and George K. Gittes

The differentiation of pancreatic exocrine AR42J cells into insulin-expressing endocrine cells has served as an important model for both endogenous in vivo β-cell differentiation as well as potential application to β-cell engineering of progenitor cells. Exogenous activin, possibly working through intracellular smad 2 and/or smad 3, as well as exogenous exendin-4 (a long-acting glucagon-like peptide-1 agonist) have both been shown to induce insulin-positive/endocrine differentiation in AR42J cells. In this study, we present evidence of significant interplay and interdependence of these two pathways as well as potential synergy between the pathways. In particular, insulin-positive differentiation seems to entail an exendin-4–induced drop in smad 2 and elevation in smad 3 in RNA levels. The latter appears to be dependent on endogenous transforming growth factor (TGF)-β isoform release by the AR42J cells and may serve as a mechanism to promote β-cell maturation. The drop in smad 2 may mediate early endocrine commitment. The coapplication of exogenous exendin-4 and, specifically, low-dose exogenous TGF-β1 led to a dramatic 20-fold increase in insulin mRNA levels, supporting a novel synergistic and codependent relationship between exendin-4 signaling and TGF-β isoform signaling. *Diabetes* 53:2824–2835, 2004

Mechanisms underlying the differentiation of pancreatic precursor cells into insulin-positive cells are of paramount importance toward our goal of engineering cells to become glucose-responsive β-cells for the curative therapy of diabetes. Over the last several years, a well-validated model of such differentiation has been developed using rat AR42J pancreatic epithelial cells (1–3). Early reports used either activin, or activin in combination with hepatocyte growth factor or β-cellulin, to generate either endocrine (activin alone) or insulin-positive (activin in combination) cell lines. Activins are members of the transforming growth factor (TGF)-β superfamily that form hetero- or homodimers and bind specific type I and type II TGF-β superfamily receptors (4). In most cases, activin ligand signaling induces phosphorylation and activation of smad 2 and/or smad 3 transcription factors to initiate downstream signaling (5). Based on the above initial observations of the effects of activin, subsequent AR42J studies showed a potential role for intracellular smad signaling in mediating these activin-induced pathways. Early findings were that smad 2 was necessary for insulin-positive differentiation, but forced overexpression of smad 2 in AR42J cells was not sufficient to induce insulin-positive differentiation (6). More recently, given the incretin (insulin release) and insulinotrophic (insulin cell growth) effects of glucagon-like peptide-1 (GLP-1) (7–15), either GLP-1 protein or a long-acting form of GLP-1 (exendin-4) was used to induce an endocrine phenotype in AR42J cells. Here, multiple islet cell types were seen, with expression of cell-specific markers and the acquisition of an endocrine morphology in culture (14).

Given the similar effect of these two different signaling pathways, TGF-β and GLP-1, we hypothesized that TGF-β superfamily/smad signaling may play an important role in mediating the GLP-1–induced effect. We first found that expression of several of the TGF-β isoforms, their receptors, and downstream smad molecules changed in response to exendin-4. Furthermore, the acquisition of a β-cell phenotype, including insulin mRNA and protein expression, islet amyloid polypeptide (IAPP) expression, and pdx-1 expression, were dependent on smad 2 and TGF-β isoform signaling. Changes in smad mRNA levels were accompanied by parallel changes in smad protein levels. Interestingly, important but distinct roles for smad 2 and smad 3 were also identified. These results imply a necessary and potentially important role for TGF-β superfamily signaling in mediating GLP-1 signaling and in GLP-1–induced insulin-positive differentiation. Also, a surprising synergistic effect of TGF-β isoform signaling on GLP-1–induced insulin-positive differentiation was seen.

RESEARCH DESIGN AND METHODS

Reagents and kits. Exendin-4 was obtained from Sigma-Aldrich (St. Louis, MO). RNeasy Mini Kit, Sensiscript Reverse Transcriptase Kit, and QIAquick Gel Extraction Kit were all from Qiagen (Valencia, CA). AmpliTaq Gold with GeneAmp 10× PCR buffer and $MgCl_2$ solution was from Applied Biosystems (Foster City, CA). F-12K Nutrient Mixture (Kaighn's modification) was from Gibco/Invitrogen (Carlsbad, CA).

From the Laboratory of Surgical Organogenesis, Children's Mercy Hospital, Kansas City, Missouri.

Address correspondence and reprint requests to George Gittes, MD, Children's Mercy Hospital, Lab. of Surgical Organogenesis, HHC620, 2401 Gillham Rd., Kansas City, MO 64108. E-mail: ggittes@cmh.edu.

Received for publication 22 June 2004 and accepted in revised form 30 July 2004.

BMP, bone morphogenic protein; GLP-1, glucagon-like peptide-1; IAPP, islet amyloid polypeptide; TGF, transforming growth factor.

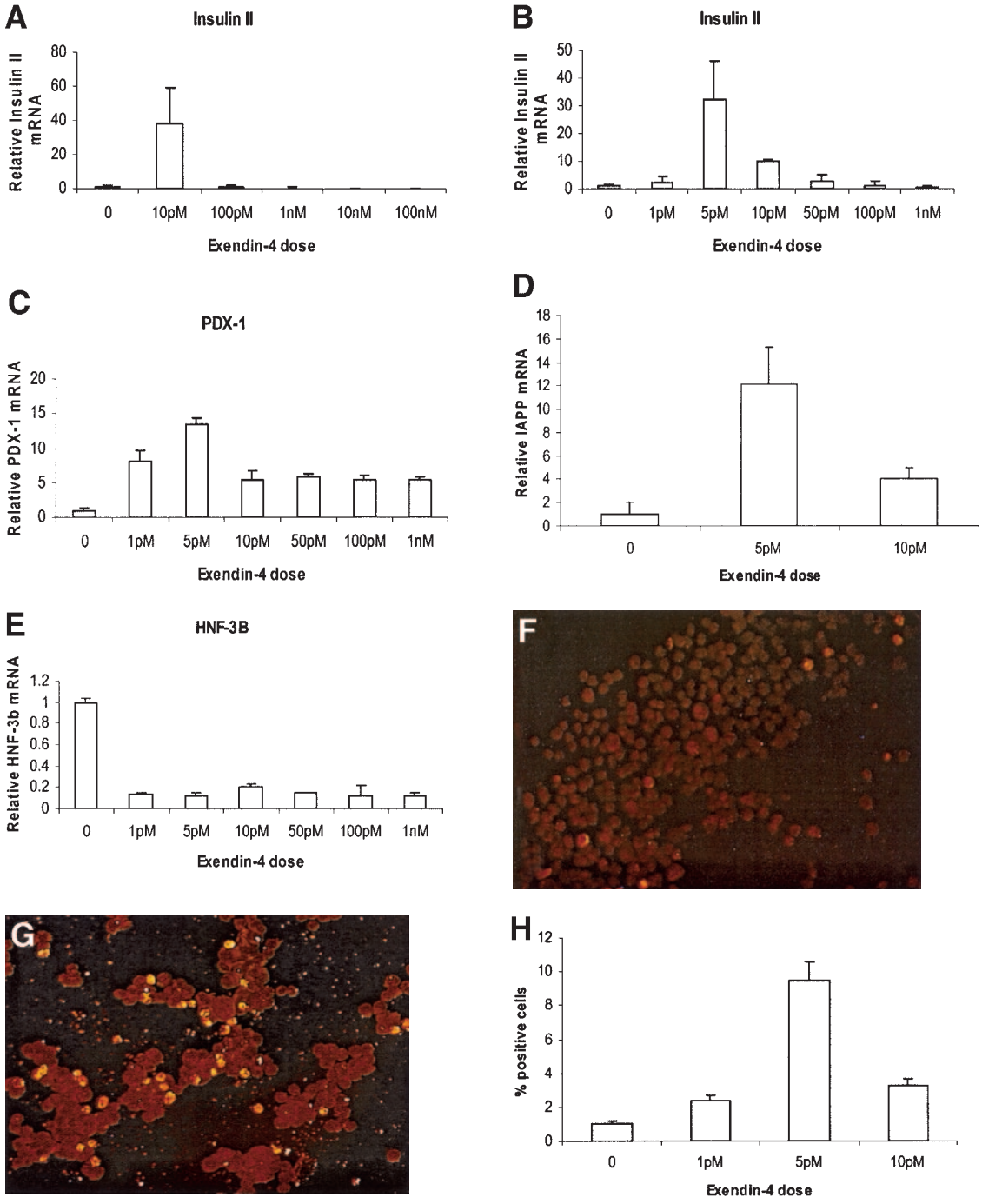

FIG. 1. Quantitative effects on β-cell marker transcription and insulin-positive staining. *A*: Broad-range exendin-4 dose-response shows a maximal effect in the 10-pmol range. *B*: A narrower window around the 10-pmol range of exendin-4 shows maximal insulin mRNA levels with 5 pmol exendin-4. *C* and *D*: Maximal pdx-1 and IAPP mRNA levels are also achieved with this same dose of exendin-4 (other doses of exendin-4 for IAPP not shown). *E*: HNF-3β as a marker of endoderm is not elevated in response to exendin-4 and is in fact markedly decreased, more like βtc cells. *F* and *G*: Immunostaining of AR42J cells with and without exendin-4 treatment for insulin shows marked upregulation of insulin-positive staining. *H*: Quantification of the immunocytochemistry in *F* and *G* reflects an increased number of positive cells and an increased percentage of positive cells.

Antibodies. Normal donkey serum and Rhodamine (tetrarhodamine isothiocyanate)-conjugated AffiniPure donkey anti-sheep IgG antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Sheep anti-human insulin (IgG fraction) was from the Binding Site (Birmingham, U.K.). In additional experiments, a pan-specific TGF-β neutralizing antibody (AB-100-NA; R&D Systems) was added at a final concentration of 30 μg/ml, which has been established by the manufacturer to inhibit 80% of TGF-β activity.

Morpholino oligos. Antisense morpholino oligos complementary to smad 2 (5′-CCCCGCCCACTCACCTTGGTATGGT-3′), smad 3 (5′CAAGCTGGTCACAT TCATAGCGGCT-3′), and missense control oligos were obtained from Gene Tools (Philomath, OR). Scrape delivery method was performed, and the scraped cells along with morpholino oligos were incubated over the weekend in a CO_2 incubator, which gives the best results in our system.

Cell culture and treatment. AR42J cells, purchased from American *Type Culture* Collection (Manassas, VA), were grown in Kaighn's modification of Ham's F12K medium with 2 mmol/l L-glutamine, 250 μg/ml Amphotericin, 100 units/ml penicillin, 100 μg/ml streptomycin, and 20% fetal bovine serum at 37°C under a humidified condition of 95% air and 5% CO_2. Cells were plated at a density of ~10^5 cells/ml in 12-well plates. Morpholino antisense or missense control was added separately to culture media at 20 μmol/l. Cells were then cultured with exendin-4 at doses of 1, 5, and 10 pmol/l for 3 days.

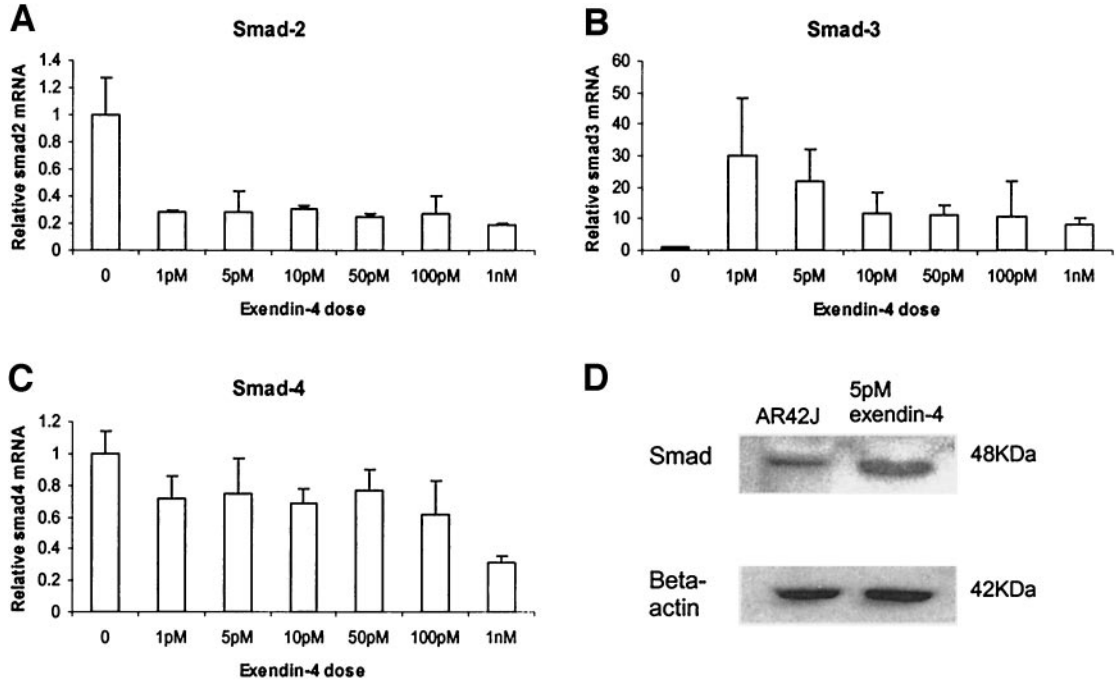

FIG. 2. TGF-β/activin smad response to exogenous exendin-4 in AR42J cells. *A*: Smad 2 levels are seen to decrease across the board in response to even the lowest doses of exendin-4. *B*: Smad 3 conversely shows marked elevation of mRNA levels in response to exendin-4, particularly at the lower doses. *C*: Smad 4, which serves as a common binding partner for smad 2 or 3 and can potentially serve as a rate-limiting factor, was not found to be significantly altered by exogenous exendin-4 up until very high levels. *D*: Western blotting confirms effect of exendin-4 on smad 3 levels in AR42J cells.

Immunocytochemistry. Cells cultured on noncoated glass coverslips (Fisher Scientific, Pittsburgh, PA) at a density of ~10^5 cells/ml were fixed with 4% phosphate-buffered paraformaldehyde for 15 min at room temperature. After fixation, the cells were permeabilized with 0.2% Triton X-100 (Sigma, St. Louis, MO) for 5 min at room temperature, blocked with normal donkey serum for 30 min, and then incubated with primary sheep anti-human insulin antibody in a moist chamber for 2 h at room temperature. The cells were rinsed with PBS and incubated with secondary rhodamine-conjugated donkey anti-sheep IgG for 2 h at room temperature in a dark cabinet. After several washes, coverslips containing cells were mounted onto slides in aqueous mounting medium with anti-fading agents (Biomeda, Foster City, CA). Fluorescence digital images were captured using an Olympus BX60 (Melville, NY) microscope attached with an Olympus U-PMTVC camera adaptor. Optronics DEI-750 (Goleta, CA) software was used to acquire and analyze images.

RT-PCR (nonquantitative). Total RNA was extracted from cells and treated with DNase. RNA was subjected to reverse transcription. cDNA was then amplified by PCR for 40 cycles (Table 1). All PCR products were separated by electrophoresis in 2% agarose gel. PCR cycles were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and final extension at 72°C for 10 min.

SYBR green real-time quantitative PCR. PCR amplifications were performed using a Bio-Rad iCycler (Hercules, CA) sequence detection system. Reactions were performed in a 50-μl reaction mixture including 10× Gold Buffer, 25 mm $MgCl_2$, 2.5 mmol/l dNTPs, 10× SYBR green, TaqGold polymerase, dH_2O, DNA template, and 10 μmol/l of each primer. Amplication was performed by initial polymerase activation for 10 min at 95°C, 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 20 s, and elongation for 30 s at 72°C.

Western blot analysis. Proteins were separated on a 10% Tris-Hcl Ready Gel (Bio-Rad, Hercules, CA), transferred onto nitrocellulose membranes, and incubated with either β-actin antibody (at a dilution of 1:5,000) or anti–smad 3 antibody (at 4 μg/ml) overnight at 4°C. After incubation, the membranes were washed twice for 15 min in washing buffer (PBS 0.05% Tween 20) and incubated with a secondary anti-mouse (β-actin) or anti-rabbit (smad 3) antibody coupled to horseradish peroxidase (Vector Labs, Burlingame, CA) for 1 h at room temperature. Then, the membranes were washed three times for 15 min in washing buffer, and immunoreactivity was normalized by chemiluminescence (ECL+Plus kit, RPN2132; Amersham) according to the manufacturer's instructions.

RESULTS

A narrow window of exendin-4 induces optimal insulin-positive differentiation. To expand on previous observations that the GLP-1 agonist exendin-4 was able to induce insulin-positive differentiation (14), we first performed a broad dose-response of GLP-1 in the AR42J cells

TABLE 1
List of PCR primer sequences

Genes	Left primer	Right primer
β-Actin	CTAAGGCCAACCGTGAAAAG	ACCCTCATAGATGGGCACAG
Insulin II	GGGAGCGTGGATTCTTCTAC	CAGTGCCAAGGTCTGAAGGT
Pdx-1	GAAATCCACCAAAGCTCACG	GAATTCCTTCTCCAGCTCCA
Pax4	CTCGAATTGCCCAGCTAAAG	CCCAAGGACTCGATTGATA
Pax6	TCAGCTTGGTGGTGTCTTTG	ACTCACACATCCGTTGGACA
Hnf-3β	AGGCTGAGTGGAAACATTGG	ATCAGTGTGGCCCAGCTATT
IAPP	ACATGTGCCACACAACGTCT	GCCACATTCCTCTTCCCATA
Smad 2	GCCCCAACTGTAACCAGAGA	GCCAGAAGAGCAGCAAATTC
Smad 3	GGCTTTGAGGCTGTCTACCA	GGTGCTGGTCACTGTCTGTC
Smad 4	TGGATGAAGTCCTGCACACC	AGTCCACCATCCTGGAAATG

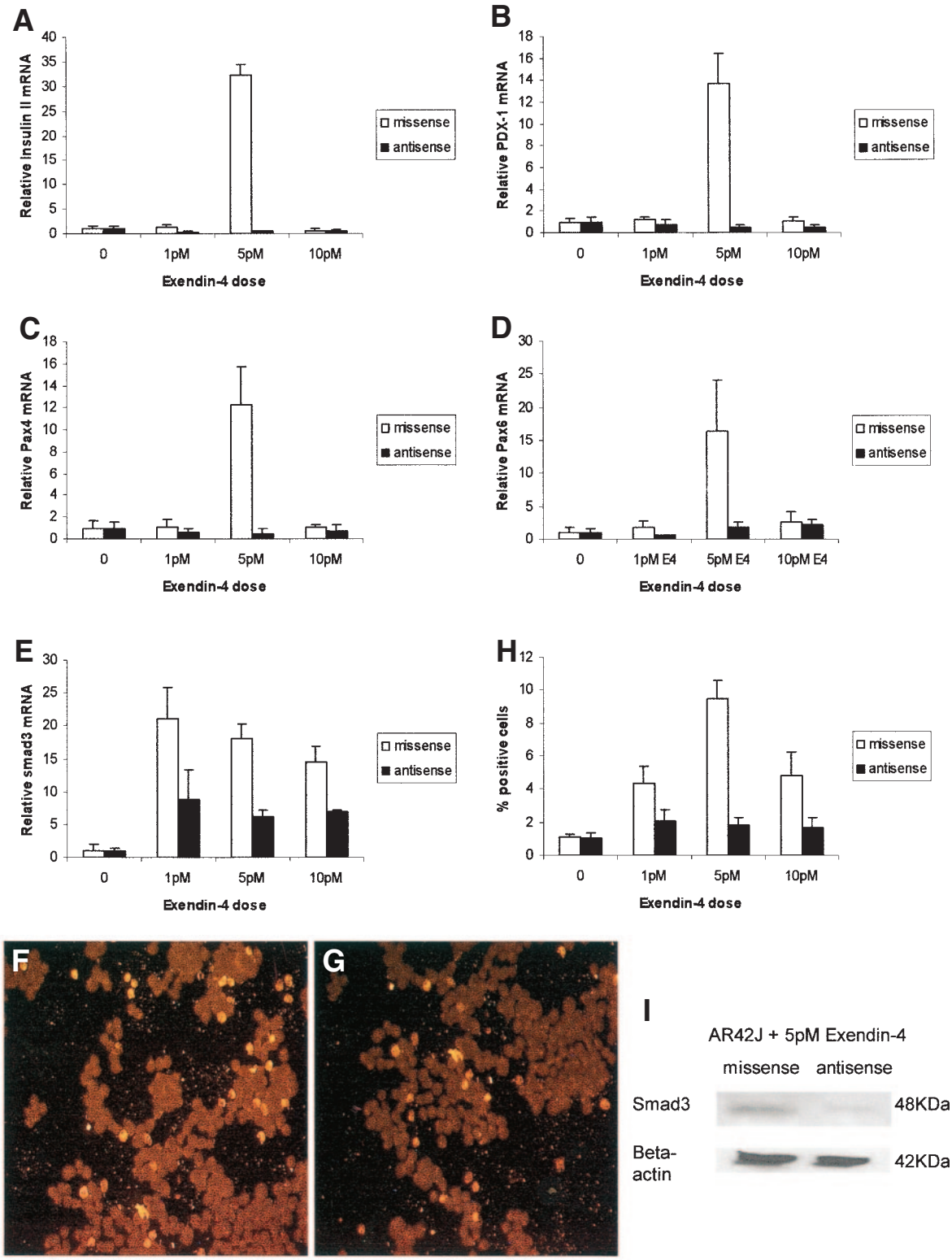

FIG. 3. Effect of smad 2 morpholino antisense on insulin-positive differentiation. *A*: The increase in insulin II mRNA levels in response to exendin-4 is completely ablated by the smad 2 antisense. *B–D*: Similar effects were seen on markers of β-cell and endocrine differentiation including pdx-1 (*B*), pax-4 (*C*), and pax-6 (*D*). *E*: Levels of smad 3 mRNA were inhibited by smad 2 antisense, suggesting a downstream role for smad 3 after smad 2 actions. *F–H*: Immunocytochemical analysis of insulin in AR42J cells through exendin-4 and smad 2 antisense. *F*: Missense treatment shows no obvious difference from exendin-4 treatment alone. *G*: An inhibition of the number of insulin-positive cells back to near- baseline levels, suggesting a critical role for smad 2 in insulin-positive differentiation. *H*: Quantitation of the percentage of positive cells in response to exendin-4 (number of positive cells shows similar results, data not shown). *I*: Western blot for smad 3 in exendin-4–treated cells with smad 3 antisense or missense.

and found that exendin-4 in the 1- to 10-pmol/l range gave maximal insulin II mRNA levels, as determined by semi-quantitative RT-PCR (Fig. 1*A*). Further dose-responses over a narrower range showed that 5 pmol/l specifically gave the highest levels of insulin II mRNA expression in our hands (Fig. 1*B*). To further analyze the nature of this

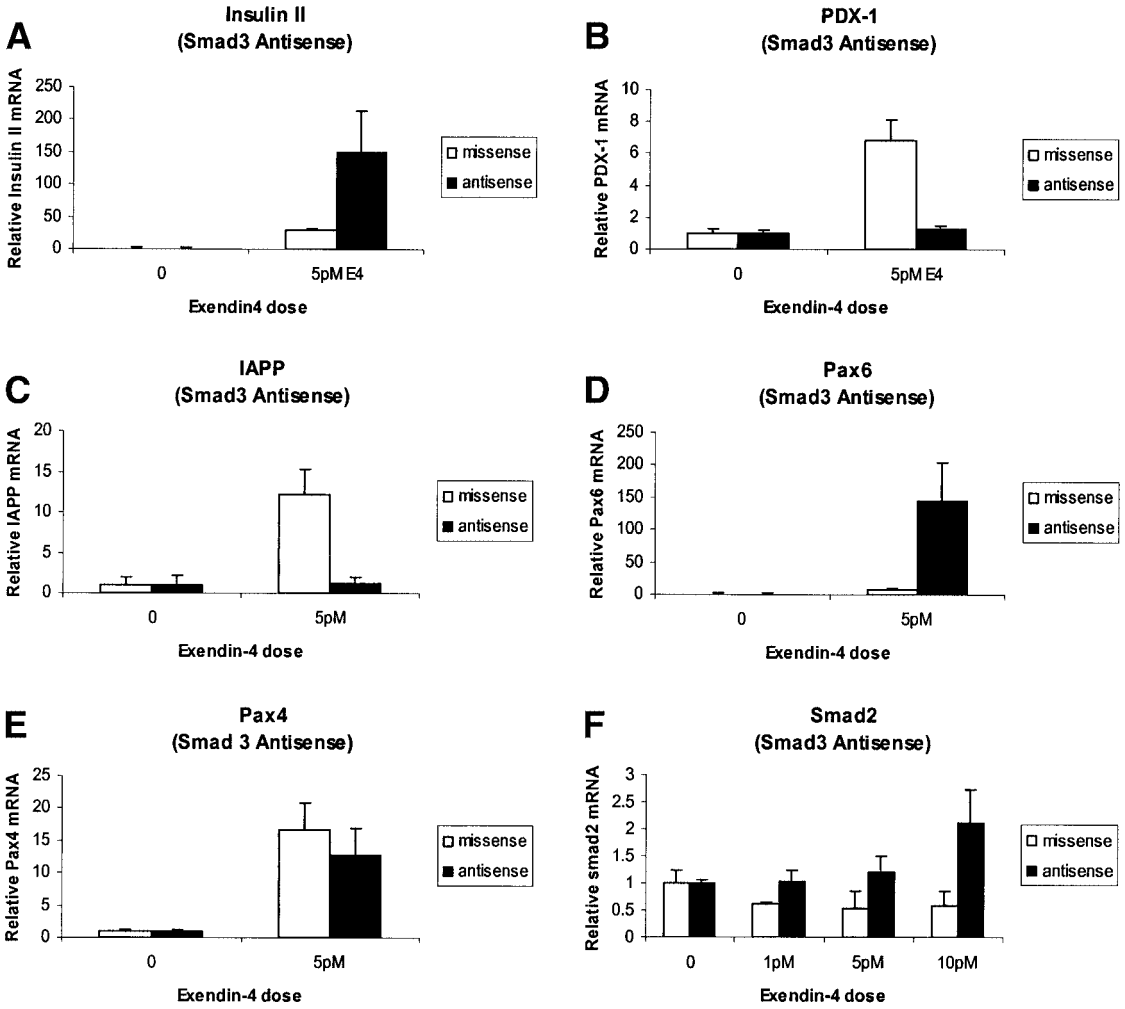

FIG. 4. Role of smad 3 in insulin-positive differentiation of AR42J cells in response to exendin-4. *A*: Smad 3 morpholino antisense treatment dramatically enhances levels of insulin II mRNA. *B*: Unlike previous elevations of insulin II mRNA, smad 3 morpholino-induced insulin II mRNA elevation is not accompanied by elevations in pdx-1 mRNA and in fact is accompanied by a marked suppression of pdx-1 mRNA levels back to baseline. *C*: Similar results were obtained for IAPP, another marker of mature β-cells like pdx-1. *D*: Pax-6, a purported marker of immature endocrine cells, is markedly elevated by the smad 3 morpholino antisense, suggesting augmentation of a precursor population of relatively immature β-cells. *E*: No effect on pax-4 was seen. *F*: Modest-to-minimal increases in smad 2 mRNA levels were seen in response to smad 3 morpholino, suggesting that smad 2 effects of the smad 3 morpholino are unlikely to be critical. *G–I*: Quantitative immunocytochemistry for insulin in AR42J cells treated with exendin-4 and either missense (*G*) or antisense (*H*) for smad 3. *I*: Similar to the mRNA levels, the number of insulin-positive cells and the percentage of insulin-positive cells (data not shown) were increased significantly.

effect, we studied levels of pdx-1 mRNA, a transcription factor that is expressed at low levels in early pancreatic progenitor epithelial cells but at high levels in differentiated β-cells. Like insulin, pdx-1 was found to have the highest expression with the same doses of exendin-4, implying a similarly affected population of cells and implying that the insulin-positive cells may be becoming mature β-cells (Fig. 1*C*). Similarly, IAPP mRNA also was maximal at this dose (Fig. 1*D*), again indicating mature β-cell differentiation. However, HNF-3β mRNA, a marker of endoderm, was suppressed by exendin-4, suggesting that HNF-3β enhancement is neither necessary nor responsible for the increased pdx-1 (though the pdx-1 gene has an HNF-3β responsive element) (Fig. 1*E*). Furthermore, immunocytochemistry for insulin revealed that only a few untreated AR42J cells were insulin positive, whereas many insulin-positive cells were seen after exendin-4 treatment of the AR42J cells (Fig. 1*F–H*).

Opposing effects of activin/TGF-β receptor-specific smads. In light of the known role for activin in inducing an endocrine phenotype in AR42J cells (1,2,16), we next determine whether the activin/TGF-β isoform-specific smads (smad 2 and 3) may mediate the effect of exendin-4. We first analyzed levels of smad mRNAs. Interestingly, smad 2 mRNA was found to be decreased significantly upon stimulation of AR42J cells with exendin-4 at all doses tested (Fig. 2*A*). Conversely, smad 3 was found to be strongly upregulated after exendin-4 treatment (Fig. 2*B*). To determine whether levels of these two smads may be secondarily dependent on levels of smad 4, which serves as a common binding partner for smad 2 or 3 and can potentially serve as a rate-limiting factor, we found minimal change of smad 4 levels with or without exendin-4 treatment (Fig. 2*C*). The changes in the levels of these receptor-specific smads (2 and 3) may be a key point of regulation. Similarly, by Western blotting, levels of the proteins were found to correlate with the mRNA.

Smad 2 and 3 sequentially regulate exendin-4–mediated insulin-positive differentiation. To determine whether the observed changes in smad 2 and 3 levels

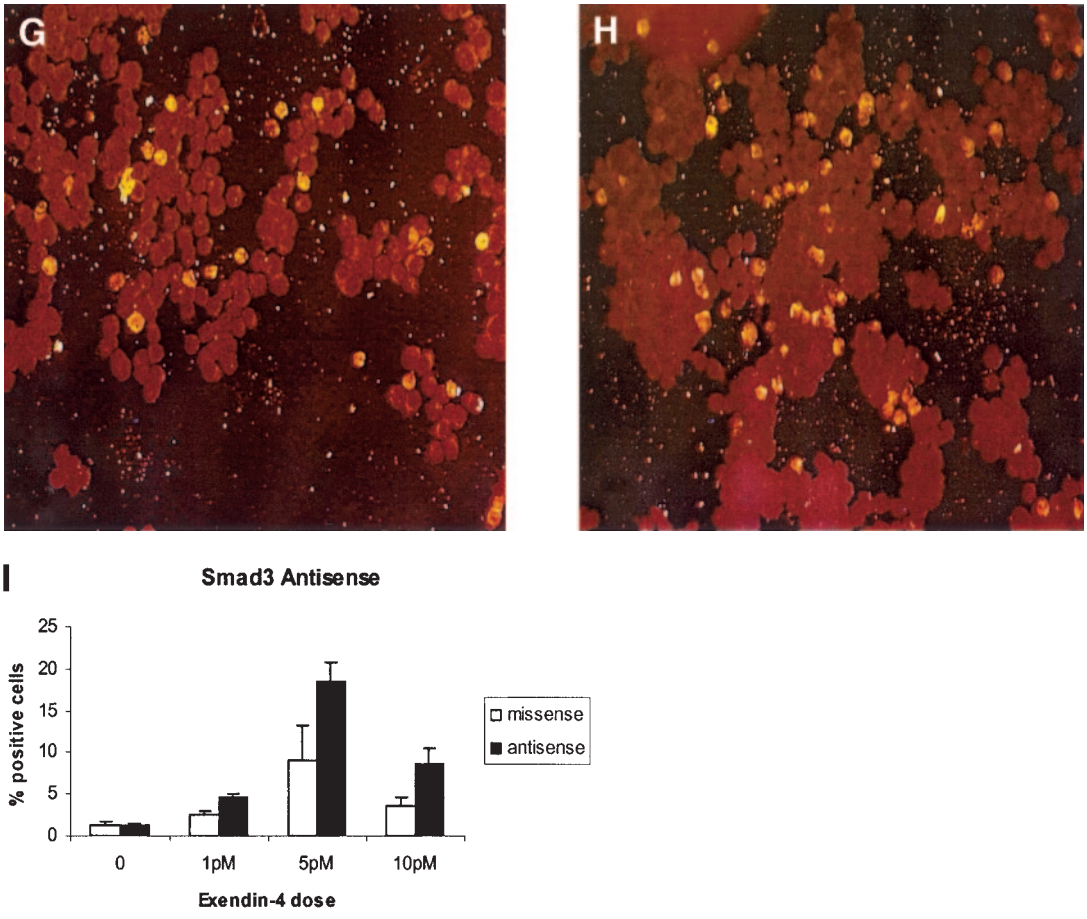

FIG. 4. Continued.

mediate the insulin-positive differentiation seen with exendin-4 treatment, we used a morpholine ring antisense approach to inhibit production of smad 2 and/or smad 3 protein in the AR42J cells. In the presence of smad 2 antisense, no increase in insulin II mRNA or pdx-1 mRNA was seen, suggesting that smad 2 is necessary for β-cell differentiation (Fig. 3*A* and *B*). In an effort to better define the differentiation state of the endocrine cells that were present, levels of pax 4 and pax 6 mRNA were assayed. Levels of both of these mRNAs were reduced to baseline by smad 2 antisense (Fig. 3*C* and *D*), suggesting a complete absence of endocrine cells. In addition, smad 3 mRNA levels were decreased as a result of smad 2 inhibition, suggesting that smad 2 in AR42J cells may be necessary both for insulin-positive differentiation as well as for full smad 3 upregulation (Fig. 3*E*). Immunocytochemistry confirmed the decrease in insulin, though the number of positive cells was not as dramatically decreased as the insulin II mRNA levels, suggesting that the number of mRNA molecules per insulin-positive cell was inhibited in addition to the decreased number of insulin-positive cells (Fig. 3*F–H*). To confirm the inhibiting effects of the morpholino ring antisense on smad levels, Western blotting was performed in morpholino ring antisense-treated and sense-treated controls, confirming sequence-specific decreases in target smads by antisense (Fig. 3*I*).

When smad 3 antisense was used instead of smad 2, there was a surprising fivefold increase in insulin II mRNA levels (Fig. 4*A*) and a doubling of the number of insulin-positive cells by immunocytochemistry (Fig. 4*G–I*). Unlike the previous elevations of insulin II mRNA, however, there was a decrease in pdx-1 and IAPP mRNA levels (Fig. 4*B* and *C*), implying that the elevated levels of insulin expression in the absence of smad 3 could represent an immature, possibly proliferative β-cell progenitor. In addition, levels of nestin mRNA expression were also elevated (data not shown), suggesting that these smad 3 antisense-treated cells may be frozen in a predifferentiated state, with low levels of IAPP and pdx-1. Pax6 mRNA showed a 10- to 15-fold increase (Fig. 4*D*), again suggesting amplification of a precursor population of relatively immature β-cells. Minimal effects were seen on pax4 mRNA levels (Fig. 4*E*). Together, these results suggest that smad 3 antisense induced an increased number of endocrine precursor cells. Smad 2 mRNA levels were only modestly increased (Fig. 4*F*). Immunocytochemistry correlated with the RT-PCR results, except to a less dramatic degree, suggesting that either the number of insulin mRNA molecules per cell was lower or that there were numerous insulin-positive cells below the threshold of detection of immunocytochemistry.

Given the opposing effects of smad 2 or 3 antisense inhibition, we then blocked both smad 2 and 3 simultaneously, using double antisense treatment of AR42J cells (Fig. 5). Here, the results were essentially identical to those obtained with smad 2 antisense alone. Thus, it appears that smad 3 effects are probably downstream or dependent on smad 2 effects. It also suggests that smad 2 may initially be

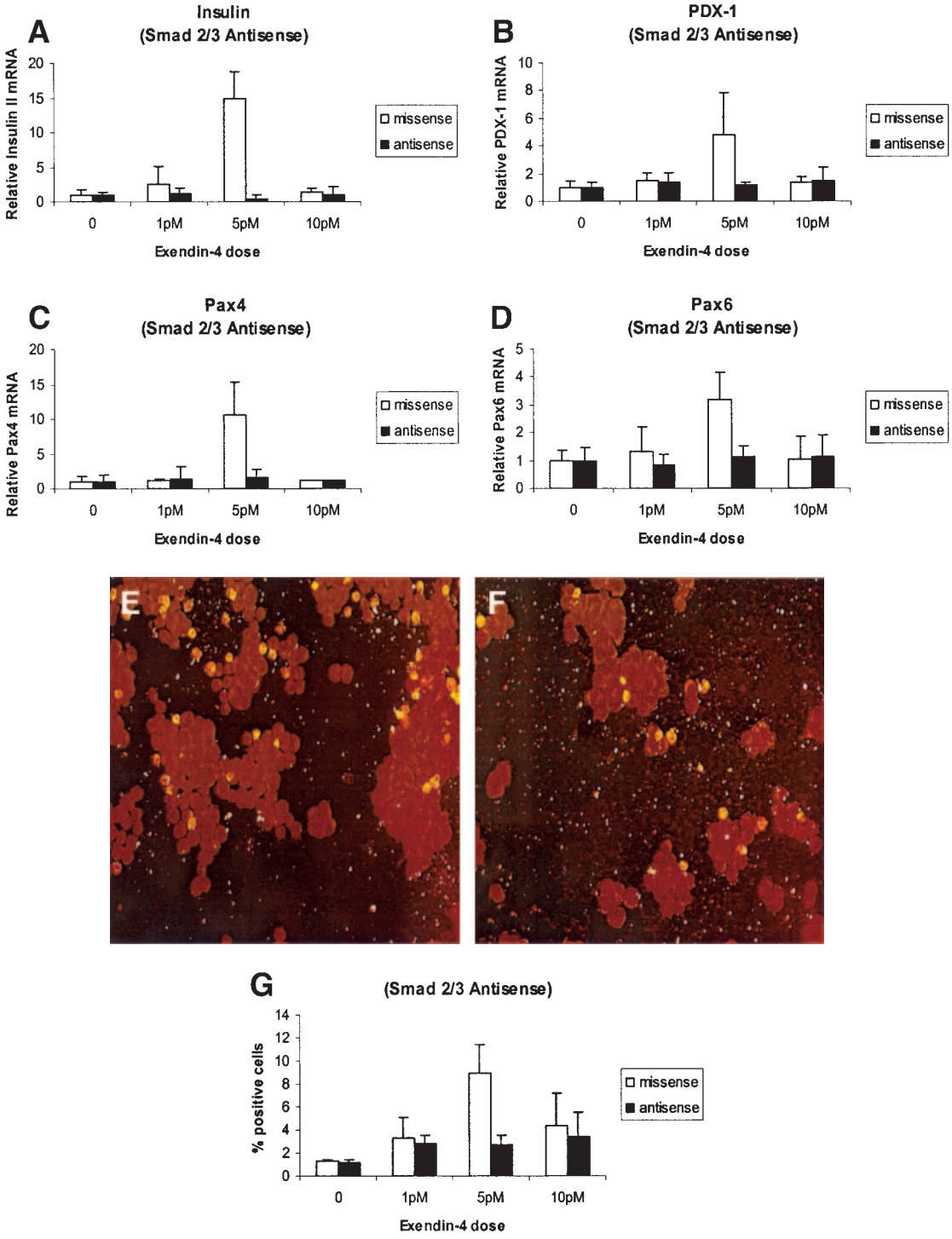

FIG. 5. Insulin-positive differentiation response to smad 2 and 3 double antisense. *A*: Insulin II mRNA response to exendin-4 is blocked by smad 2 and 3 double antisense, consistent with the results seen for smad 2 alone and opposite the results seen with smad 3 alone, suggesting that smad 2 is working upstream of smad 3 effects. *B–D*: A similar suppression is seen for pdx-1 (*B*), pax-4 (*C*), and pax-6 (*D*). *E–G*: Immunohistochemical staining shows insulin-positive cells in response to the double antisense (*F*) compared with controls (*E*), and these results are supported by quantitative cell counting (*G*).

necessary for elevation of insulin levels and then subsequently may lower insulin levels, possibly as part of β-cell maturation.

Role of TGF-β superfamily ligand receptor signaling in exendin-4–induced β-cell differentiation. Given the apparent necessity for smad 2 and 3 in properly regulated, exendin-4–induced insulin-positive differentiation in AR42J cells, we presumed that activin and/or TGF-β isoform signaling may be playing an important role in mediating the exendin-4–induced effects. Thus, we performed a screen for differentially expressed TGF-β isoform ligands and activin ligands, as well as potential type I receptors for those ligands. The screen was performed in AR42J cells, with and without exendin-4 treatment (Fig. 6). From this

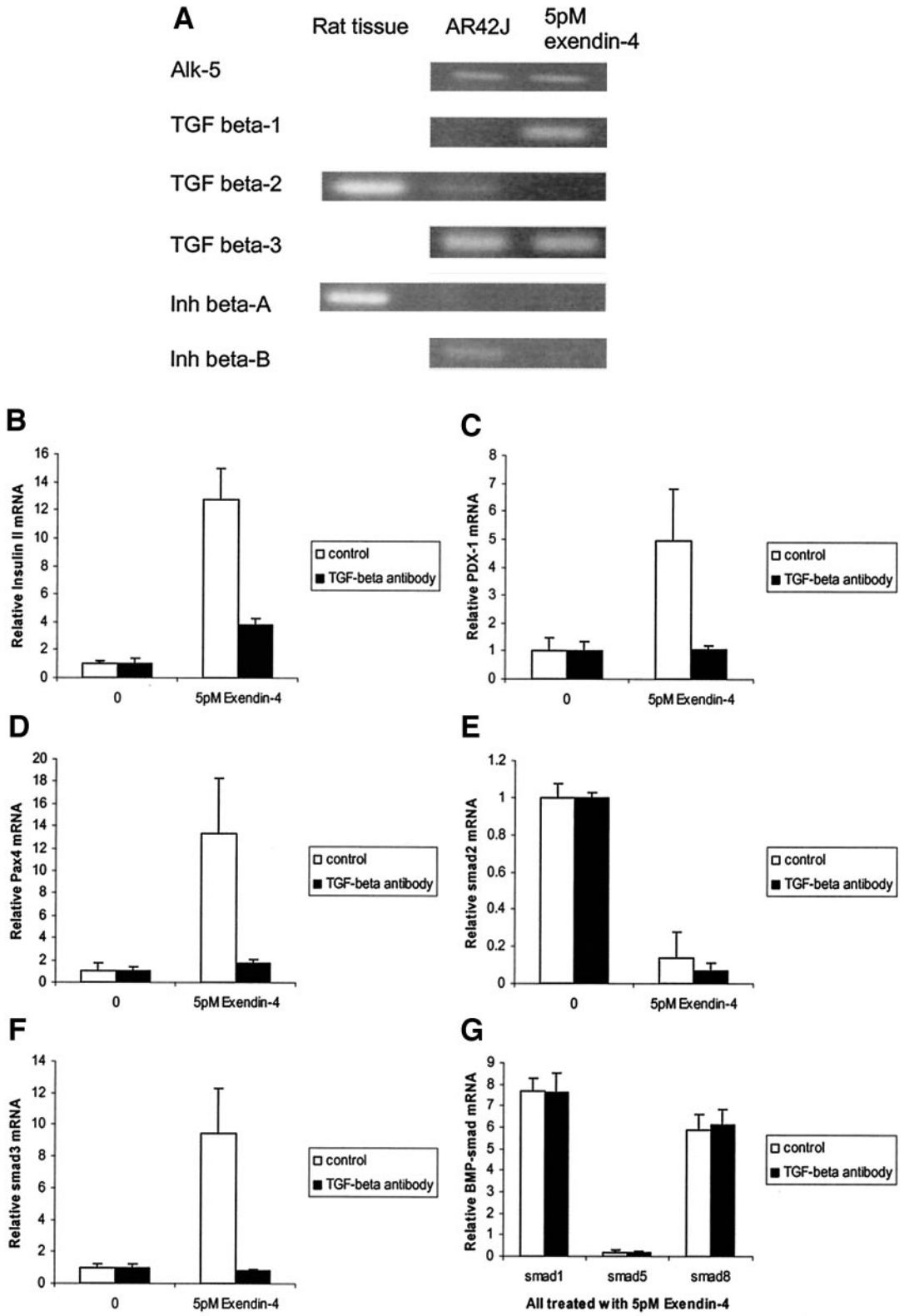

FIG. 6. *A*: **Screening for activin and TGF-β ligands and potential type I receptors. Simple PCR was performed to look for activin ligands (inhibin A and B). Neither was present in exendin-4–stimulated cells, and inhibin B monomer was only weakly present in the baseline AR42J cells. TGF-β1 and 3 were clearly present in exendin-4–stimulated AR42J cells, and TGF-β3 was present in unstimulated AR42J cells. TGF-β2 was minimally present in AR42J cells and not present in exendin-treated cells. Here the type I receptor for TGF-β (alk5) was present in both unstimulated and exendin-4–stimulated AR42J cells. For inhibin A and TGF-β2, because of the weak band in the AR42J cells, positive control rat tissues (foregut-derived tissues) were also used.** *B*: **Insulin II mRNA levels are markedly reduced in the presence of TGF-β pan-neutralizing antibodies (controls included no antibody as shown in the figure and HNF-3β irrelevant antibody, data not shown).** *C* **and** *D*: **Similar effects were seen on pdx-1 and pax-4 mRNA, suggesting that TGF-β isoform signaling was necessary for mature β-cell formation.** *E* **and** *F*: **The effects of TGF-β neutralizing antibody on smad 2 and 3 are shown. Smad 2 levels interestingly were not affected in the untreated cells, but importantly the smad 3 elevation normally seen with exendin treatment was blocked by the TGF-β pan-neutralizing antibodies (*F*).** *G*: **As a control, other receptor-specific smads (BMP smad 1, 5, and 8) were analyzed for a response to TGF-β antibody. The mRNA levels of all of these remained stable with exendin-4 treatment in the presence of the TGF-β pan-neutralizing antibody.**

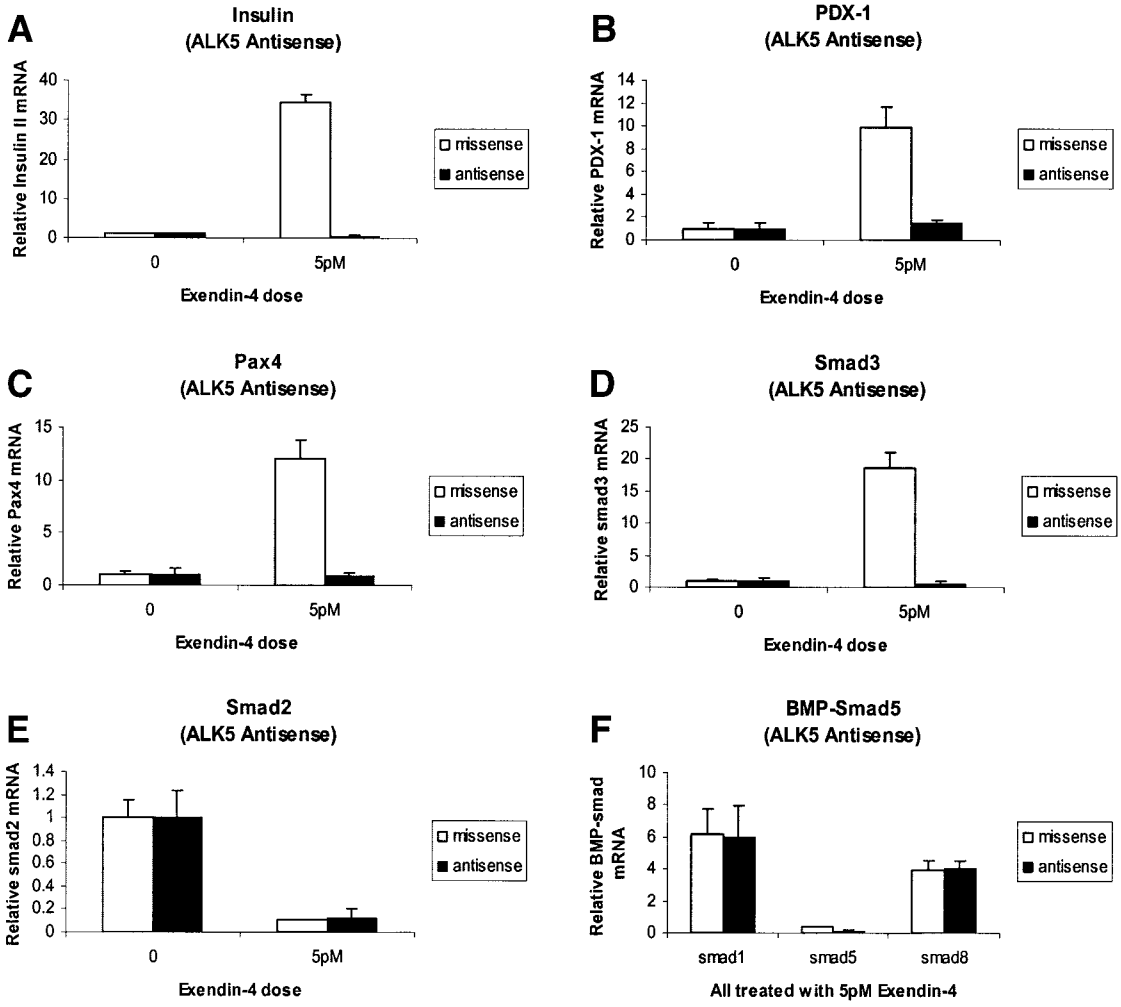

FIG. 7. Role for TGF-β type I receptor alk5 in mediating exendin-4–induced insulin differentiation. *A*: Insulin II mRNA elevations in response to exendin-4 are completely abolished by alk5 morpholino antisense. *B* and *C*: Similar effects are seen on pdx-1 and pax-4 mRNA. *D*: Smad 3 mRNA levels are suppressed in the alk5 antisense-treated cells, suggesting that alk5 is the responsible type I receptor for inducing smad 3 transcription. *E*: No effect on baseline smad 2 is seen with alk5 treatment or with exendin-4 treatment, suggesting that smad 2 may be not critically regulated by alk5 antisense. *F*: Except possibly for smad 5, the BMP-specific smads do not appear to be greatly affected by alk5 antisense in the presence of exendin-4.

screen, it appeared that the activin monomers (inhibin A and B) were minimally, if at all, expressed in exendin-treated AR42J cells. TGF-β1 and -β2, however, were expressed, and TGF-β1 was upregulated with exendin-4 treatment. (It was essentially not expressed in untreated AR42J cells.) In addition, the TGF-β type I receptor (alk5), which phosphorylates and activates both smad 2 and 3, was clearly expressed in AR42J cells, either with or without exendin-4 treatment. Activin-binding type I receptors were present (data not shown), but TGF-β isoforms do not typically bind to activin type I receptors (4).

Therefore, we next tested the possibility that TGF-β isoform ligands were mediating the exendin-4–induced insulin-positive differentiation. A specific, well-characterized TGF-β pan-neutralizing antibody was able to almost completely abolish exendin-4–induced insulin-positive differentiation (Fig. 6*B*–*G*), as measured by insulin II mRNA, pdx-1 mRNA, and pax4 mRNA, suggesting that TGF-β isoform signaling was necessary for mature β-cell formation. Interestingly, both smad 2 and 3 mRNA levels were decreased, suggesting that exendin-4 inhibits smad 2 independent of TGF-β isoform signaling but that the secondary elevation in smad 3 levels seen with exendin-4 treatment, as well as the insulin-positive response, are TGF-β isoform dependent. Bone morphogenic protein (BMP) smads (smad 1, 5, and 8) were unaffected in the TGF-β pan-neutralizing antibody-treated cells. An irrelevant antibody against the transcription factor HNF-3β (data not shown) had no effect on any of these parameters.

In an effort to both confirm the specific role of TGF-β isoform signaling and better delineate the overall signaling mechanisms, we used antisense against the TGF-β type I receptor (alk5). The alk5 antisense had a dramatic inhibitory effect on exendin-4 induction of insulin, pdx-1, and pax4 mRNA (Fig. 7). Alk5 antisense specifically blocked the upregulation of smad 3, suggesting that alk5 is a type I receptor necessary for inducing smad 3 transcription. No apparent effect on the inhibition of smad 2 was seen, suggesting that smad 2 may not be critically regulated by alk5.

Synergistic effect of exogenous TGF-β and exendin-4 treatment. To determine whether the TGF-β isoforms may play a synergistic role with exendin-4, and perhaps with GLP-1, in the induction of insulin-positive differentia-

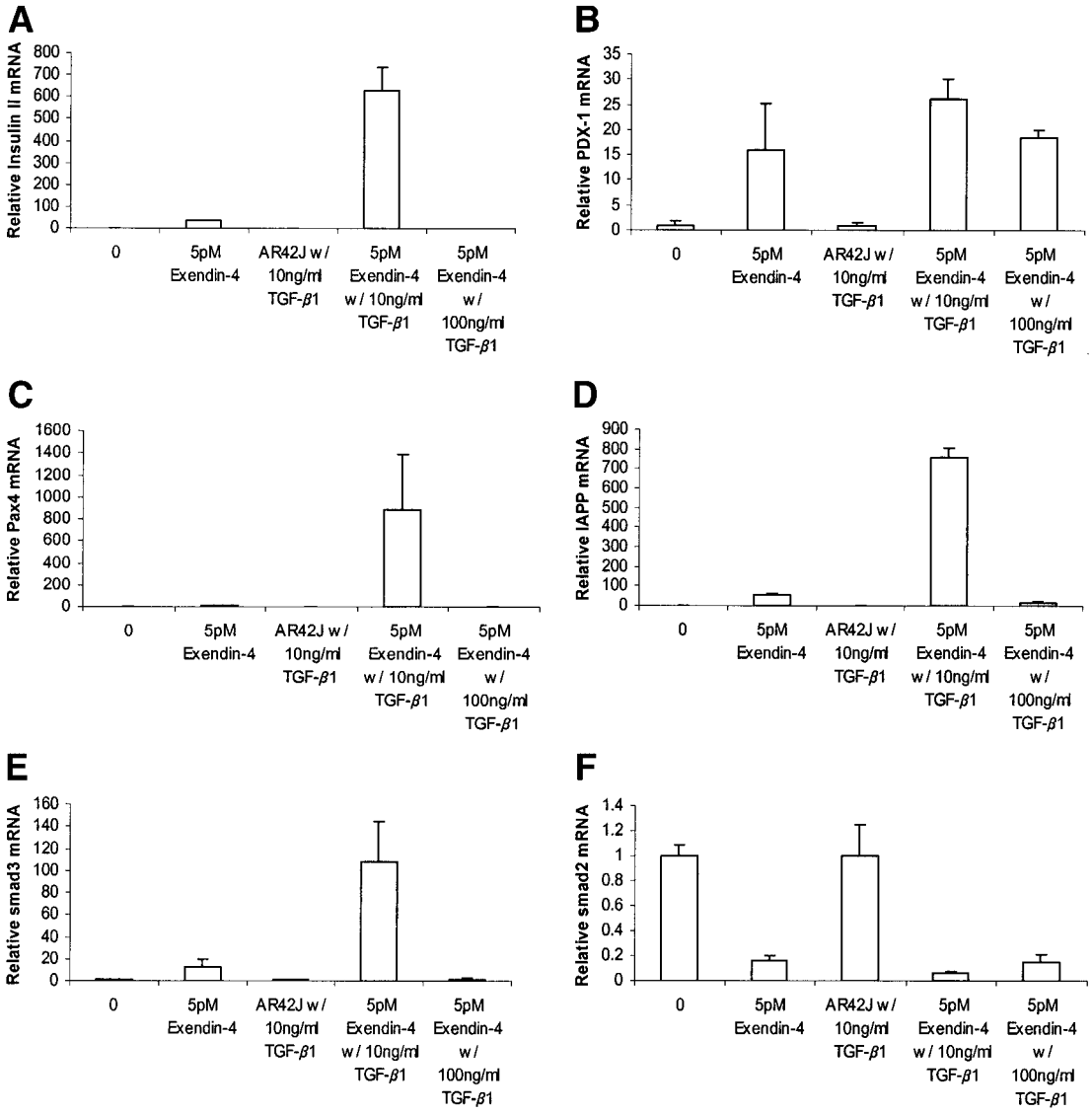

FIG. 8. Synergistic effects of exogenous exendin-4 and TGF-β1 on insulin-positive differentiation. *A*: Insulin II mRNA levels are dramatically increased by the combination of low-dose (10 ng/ml) TGF-β1 and 5 pmol exendin-4. Here the scale has been reduced such that the elevation of insulin II mRNA in response to 5 pmol exendin-4 alone does not appear to be as high, but the increase was similar to that in previous experiments. The TGF-β1 synergistically further increased the insulin II mRNA levels by 20-fold. *B–D*: Synergy of low-dose TGF-β1 with exendin-4 was not seen for pdx-1 (*B*) but was seen for pax-4 (*C*) and IAPP (*D*). As in *A*, elevation of mRNA levels after picomoler exendin treatment alone were similar to previous studies, but the scale of the graph had to be reduced due to the high elevation in the synergistic treatment. *E*: Smad 3 showed a similar synergistic effect, again suggesting that the mechanism by which TGF-β1 is augmenting insulin-positive differentiation is through smad 3. *F*: Exogenous TGF-β1 appears to have no augmenting effect on smad 2 levels, consistent with the suppression of smad 2 seen after exendin-4 treatment alone.

tion and insulin expression, exogenous TGF-β1 was added to AR42J cells with and without exendin-4 treatment (Fig. 8). Here there was a dramatic increase in insulin mRNA levels when 10 ng/ml TGF-β1 ligand was added with 5 pmol/l exendin-4. TGF-β1 synergistically increased the insulin II mRNA levels by 10- to 15-fold over exendin-4–induced insulin alone. Pax4, IAPP, and pdx-1 were similarly elevated. TGF-β1 ligand alone essentially had no proinsulin effect on the AR42J cells, and higher doses of TGF-β1 ligand (100 ng/ml) with exendin-4 were inhibitory. Smad 3 levels were elevated with 10 ng/ml TGF-β1 treatment in the exendin-treated AR42J cells, suggesting that smad 3 may be a key mediator in regulating the maturation of insulin-positive cells.

DISCUSSION

Understanding the mechanisms by which precursor cells can be induced to differentiate into insulin-positive β-cells is critical if we are to successfully engineer cells for the treatment of diabetes. Over the last few years, two extracellular signaling pathways have been shown to play a role in the induction of insulin-positive differentiation in several cell systems. GLP-1–induced insulin-positive differentiation in certain transformed cell lines (7–15) and some TGF-β superfamily signaling molecules have been found to control endocrine and insulin-positive differentiation in some pancreatic cell lines and tissues (7–15,17,18). Of particular interest, both GLP-1 and a combination of activin and either HGF or β-cellulin have the capacity to induce the pancreatic exocrine cell line, AR42J, to differentiate

into insulin-positive endocrine cells (7–15). Here we found that there is an interaction of the two signaling pathways to induce insulin-positive differentiation. The interaction was demonstrated by the fact that inhibition of TGF-β signaling at any of several levels blocked GLP-1–induced insulin-positive differentiation, whereas TGF-β ligands alone were unable to induce insulin-positive differentiation in the absence of exendin stimulation. Further interactions were implied by the synergistic effect of low-dose exogenous TGF-β ligands with exendin-4.

The canonical TGF-β signaling pathway entails phosphorylation of receptor-specific smads and subsequent translocation to the nucleus of a smad heteromeric complex that directly activates gene expression (4,5). However, several studies have documented complex intracellular interactions of other pathways on smad signaling pathways. In particular, the G-protein signaling pathways (of which GLP-1 receptor is included) have been shown to significantly interact with smad signaling (19). Also, notch signaling (20) and the JAK/STAT pathway (21) have significant effects on smad pathways. In many cases, these pathways can act synergistically with the smads, as may be occurring here in AR42J cells.

We found that smad 2 expression was necessary for exendin-4–induced insulin-positive differentiation but also that smad 2 expression was strongly downregulated with exendin-4. The necessity for smad 2 is consistent with earlier findings by Zhang et al. (6) that smad 2 was necessary for the endocrine commitment of AR42J cells induced by activin alone. Therefore, it seems plausible that while smad 2 expression is necessary for endocrine lineage commitment, further differentiation requires downregulation of smad 2. Zhang et al. found that with activin stimulation alone, there was persistent expression and accumulation of phosphorylated smad 2, resulting in apoptosis.

Smad 2 and 3 are well known to act competitively in several differentiation systems (22–24). We found that smad 3 antisense resulted in a greatly increased number of insulin-positive cells and in increased insulin mRNA but without a concomitant increase in expression of β-cell–specific markers, such as IAPP. Based on these data, it appears that smad 3 may support maturation of β-cells and inhibition of proliferation but only after smad 2 has initiated a proendocrine differentiation program. This possibility is supported by the fact that the smad 3 antisense had no effect if cells were also treated with smad 2 antisense, suggesting that smad 2–mediated effects are a necessary preparation for smad 3 actions. A decrease in smad 2 levels in the absence of any change in smad 4 may be necessary to allow smad 3 binding to smad 4. Also, it appears that the drop in smad 2 levels may be directly related to GLP-1 signaling, since the drop occurred with exendin-4 treatment alone, even in the presence of TGF-β neutralizing antibodies. The mechanism by which exendin-4 may lead to smad 2 downregulation is unclear.

One logical area of interplay between the GLP-1 and TGF-β signaling systems may be the induction of TGF-β expression and secretion by AR42J cells to act by either autocrine or paracrine pathways to initiate secondary aspects of insulin-positive differentiation. This role for TGF-β would be consistent with known effects of TGF-β in cultured embryonic pancreas (17). It seems unlikely, however, that GLP-1 plays an important role in the normal endogenous differentiation of early endocrine cells because GLP-1 is not expressed significantly in the embryonic pancreas until later in gestation (10).

The observed synergistic effect of low-dose TGF-β ligands with exendin-4 is of interest, not only with respect to potential cellular engineering applications but also because of implications regarding mechanisms of differentiation. The combination of TGF-β ligand and exendin-4 gave low smad 2 and high smad 3 levels as well as high insulin and high pdx-1, suggesting the presence of mature β-cells. Smad 3 antisense, however, gave high levels of insulin but not pdx-1. From these data, we conclude that smad 3 plays a role in the maturation of β-cells with the acquisition of pdx-1 expression. GLP-1 appears to be necessary for the high levels of insulin, but the intracellular molecules downstream of TGF-β signaling necessary for this synergistic action remain to be determined.

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