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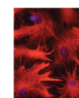

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Epithelial-to-Mesenchymal Transition Generates Proliferative Human Islet Precursor Cells

Marvin C. Gershengorn,* Anandwardhan A. Hardikar, Chiju Wei, Elizabeth Geras-Raaka, Bernice Marcus-Samuels, Bruce M. Raaka

Insulin-expressing beta cells, found in pancreatic islets, are capable of generating more beta cells even in the adult. We show that fibroblast-like cells derived from adult human islets donated postmortem proliferate readily in vitro. These mesenchymal-type cells, which exhibit no hormone expression, can then be induced to differentiate into hormone-expressing islet-like cell aggregates, which reestablishes the epithelial character typical of islet cells. Immunohistochemistry, in situ hybridization, and messenger RNA measurements in single cells and cell populations establish the transition of epithelial cells within islets to mesenchymal cells in culture and then to insulin-expressing epithelial cells.

A goal of diabetes research is to generate large numbers of cells from islets of Langerhans (beta cells) for replacement therapy (*1–3*). Although beta cells proliferate in vivo (*4*) and in vitro (*5*), well-differentiated cells do not proliferate rapidly (*6*). It is likely that expansion of mature islet cells would not yield adequate cell numbers. Stem cells with potential for extensive proliferation and differentiation have been postulated but have not been identified definitively within the adult pancreas (*7–13*). Epithelial cells within the adult pancreas, however, could be isolated in vitro as undifferentiated cells and could be induced to expand and redifferentiate, thereby serving as islet precursors. Indeed, epithelial-to-mesenchymal transition (EMT) has been documented in vitro and in vivo during development and carcinogenesis (*6*, *14–16*). We show that cells from adult human islets undergo reversible EMT to produce proliferating precursors of islet-like cell aggregates (ICAs). The unexpected plasticity of human islet-derived precursor cells (hIPCs) may be exploited to generate cells for replacement therapy.

We demonstrated previously (*17*) that clonal human pancreatic cancer (PANC-1) cells and hIPCs can transition into hormone-expressing ICAs. PANC-1 cells transitioned from epithelial cells that proliferate in adherent monolayers into spherical ICAs after a change from serum-containing medium (SCM) to serum-free medium (SFM). In SCM, PANC-1 cells exhibit a "ductal" phenotype, expressing cytokeratins-7 and -19 (Ck-7/19) but no insulin or glucagon, whereas ICAs exhibit an endocrine phenotype with cells expressing insulin or glucagon. PANC-1 ICAs could be maintained in SFM for several weeks, and the phenotype could be reversed by reexposure to SCM (fig. S1A) (*18*). ICAs deaggregated rapidly with cells flattening and migrating out within 18 hours (movie S1) to recreate a population indistinguishable from parental cells by 48 hours (fig. S1A). After 12 hours in SCM, most cells that had migrated expressed Ck-7/19 and about 10% of these cells co-expressed insulin, which suggests that endocrine cells were transitioning to the ductal phenotype (movie S2). PDX1, a factor involved in insulin gene transcription (*19*), was observed primarily within ICAs.

During ICA formation in SFM, Ck-19 mRNA decreased by a factor of ~10, whereas proinsulin mRNA increased by a factor of at least 1000 from an initially undetectable level (Fig. 1A). Low levels of proglucagon transcript were detected in ductal cells and increased about 100-fold during transition. When 30-day ICAs were exposed to SFM supplemented with fetal bovine serum or epidermal growth factor (EGF) (Fig. 1A), deaggregation was associated with decreases in proinsulin and increases in Ck-19 transcript levels. When these cells were reexposed to SFM without EGF, proinsulin mRNA was up-regulated and Ck-19 mRNA was down-regulated. Furthermore, when PANC-1 ICAs were returned to SCM, some cells that migrated were positive for proinsulin mRNA and Ck-7/19 peptides (Fig. 1B), which indicated that the transition from ductal to endocrine phenotype was reversible. Transcript levels for the intermediate filament protein Ck-19 remained relatively high even when it was not detectable by immunostaining. This may be because Ck-19 stains better when in filamentous form in migratory cells rather than in nonfilamentous form in ICAs.

Transition from one epithelial phenotype to another may involve a mesenchymal intermediate (*16*). Vimentin, another intermediate filament protein, is used as a marker of mesenchymal cells (*16*). After 3 hours in SFM, PANC-1 cells migrating into ICAs (*17*) expressed vimentin in filaments, whereas parental PANC-1 cells in SCM did not (fig. S1B). Thus, PANC-1 cells appear to undergo epithelial-to-mesenchymal-to-epithelial transitions.

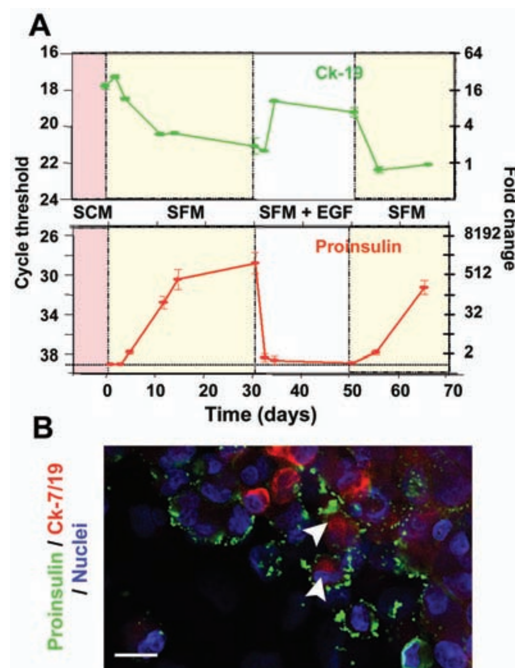

Fig. 1. PANC-1 cells undergo reversible epithelial-to-mesenchymal-to-epithelial transition. (**A**) Transition from "ductal" to endocrine phenotype. Cells were incubated in SCM and changed to SFM as described (*17*) on day 0, to SFM + EGF on day 30, and to SFM on day 50. Proinsulin and Ck-19 mRNAs were measured by quantitative RT-PCR. (**B**) Cells expressing proinsulin mRNA or Ck-7/19 peptide or both (arrowheads) were present 18 hours after ICAs were exposed to SCM. Scale bars, 10 μm.

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Like PANC-1 cells, hIPCs are proliferative cells that can be induced by serum deprivation to differentiate into hormone-expressing ICAs (see below). Having observed reversible EMT in PANC-1 cells, we hypothesized that hIPCs derived from islet epithelial cells by EMT, specifically from a heterogeneous population of adherent cells that emerge from islets (fig. S2A). After 2 days in culture, more than 40% of cells were positive for C-peptide (peptide derived from the connecting region), and 3% were positive for vimentin (Fig. 2A); cells expressing both proteins were not observed. By day 7, 28% were positive for vimentin, C-peptide–positive cells decreased to 36% and, most important, 3% were positive for both proteins. The trend of increasing vimentin and decreasing C-peptide expression continued through day 14. It is noteworthy that cells positive for both proteins were not observed at the later time, which suggests that double-positive cells may reflect a transient state as C-peptide–positive cells transition to vimentin-positive hIPCs. In general, insulin-positive cells were smaller than vimentin-positive cells. Size is a characteristic of different phenotypes within a single hIPC population, because cultures of "small" cells contained cells of both sizes within 3 days (fig. S3, A and B).

In situ hybridization (*18*) showed that some adherent cells emerging from islets were positive for proinsulin mRNA and for vimentin protein (Fig. 2B), even though most cells were vimentin-negative (Fig. 2A). Vimentin staining was filamentous as in mesenchymal cells (*6*) and migratory PANC-1 cells (fig. S1B). Similarly, cells emerging from islets after 4 days express filamentous arrays of nestin, smooth muscle actin, and vimentin (fig. S2B). These data suggest that hIPCs are derived from insulin-expressing cells by EMT. To test this hypothesis, we measured proinsulin transcript in randomly selected, adherent single cells during the first 17 days of culture (Fig. 2C). During days 2 through 8, when the number of viable cells remained constant, most cells were positive

Table 1. Expression of representative epithelial and mesenchymal mRNAs in human islets and hIPCs. Total RNA was prepared from human islets within 3 days of organ donation from a single donor (Single) or pooled from 3 donors (Pooled) and from hIPCs derived from 3 individual donors (A, B, or C) at passages 8 (p8), 10 (p10), or 16 (p16). INS, proinsulin; GCG, proglucagon; GCK, glucokinase; PDX1, insulin promoter factor 1; GLP1R, glucagon-like peptide 1 receptor; CDH1, E-cadherin; CLDN3, claudin 3; CLDN4, claudin 4; OCLN, occludin; PECAM1, platelet/endothelial cell adhesion molecule (CD31); VIM, vimentin; NES, nestin; ACTA2, smooth muscle actin alpha 2; ACTG2, smooth muscle actin γ 2; ENG, endoglin (CD105); MMP2, matrix metalloproteinase 2; SNAI1, snail homolog 1; SNAI2, snail homolog 2; THY1, Thy-1 cell surface antigen; P4HA1, prolyl 4-hydroxylase alpha subunit. ND, not determined.

	qRT-PCR cycle threshold				
	Human islets		hIPCs		
	Single	Pooled	A, p8	B, p16	C, p10
Epithelial					
INS	14	14	27	>38	>38
GCG	19	20	33	35	>38
GCK	23	24	>38	>38	ND
PDX1	23	24	34	>38	ND
GLP1R	24	25	>38	>38	ND
CDH1	20	21	30	30	30
CLDN3	23	24	31	31	34
CLDN4	18	18	27	28	30
OCLN	20	21	26	26	27
PECAM1	24	24	34	31	35
Mesenchymal					
VIM	18	19	14	15	16
NES	26	27	24	24	23
ACTA2	24	25	17	18	16
ACTG2	33	31	23	21	23
ENG	25	25	21	22	22
MMP2	23	22	17	17	17
SNAI1	26	26	24	26	24
SNAI2	25	25	21	22	22
THY1	25	26	18	18	19
P4HA1	22	24	20	20	21

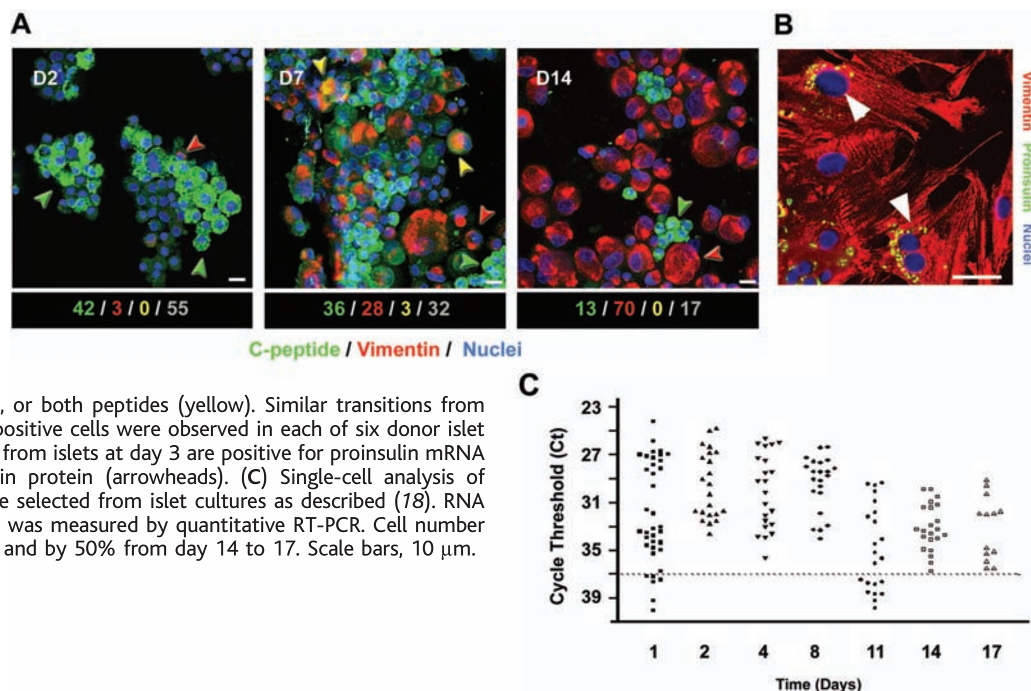


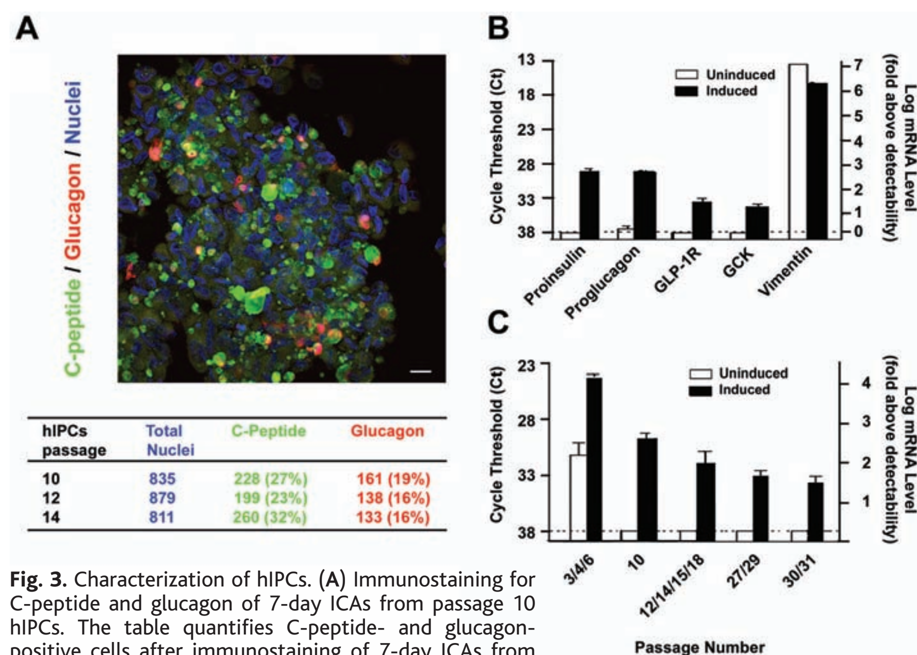
Fig. 2. hIPCs are derived from insulin-expressing cells by epithelial-to-mesenchymal transition in vitro. (**A**) Cells migrating out from adult human islets lose expression of C-peptide and express vimentin protein in filaments. Islet cultures were harvested with trypsin at days 2 (D2), 7 (D7), and 14 (D14); cytospun; and immunostained for C-peptide and vimentin. From >400 cells, the percentage positive for C-peptide (green), vimentin (red), both peptides (yellow), or neither (gray) is shown below each panel. Arrowheads identify cells expressing C-peptide (green), vimentin (red), or both peptides (yellow). Similar transitions from mostly negative to mostly vimentin-positive cells were observed in each of six donor islet cultures. (**B**) Some cells migrating out from islets at day 3 are positive for proinsulin mRNA by in situ hybridization and vimentin protein (arrowheads). (**C**) Single-cell analysis of proinsulin mRNA. Individual cells were selected from islet cultures as described (*18*). RNA was isolated and proinsulin transcript was measured by quantitative RT-PCR. Cell number increased twofold from day 11 to 14 and by 50% from day 14 to 17. Scale bars, 10 μm.

for proinsulin, although transcript levels were distributed over three orders of magnitude. This suggests that our culture conditions select for proinsulin mRNA-positive cells. From day 11 to 17, the level of proinsulin transcript declined in individual cells. Most important, as the cell number doubled from day 11 to 14 and increased again by one-half from day 14 to 17, the percentages of proinsulin-positive cells were 95% (day 14) and 100% (day 17). If proliferative hIPCs had arisen from proinsulin transcript-negative cells, e.g., "stem cells," a doubling of cell number would have decreased proinsulin transcript–expressing cells to 50%, and a further increase in cell number by one-half would have decreased proinsulin-expressing cells to 33%. This did not occur. In two other islet preparations, proinsulin mRNA remained detectable in 79% and 74% of cells after 14 days in culture. To demonstrate directly that insulin-expressing cells were proliferating, we labeled cells after 7 days in culture with BrdU for 40 hours and co-stained them with antibodies to BrdU and to C-peptide (fig. S4, A and B). Of the 38% of the cells in this experiment that were C-peptide–positive, more than one-fifth were also positive for BrdU, which indicated that these cells were proliferating. Taken together, our findings are most consistent with the conclusion that proliferating hIPCs originate by EMT from cells initially expressing insulin.

After 2 weeks in culture, islets had flattened to generate a monolayer of cells; residual "islets" were comprised of granular, dead cells. Harvested and reseeded cells displayed a nearly homogeneous, fibroblast-like morphology (fig. S5A). hIPCs at this stage, about 14 days after islets were placed into culture, were defined as passage 0. In 3 to 4 days, the culture reached confluence (fig. S5A). During the transition from cells within islets to hIPCs, markers for epithelial cells including E-cadherin, claudins 3 and 4, occludin, and PECAM1, as well as those specific for endocrine cells including proinsulin, proglucagon, glucokinase, PDX1, and GLP1R, decreased, whereas markers of mesenchymal cells including vimentin, nestin, smooth muscle actins α 2 and γ 2, endoglin, matrix metalloproteinase 2, snail homologs 1 and 2, Thy-1 cell surface antigen, and prolyl 4-hydroxylase increased (Table 1). Cells isolated by Beattie *et al.* (*20*) and Bouckenooghe *et al.* (*21*) appear similar to hIPCs, and they may have arisen by EMT also. Unlike many other primary cell populations derived from human or rodent islets (*10*, *20*), hIPCs exhibit substantial proliferative potential for about 90 days (doubling time of 60 hours) (fig. S5B). Cells isolated from islets by Habener and colleagues (*7*, *22*) proliferated well and may be similar to hIPCs. Cryopreserved cells resumed growth after a brief lag period at rates similar to those of cells never frozen. During the initial 3 months in culture, hIPCs expanded by almost 10^{12}. As a primary culture, hIPC proliferation slowed at later passages. hIPCs from three different islet preparations at passages 4 to 14 exhibited normal karyotypes. At early passages, hIPC populations are positive for proinsulin mRNA, but the level decreased continuously and became undetectable by passage 10 (fig. S5B). The gradual loss of proinsulin transcript may reflect the long half-life of proinsulin mRNA, estimated to be about 30 hours in rodents (*23*). Other endocrine-specific transcripts, including proglucagon, glucagon-like peptide 1 receptor, and glucokinase, also decreased and were undetectable by passage 10 (Table 1).

Up to passage 30, hIPCs could differentiate into ICAs when deprived of serum. Before differentiation, hIPCs showed immunostaining for vimentin (94% of cells), nestin (75%), and smooth muscle actin (98%) in prominent filaments (fig. S2B) like mesenchymal cells (*6*, *16*) and were negative for C-peptide. In contrast, cells within ICAs expressed C-peptide and glucagon (Fig. 3A). C-peptide staining was used to exclude detection of insulin in SFM (*24*). Immunostaining for C-peptide and glucagon of 7-day ICAs from passages 10, 12, or 14 showed that 27 ± 4% of cells stained positively for C-peptide and 17 ± 2%, for glucagon (Fig. 3A). The transition of hIPCs into ICAs increased proinsulin mRNA at least 1000-fold over initially undetectable levels and proglucagon mRNA over 100-fold (Fig. 3B). Transcripts for glucagon-like peptide 1 receptor and glucokinase also increased more than 10-fold. Thus, endocrine-specific transcripts increased when mesenchymal hIPCs transitioned into epithelial ICAs. Expression of claudin 3 and 4 mRNAs (*25*) increased whereas expression of smooth muscle actin alpha2 and gamma2 mRNAs (*16*) decreased in ICAs, further supporting the epithelial transition.

Proinsulin transcript induction was compared at different passages. At passages 3, 4, or 6, proinsulin transcript increased about 10-fold over initially detectable levels (Fig. 3C) whereas at passages 10 through 18, it increased at least 100- to 1000-fold over initially undetectable levels. At passages later than 27, smaller increases in proinsulin transcript were observed. Induction of proinsulin transcript by 100-fold or more occurred consistently in ICAs generated from mid-passage hIPCs from six separate donor islets (fig. S6). Although hIPC ICAs reproducibly exhibited marked induction of proinsulin mRNA expression, the level attained was less than 0.02% of that in human islets. Thus, hIPC ICAs are not comparable to islets in the levels of insulin (or glucagon) expression. However, cells within hIPC ICAs exhibit the following features of islets: Insulin C-peptide is detected by immunostaining (Fig. 3A); in preliminary experiments, ICAs secreted C-peptide under basal and stimulated conditions in vitro (*26*) and human C-peptide was measured in blood from three of six SCID mice implanted with ICAs under their kidney capsules, and after 14 days, implants



hIPCs passage	Total Nuclei	C-Peptide	Glucagon
10	835	228 (27%)	161 (19%)
12	879	199 (23%)	138 (16%)
14	811	260 (32%)	133 (16%)

Fig. 3. Characterization of hIPCs. (**A**) Immunostaining for C-peptide and glucagon of 7-day ICAs from passage 10 hIPCs. The table quantifies C-peptide- and glucagon-positive cells after immunostaining of 7-day ICAs from three hIPC preparations at the indicated passages. (**B**) Proinsulin, proglucagon, glucagon-like peptide-1 receptor (GLP-1R), and glucokinase (GCK) mRNAs increased, and vimentin mRNA decreased, during induction of ICA formation. (**C**) Induction of proinsulin mRNA at different hIPC passages. Scale bar, 20 μm.

from these three mice immunostained for human C-peptide. The blood levels of human C-peptide in the three mice were 0.22, 0.51, and 0.91 ng/ml and similar levels were found to reverse hyperglycemia in mice transplanted with insulin-expressing cells differentiated from human fetal liver progenitor cells (*27*).

In most previous attempts to generate beta cells in culture from adult islets, maintenance of insulin expression during culture was attempted (*5*, *20*, *21*). The cells obtained in these experiments did not expand well nor did they exhibit marked induction of insulin expression. Another approach was to select for cells that expressed genes, e.g., nestin, that were thought to identify precursor cells (*7*, *22*). Although the origin of these cells was not considered, they were derived from adherent islet cells and are likely similar to hIPCs, because about 75% of hIPCs are immunopositive for nestin. We show that hIPCs are "true" endocrine pancreas precursor cells that exhibit a mesenchymal phenotype before transition into epithelial clusters containing cells expressing insulin or glucagon. Indeed, hIPCs are highly proliferative and can be expanded by a factor of $>10^{12}$ and, therefore, could serve as cells for replacement therapy for diabetes if their insulin output, in particular that in response to glucose, could be optimized and they could be shown to be safe and effective upon implantation.

The origin of hIPCs is important because it provides information about the potential plasticity of insulin-expressing cells, and perhaps of other epithelial cell types, at least after culture in vitro. In contrast to the prevailing view that the source of pancreas-derived precursor cells is adult stem cells, we provide strong evidence that hIPCs are derived from insulin-expressing cells by EMT. This conclusion would be strengthened by permanently marking insulin-expressing cells in situ for cell lineage analysis as performed in mouse models (*4*, *28*), but these experiments are not possible in humans. Our studies, however, do not negate the possibility that adult stem cells are present within islets and contribute to beta cell generation in vivo.

Last, although our observations regarding EMT were made with insulin-expressing cells in vitro, a similar phenomenon may occur in vivo. Using genetically engineered mice, Dor *et al*. recently provided evidence that new insulin-expressing beta cells are derived from "preexisting" beta cells in vivo (*4*). These authors concluded, "Adult pancreatic β-cells are formed by self-duplication." Another interpretation of their data, consistent with our observations, is that new beta cells are generated from preexisting beta cells by reversible EMT.

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Figs. S1 to S6
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Movies S1 and S2

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β-Arrestin 2 Regulates Zebrafish Development Through the Hedgehog Signaling Pathway

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β-arrestins are multifunctional proteins that act as scaffolds and transducers of intracellular signals from heptahelical transmembrane-spanning receptors (7TMR). Hedgehog (Hh) signaling, which uses the putative 7TMR, Smoothened, is established as a fundamental pathway in development, and unregulated Hh signaling is associated with certain malignancies. Here, we show that the functional knockdown of β-arrestin 2 in zebrafish embryos recapitulates the many phenotypes of Hh pathway mutants. Expression of wild-type β-arrestin 2, or constitutive activation of the Hh pathway downstream of Smoothened, rescues the phenotypes caused by β-arrestin 2 deficiency. These results suggest that a functional interaction between β-arrestin 2 and Smoothened may be critical to regulate Hh signaling in zebrafish development.

Hedgehog (Hh) molecules are highly conserved morphogens that play a central role in cell proliferation and embryonic patterning (*1*, *2*). In humans, inhibitory mutations of the Sonic Hedgehog (Shh) pathway result in developmental defects such as holoprosencephaly (*3*), whereas mutations that constitutively activate the pathway lead to basal cell carcinomas (*4*) and medullablastomas (*5*). Despite extensive studies of the Hh pathway, the sequence of events leading to a biological function has yet to be fully defined. In vertebrates, extracellular Shh glycoprotein binds to the 12-transmembrane-spanning protein, Patched (Ptc), and relieves the inhibitory effect of Ptc on Smoothened (*6*). Smoothened is a signaling molecule that causes downstream uncoupling of the negative regulator Su(fu) protein from the Gli transcription factors (*7*). The subsequent nuclear translocation and DNA binding of Gli1 and Gli2, and possibly of Gli3, is followed by the increased transcription of a number of genes, including *ptc* itself (*8*) and *nkx2.2* (*9*). In contrast, the proteolytic cleavage of Gli3, promoted by cAMP-dependent protein kinase (PKA) phosphorylation in the absence of a

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