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Dynamic Signaling Network for the Specification of Embryonic Pancreas and Liver Progenitors*

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Abstract

Studies of the formation of pancreas and liver progenitors have focused on individual inductive signals and cellular responses. Here we investigated how BMP, TGF β , and FGF signaling pathways converge on the earliest genes that elicit pancreas and liver induction in mammalian embryos. The inductive network was found to be dynamic, changing within hours, and different signals functioned in parallel to induce different early genes. Two permutations of signals induced liver progenitor domains, revealing flexibility in cell programming. Also, the specification of pancreas and liver progenitors was restricted by the TGF β pathway. These results findings may enhance progenitor cell specification from stem cells for biomedical purposes and explain incomplete programming in stem cell differentiation protocols.

Presently there are limitations in the ability to program stem cells in vitro, in that the differentiated cells lack the full spectrum of properties normally exhibited by their in vivo counterparts (1,2). One reason for the limitation could be a lack of understanding of how inductive signals interrelate to properly program pancreas and liver progenitors (1-3).

In the embryo, progenitors to the pancreatic beta cell and hepatocyte lineages arise from neighboring domains of ventral foregut endoderm. *Pdx1* (*Ipf1*) and *alb1* are activated at the 7-8 somite pair (7-8S) stage in nascent pancreas and hepatocyte progenitors, respectively, in mouse development (4-7). At the same stage and spanning both the pancreatic (*Pdx1*+) and hepatic (*alb1*+) domains, the regulatory genes *Hnf1 β* (*Tcf2*), *Hnf6* (*OC1*), and *Prox1* are induced; each is required for the subsequent development of both the liver and pancreatic buds (8-12). We focused on the signaling network that induces all five of these earliest genes specific to the pancreatic and hepatic lineages.

Embryonic tissue explant studies have established that the different progenitor cell domains are induced by signals emitted from overlying mesoderm cells (6,13-16). In mouse and zebrafish embryos, redundant fibroblast growth factors (FGFs) and bone morphogenetic proteins (BMPs) secreted from the cardiac mesoderm and septum transversum mesenchyme, respectively, induce *alb1* in the ventral endoderm and suppress *Pdx1* (5,17-21). How these signals coordinately feed into the induction of *Hnf1 β* , *Hnf6*, and *Prox1* is not known, nor is their relationship to TGF β signaling, which is also present in the foregut (22).

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The establishment of a foregut endoderm fate map (23) is crucial for monitoring endoderm signaling events prior to cell type specification. Paired lateral domains of endoderm cells move ventrally at the anterior intestinal portal, helping to close off the developing foregut and contribute to the liver bud (Fig. 1A, B). The liver bud is also constituted from a distinct population of medial endoderm cells (Fig. 1A, B); the prospective lateral and medial hepatic domains fuse near the heart at the 7-8S stage (Fig. 1C)(23). Ventral pancreatic progenitors reside in the caudal-most domain of lateral liver progenitors; they migrate toward the midline during gut closure and reside caudal to the liver and heart domains at 7-8S (23). To examine BMP, TGF β , and FGF signaling within ventral endoderm prior to pancreatic and liver specification, we stained embryos at different stages with antibodies specific to the phosphorylated pathway intermediates SMAD1,5,8 (for the BMP pathway), Smad2 (for the TGF β pathway), and ERK1/2 (for the MAPK pathway, downstream of FGF (17)). The staining patterns were blocked by pathway inhibitors to ensure specificity of the antibodies (Fig. S1).

Whole mount immunofluorescence of 3-4S embryos revealed that the prospective lateral hepatic progenitors contained activated FGF-MAPK signaling (Fig. 1D), as reported previously (17). The ventral-medial endoderm, also containing prospective hepatic progenitors, was P-ERK negative at 3-4S (Fig. 1D). Activated BMP signaling exhibited the exact opposite pattern: P-SMAD1,5,8 was evident in ventral-medial endoderm at 3-4S (Fig. 1G) and not in the ventral-lateral endoderm (Fig. 1G). Thus, the prospective ventral-medial hepatic progenitors have activated BMP signaling (as evidenced by SMAD1,5,8) at the 3-4S stage, but these progenitors do not yet have an active FGF/MAPK pathway, while prospective ventral-lateral hepatic progenitors have activated FGF/MAPK at the 3-4S stage, but no active BMP signalling.

A few hours later in development (5-6S), activated MAPK signaling tracked with the prospective lateral hepatic progenitors as they moved ventral-medially (Fig. 1E). During this period, *Bmp4* and *Bmp2* were expressed in septum transversum mesenchyme across the ventral-medial region, (20,24) (Fig. S2A,B) and P-SMAD1,5,8 began to spread laterally (Fig. 1H). At the time of activation of the liver program (7-9S), the two lateral hepatic progenitor domains had migrated ventrally, joining the midline progenitors, and thus the entire liver domain became positive for P-ERK and P-SMAD 1,5,8 (Fig. 1F, I).

At 3-6S stages, the prospective ventral pancreas progenitors lacked activated P-SMAD1,5,8 (Fig. 1G). However, by the 7-8S stages, the lateral cells moved ventrally and were in the domain of P-SMAD1,5,8 (Fig. 1H, I). In summary, the ventral pancreatic progenitors are initially negative for BMP signaling and several hours later become positive.

BMP signaling via P-SMAD1,5,8 utilizes SMAD4, the latter also being a partner for TGF β /activin signaling via phosphorylated SMAD2,3. Since TGF β 2 is expressed in the foregut and its receptors are expressed in the endoderm (22,25), we investigated P-SMAD2. Notably, during all stages we detected P-SMAD2 throughout the ventral foregut endoderm (Fig. 1J-L). Similarly, SMAD4 was expressed throughout the ventral foregut endoderm as well as in the cardiac mesoderm (Fig. 1M-O). In the dorsal endoderm, we detected SMAD4 and P-SMAD2, but not P-ERK or P-SMAD1,5,8 (Fig. 1J-L, N, O). Several hours later (11S), ventral SMAD4 expression was confined to the ventral-medial endodermal domain, extending anteriorly to the pharyngeal region of the foregut (Fig. 2A).

To investigate the role of each pathway, we employed genetic and pharmacologic perturbants. We used a *FoxA3*^{CRE} transgene, which becomes functional in the ventral foregut endoderm at the 5-6S stage (17,26) and a *Smad4* conditional allele (*Smad4*^{CA}) (27) to impair SMAD1,5,8 (BMP) and SMAD2,3 (TGF β) signaling. As anticipated, ventral foregut expression of SMAD4 was completely ablated in *FoxA3*^{CRE};*Smad4*^Δ/*Smad4*^{CA} embryos, while persisting in

the pharyngeal region (Fig. 2A, B). We also developed a new half-embryo culture system that allows foregut development (28). Briefly, yolk sacs and tissues caudal to the first somite were removed from 2-6S embryos and the anterior half-embryos were cultured 24h while floating. These half-embryos reproducibly underwent gut tube closure, induction of early liver and pancreatic markers, and formed a large, beating heart (Fig. 2D, E). We processed the ventral gut domain for mRNA analysis by qRT-PCR. Gene expression in 24h cultures was normalized to *Gapdh* and compared to that in ventral foregut tissues isolated from intact embryos at 5S, 9S, and 13S stages, with the 5S stage as a reference. *Foxa2* expression, a marker of foregut endoderm (26), was consistent throughout the embryonic stages and in 24h half-embryo cultures, while *Pdx1*, *Hnf1b*, *Hnf6*, *Prox1*, and *alb1* were activated in half-embryos to levels observed in 9-13S native embryos (Fig. S3). Thus, the half-embryo explants recapitulate the induction of the earliest known genes for the pancreatic and hepatic programs.

Treating half-embryos isolated at the 3-4S stage with the BMP inhibitor noggin suppressed induction of the hepatocyte early gene *alb1*, while exogenous BMP4 treatment slightly enhanced *alb1* induction (Fig. 2F). With half-embryos isolated at the 5-6S stage, stronger effects were observed (Fig. 2F). Similarly, noggin inhibited *Prox1* induction whereas BMP4 enhanced it (Fig. 2G). Consistent with these latter results, the embryos in which *Smad4* had been deleted in the endoderm at 5-6S exhibited a complete loss of *alb1* induction and impaired *Prox1* induction (Fig. 2F, G), though some *Afp*-positive cells were eventually produced (see Fig. 3F below). Thus, BMP signaling initially is pro-hepatic with this experimental system, as seen in our previous studies and elsewhere (1,20,21).

Treating half-embryos isolated at 3-4S with noggin induced the pancreatic beta cell early gene, *Pdx1*, and treatment with BMP4 inhibited it (Fig. 2J), implying a reciprocal effect compared to liver induction, as we saw previously (18,20). Similarly, at the 3-4S stage, *Hnf6* was induced by noggin and inhibited by BMP (Fig. 2H).

In contrast, half-embryo cultures established several hours later, at the 5-6S stage, consistently exhibited the opposite phenotype. Noggin suppressed *Pdx1* and *Hnf6* expression whereas exogenous BMP4 enhanced it (Fig. 2H, J). These results were confirmed by genetics, as deletion of *Smad4* at 5-6 somites wiped out *Pdx1* induction and impaired *Hnf6* induction (Fig. 2H, J), underscoring the rapidity with which programming signals naturally change in their inductive effects. These latter findings are consistent with studies on endoderm explants from later stage chick embryos (29).

The gene expression phenotypes in the *FoxA3^{CRE};Smad4^Δ/Smad4^{CA}* embryos were accompanied by profound organogenic defects. Immunofluorescence staining revealed virtually no *Pdx1* positive cells at E9.5 in the ventral gut; nor was there morphological evidence of a ventral pancreatic bud (Fig. 3C,D). By E10.5, a few ventral *Pdx1* positive cells were evident (Fig. 3G, H). Liver development was also impaired (Fig. 3E, F). However, there was only a weak effect of *Smad4* deletion on dorsal pancreatic development (Fig. 3A,B,G,H), consistent with our observation that P-SMAD1,5,8 was not observed in the dorsal endoderm (Fig. 1G-I). Interestingly, another study used *Pdx1^{CRE}*, which is activated after 7-8S, to delete a conditional *Smad4* allele and observed no phenotype on dorsal or ventral pancreatic development (30). Taken together, it appears that BMP-SMAD4 signaling is crucial solely for a several hour period of ventral pancreatic development, from the 5-6S stage to the 7-8S stage, when *Pdx1* becomes activated.

To assess the role of activated P-SMAD2,3 (TGF β signaling) throughout the foregut endoderm (Fig. 1J-L), we treated half-embryos at 3-4S and 5-6S stages with SB431542, an inhibitor of the activin receptor-like kinases (ALK) 4,5,7, as well as treated half-embryos with exogenous TGF β 2. TGF β signaling was strongly inhibitory to pancreatic *Pdx1* and *Hnf1b* and modestly

inhibitory to the hepatic *alb1* and *Hnf6* at 5-6S (Fig. 2F-J). Since *Hnf1b* was strongly induced in the *Smad4* null embryos and not affected by the other signaling pathways, it appears that *Hnf1b* suppression could be a primary effect of TGF β signaling.

The extensive inhibitory effect of TGF β signaling suggested that it could be restraining cell type specification in the foregut. To test this, we marked nascent pancreatic progenitor cells by pulsing pregnant females from a *Pdx1^{cre-ER} × Rosa26R* cross (31) with tamoxifen at E7.5, isolating *Pdx1^{cre-ER}; Rosa26R* embryos at E8.25, and setting up 5-6S half-embryo cultures with or without the ALK4,5,7 inhibitor or exogenous TGF β 2. The ALK4,5,7 inhibitor greatly increased the number of *Pdx1^{CRE}*-positive cells along the gut endoderm and excess TGF β 2 virtually prevented their appearance (Fig. 4A,C,E). To determine if the ALK4,5,7 inhibitor markedly changed the proliferative rate of the *Pdx1^{CRE}*-positive cells, we sectioned the embryo halves sagittally and stained for Ki67. Extensive Ki67-positive cells were seen within the gut endoderm, but in both the treated and untreated tissues, *Pdx1^{CRE}; Rosa26R*-positive cells exhibited little or no staining (Fig. 4B,D,F). Furthermore, the ALK4,5,7 inhibitor did not induce apoptosis (Fig. S4). Taken together, the data indicate that continuous TGF β signaling within the foregut endoderm restrains the number of ventral pancreas cells that are specified.

During foregut closure, the movement of undifferentiated ventral endoderm cells ventrally and caudally, away from pancreas-inhibitory FGF signaling from the cardiac mesoderm, is necessary for pancreatic specification (32). We speculate that TGF β signaling restrains lateral endoderm specification until the cells move sufficiently far from the heart and into a BMP signaling domain, with the latter then becoming the dominant SMAD4-dependent pathway leading to pancreatic induction.

Our findings underscore the importance of a detailed understanding of the signaling events for cell type specification. Specifically, it would be crucial to modulate the timing of BMP and TGF β signaling to optimize for pancreatic progenitors from stem cells. Our work also suggests possible flexibility in hepatic programming, in that the prospective lateral hepatic progenitors receive FGF signaling prior to that for BMP, whereas the prospective medial hepatic progenitors receive BMP signaling prior to that for FGF. Regardless of this marked difference, descendants of both progenitor cell types activate *Afp* and *Hnf4* in the nascent liver bud (23).

Finally, we find that the signals that induce the earliest hepatic and pancreatic genes operate in parallel, independently tracking to different gene targets that together are crucial for cell specification (Fig. 4G). The parallel model explains incomplete cell programming in stem cell studies, in that if a key signal is not provided, or not provided at the appropriate time, various markers of correct differentiation could still be induced, but not all of them. A careful investigation of the inductive networks at each step of hepatocyte or beta cell differentiation should allow prospective cell derivation that is useful for research and therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Dynamic signal transduction molecule activation in the foregut endoderm revealed by whole mount immunofluorescence (IF). A-C. Fate map of foregut endoderm at different stages (23). D-O. Images looking into foregut. Arrows indicate regions with positive signal; brackets indicate regions lacking signal. m.e., medial endoderm; l.e., lateral endoderm; d.e., dorsal endoderm; hf, head fold; ht, heart; fg, foregut; liv., liver; v.p., ventral pancreas.

Fig. 2.

Genetic and pharmacologic perturbation of signaling pathways. A, B. Whole mount IF of designated genotypes. Red arrow indicates presence of SMAD4 in ventral foregut endoderm; dashed arrow indicates lack of SMAD4. C-E. Images of 5S half embryo prior to (C) or after (D,E) culture. F-J. qRT-PCR on endoderm from half-embryos +/- inhibitors or from *FoxA3^{cre}; SMAD4^{CA/Δ}* (*Smad4* null) embryos at 11S compared to that from 11S wild type embryos. N=2 to 8, each sample analyzed in triplicate; an asterisk indicate $p \leq 0.05$ determined by t-test; two asterisks, $p \leq 0.005$. "nd", not determined. Error bars indicate standard deviation (SD) calculated with ΔC_t method.

Fig. 3. Defective ventral pancreatic development in embryos deficient for *Smad4* in the endoderm. A-F. Transverse IF sections for Pdx1 or AFP (red), and DAPI. G, H. Whole mount IF for Pdx1/DAPI.

Fig. 4. TGFβ signaling restrains the specification of ventral pancreatic progenitors. A,C,E. Whole mount X-gal staining (blue) for pancreatic progenitors. B, D, F. Sagittal sections, counterstained for Ki67 (brown). G. Dynamic network inducing the earliest genes for liver and pancreas specification. Arrows, positive signaling; barred lines, negative. The thicker lines for BMP and thinner lines for TGFβ at 5-6S indicate BMP as the dominant *Smad4*-dependent pathway for *alb1*, *Hnf6*, and *Pdx1*.