Glucocorticoid-dependent transdifferentiation of pancreatic progenitor cells into hepatocytes is dependent on transient suppression of WNT signalling

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Summary
Developmentally, the pancreas and liver are closely related and pathological conditions – including elevated glucocorticoid levels – result in the appearance of hepatocytes in the pancreas. The role of the WNT signalling pathway in this process has been examined in the model transdifferentiating pancreatic acinar AR42J-B-13 (B-13) cell. Glucocorticoid treatment resulted in a transient loss of constitutive WNT3a expression, phosphorylation and depletion of β-catenin, loss of β-catenin nuclear localisation, and significant reductions in T-cell factor/lymphoid enhancer factor (Tcf/Lef) transcriptional activity before overt changes in phenotype into hepatocyte-like (B-13/H) cells. A return to higher Tcf/Lef transcriptional activity correlated with the re-expression of WNT3a in B-13/H cells. β-catenin knock down alone substituted for and enhanced glucocorticoid-dependent transdifferentiation. Overexpression of a mutant β-catenin (pt-Xβ-cat) protein that blocked glucocorticoid-dependent suppression of Tcf/Lef activity resulted in inhibition of transdifferentiation. A small-molecule activator of Tcf/Lef transcription factors blocked glucocorticoid-dependent effects, as observed with pt-Xβ-cat expression. Quercetin – a Tcf/Lef inhibitor – did not promote transdifferentiation into B-13/H cells, but did potentiate glucocorticoid-mediated transdifferentiation. These data demonstrate that the transdifferentiation of B-13 cells into hepatocyte-like cells in response to glucocorticoid was dependent on the repression of constitutively active WNT signalling.

Key words: WNT3a, GSK3, β-catenin, Tcf/Lef, C/EBP-β, Liver, Pancreas

Introduction
The pancreas and liver are derived from the embryonic endoderm (Lemaigre and Zaret, 2004). Hepatocytes (and biliary epithelial cells) are thought to differentiate from hepatoblasts, which are directed from a default pancreatic differentiation program by factors released from the embryonic mesenchyme (Lemaigre and Zaret, 2004). A close developmental relationship therefore exists between the liver and pancreas; this might account for the appearance of hepatocytes in damaged or diseased rodent pancreata (Rao et al., 1988; Yeldandi et al., 1990; Krakowski et al., 1999; Yamaoka et al., 2002). The mechanism(s) involved have not been determined, but are thought to be dependent on the proliferation and aberrant differentiation of progenitor cells in ductal regions of the pancreas (Makino et al., 1990; Dabeva et al., 1997).

An alternative or additional mechanism by which hepatocyte-like cells appear in the pancreas could be through transdifferentiation. Transdifferentiation is a form of metaplasia and describes the irreversible switching of cellular differentiation that sometimes occurs in fully differentiated cells in disease (Burke and Tosh, 2005). The AR42J-B-13 (B-13) rat pancreatic exocrine cell line is a model system that is used to study the process of pancreatic transdifferentiation into hepatocyte-like cells in vitro (Shen et al., 2000; Marek et al., 2003; Lardon et al., 2004). To date, the mechanism has only been shown to be dependent on the induction of the CCAAT-enhancer-binding protein-β (C/EBP-β) (Shen et al., 2000).

Recent data indicate that AR42J-B-13 cells express Wnt genes, WNT receptor proteins (frizzled) and receptor-interacting proteins (dikkopf, Lrp5, sFRP), signalling components [glycogen synthase kinase 3 (GSK3) and β-catenin] and several distal T-cell factor/lymphoid enhancer factor (Tcf/Lef) transcription factors (Wallace et al., 2009). The expression of many of these genes is modulated by glucocorticoids in such a way as to suggest that WNT signalling activity is probably suppressed in AR42J-B-13-derived hepatocyte-like cells (Wallace et al., 2009).

Glucocorticoids are steroid hormones that bind and modulate the transcriptional function of nuclear receptors such as the glucocorticoid receptor (Munck et al., 1984). Because nuclear receptors are able to directly interact with elements of the WNT signalling pathway (for a review, see Mulholland et al., 2005), we hypothesis that changes in WNT signalling activity play a crucial role in the process of AR42J-B-13 cell transdifferentiation.

This paper demonstrates for the first time that glucocorticoid-mediated transdifferentiation of AR42J-B-13 cells is associated with changes in β-catenin phosphorylation, loss of nuclear β-catenin localisation and loss of Tcf/Lef transcriptional activity. Small interfering (siRNA)-mediated β-catenin silencing and β-catenin overexpression substituted for and inhibited glucocorticoid-dependent transdifferentiation, respectively, which occurred upstream of changes in C/EBP-β expression. However, direct Tcf/Lef inhibition did not substitute for glucocorticoid, suggesting that a β-catenin-dependent, Tcf/Lef-independent mechanism was also involved. WNT signalling is therefore a pivotal regulatory pathway controlling B-13 cell self-renewal and transdifferentiation.
Results
B-13 cells respond to DEX, but not to embryonic regulators FGF-2 and BMP-4

Glucocorticoids are steroid hormones normally associated with the regulation of intermediary metabolism. However, they are often incorporated into stem and progenitor cell differentiation protocols, because they probably promote maturation into a fully differentiated phenotype in vitro. We initially hypothesised that the B-13 cell line is related to an embryonic endodermal cell capable of differentiating into a liver hepatocyte or bile-duct epithelial cell. We therefore treated B-13 cells with the factors that promote embryonic endodermal differentiation into hepatocytes: fibroblast growth factor 2 (FGF-2) and bone morphogenetic protein 4 (BMP-4) (Lemaigre and Zaret, 2004). Fig. 1A demonstrates that B-13 cells expressed the embryonic endoderm marker SOX17 (also observed in rat embryo), but not the mesodermal and ectodermal markers brachyury and nestin, respectively, suggesting that the B-13 cells might be related and/or behave similarly to the embryonic endoderm. However, Fig. 1B,C demonstrates that only dexamethasone (DEX) promoted differentiation into a hepatocyte phenotype (denoted as B-13/H cells once maximal transdifferentiation has occurred after 10-14 days treatment), whereas FGF-2 and BMP-4 either alone or in combination did not. The cells did not differentiate into bile-duct epithelial cells or any other liver-cell type in response to DEX (or a range of other factors), but cultures treated with DEX always retained a population

Fig. 1. Embryonic developmental marker gene expression in B-13 cells and response to embryonic promoters of the hepatocyte phenotype. (A) Reverse transcriptase (RT)-PCR was performed on RNA isolated from the following: B-13, proliferating B-13 cells 4 days after subculture; B-13/H, B-13 cells treated for 14 days with 10 nM DEX; BMP-4, B-13 cells treated with 10 ng/ml BMP-4 for 5 days; embryo, whole rat (16 day) embryo RNA; control, RT-PCR reaction carried out in the absence of template RNA. Positive RNA controls were obtained from adult rat tissues. (B) B-13 cells were treated as indicated for 11 days prior to harvesting and analysis by western blotting. Each lane contains 32 μg total cell protein. Hepatocytes: purified rat hepatocytes freshly isolated from a collagenase perfusion. GD, glutamate dehydrogenase; CPS, carbamoyl phosphate synthase; CYP reduct, cytochrome P450 reductase. (C) B-13 and B-13/H cells were fixed and stained for DNA using DAPI (blue) and the indicated protein using a primary antibody against the protein followed by incubation with a fluorescein isothiocyanate (FITC)-conjugated (green) secondary antibody. No primary control: cells stained without addition of primary antibody. All photomicrographs were recorded under identical exposure and magnification conditions. (D) Para-nitrophenol hydroxylase activity in the indicated cells. Results are the mean and standard deviation of the activity from at least three separate cultures per time point. Hepatocytes: freshly isolated rat hepatocytes. *Significantly different (P<0.05) versus B-13 cells using Student’s t-test (two tailed). All results typical of at least three separate experiments.
of cells (~15% of the total) that remained in the B-13 phenotype and, upon subculture, gave rise to a similar (15:85) proportion of B-13 and B-13/H cells when treated with DEX (supplementary Fig. S1). The cells therefore have a pancreatic acinar phenotype and also a progenitor cell phenotype, in that they have a restricted ability to differentiate into another cell type (hepatocytes) and, in the presence of differentiating signals, undergo asymmetric division and self-renewal. Fig. 1D demonstrates that the expression of CYP2E (cytochrome P450 2E) in hepatocytes derived from B-13 cells was functional, confirming previously published work demonstrating both hepatocyte gene expression and functionality (Marek et al., 2003).

**B-13 cell transdifferentiation is associated with a transient reduction in WNT signalling activity**

WNT signalling is known to be involved in liver development (Monga et al., 2003; Micsenyi et al., 2004; Tan et al., 2006; Thompson and Monga, 2007) and also to regulate zonal hepatocyte gene expression in the adult liver (Burke et al., 2009). Fig. 2A indicates that WNT3a was constitutively expressed by both hepatocytes and B-13 cells, but was barely detectable in B-13/H cells as they reach early maximal transdifferentiation. Fig. 2A shows that hepatocytes expressed WNT3a and that extending DEX treatment of B-13/H cells resulted in the re-expression of WNT3a from around 21 days. These data suggest that there might be constitutively active autocrine or paracrine regulation of WNT signalling in B-13 cells by WNT3a and that transdifferentiation might be dependent on the temporary suppression of WNT signalling activity. To test this hypothesis, WNT signalling activity was examined during the period prior to re-expression of WNT3a and compared with its activity in parent B-13 cells.

mRNA encoding GSK3-β is constitutively expressed at similar levels in both B-13 and B-13/H cells (Wallace et al., 2009). Fig. 2B shows that GSK3-β (and potentially the less well-characterised related α form) was present as the kinase-inactive phosphorylated protein in B-13 cells. It was undetectable in B-13/H cells, despite

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**Fig. 2.** Transdifferentiation of B-13 into B-13/H cells is associated with repression of WNT3a expression, upregulation of an active form of GSK3-β, phosphorylation and depletion of β-catenin, and changes in β-catenin cellular distribution. (A) Western blot of the indicated proteins (20 μg protein/lane). (B) Western blot of the indicated proteins in extracts from B-13 and B-13/H cells (30 μg protein/lane). (C) Immunocytochemical staining for β-catenin (green) in B-13 and B-13/H cells. Nuclei are stained with DAPI (blue). All results typical of at least three separate experiments. (D) Quantitation of the percentage of cells containing nuclear β-catenin. Data are the mean and standard deviation of at least three separate experiments.*Significantly different (P<0.05) versus B-13 cells using Student’s t-test (two tailed).
the apparent induction of GSK3-β protein. These changes correlated with an increase from undetectable levels of Ser32- and Ser37-phosphorylated β-catenin, and depletion of total β-catenin levels in B-13/H cells compared with B-13 cells (Fig. 2B). In B-13 cells, β-catenin was dispersed throughout the cell, including the nucleus in ~50% of cells (Fig. 2C,D). In B-13/H cells, β-catenin had a plasma-membrane and cytosolic location, with no evidence of its presence within nuclei (Fig. 2C,D).

Fig. 3A indicates that Tcf/Lef transcriptional activity was high in B-13 cells and that activity falls with DEX treatment. Treatment with DEX for 24 hours was sufficient to inhibit Tcf/Lef activity to a level similar to that after treatment with the Tcf/Lef inhibitor quercetin (Park et al., 2005) and prior to any overt change in B-13 morphology (see Fig. 3B). With increasing treatment time, cells showed clear hepatocyte-like morphology, such that, at 16 days, between 70-90% of cells had a hepatocytic phenotype (Fig. 3B; see also supplementary material Fig. S1). This change in morphology correlated with a fall in Tcf/Lef activity, with much of the activity probably associated with remaining B-13 cells (which accounts for the majority of the activity that can be inhibited by quercetin in day 14-16 cultures). This is supported by experiments in which B-13/H cells were selectively trypsinised from day 14-16 cultures after transfection. Tcf/Lef activity was determined in adhered (primarily B-13) and suspended (primarily B-13/H) cells. Fig. 3C confirms that extracts containing high levels of CYP2E protein (and therefore enriched with B-13/H cells) contained significantly less reporter gene activity (normalised for transfection efficiency) than extracts containing primarily B-13 cells.

**Suppression of β-catenin protein is sufficient to promote C/EBP-β induction and transdifferentiation**

To determine whether components of the WNT signalling pathway are crucial for transdifferentiation, B-13 cells were transfected with siRNA designed to promote the degradation of mRNA encoding β-catenin. Fig. 4A,B demonstrates that transfection with siRNA directed against β-catenin (and knock down of β-catenin protein levels) resulted in the expression of CYP2E and albumin in B-13 cells, producing a higher level of expression of these hepatocyte markers than DEX treatment alone for the same period of time. Previous work has indicated that overexpression of C/EBP-β also promotes B-13 cell transdifferentiation into B-13/H cells.
Fig. 4B shows that knock down of β-catenin expression resulted in induction of C/EBP-β, suggesting that β-catenin is an upstream regulator of C/EBP-β expression in B-13 cells. β-catenin knock down also inhibited B-13 cell proliferation, as observed when the cells were treated with DEX (data not included).

**Overexpression of β-catenin blocks DEX-dependent B-13 cell transdifferentiation – a crucial role for β-catenin**

To firmly establish whether β-catenin levels are crucial regulators of B-13 cell differentiation, B-13 cells were treated with DEX and also transfected with vectors that drive the expression of β-catenin mutant proteins. Fig. 5A,B demonstrates that overexpression of pt-Xβ-catenin, which cannot be phosphorylated at several N-terminal sites and is resistant to degradation (Yost et al., 1996), led to similar levels of induction of Tcf/Lef reporter gene expression as the chemical ‘WNT agonist’ Tcf/Lef activator 2-amino-4-[3,4-(methylenedioxy)benzylamino]-6-(3-methoxyphenyl)pyrimidine (Liu et al., 2005). Overexpression of pt-Xβ-catenin also abrogated DEX-mediated suppression of Tcf/Lef activity, blocked induction of C/EBP-β by DEX and prevented transdifferentiation (i.e. there was no expression of CYP2E and albumin). Overexpression of β-catenin-163, a mutant protein that cannot interact with Tcf/Lef (Graham et al., 2000), failed to block DEX-dependent suppression of Tcf/Lef activity (Fig. 5B), induction of C/EBP-β and transdifferentiation (Fig. 5A). These data therefore indicate that the effects of pt-Xβ-catenin expression are unlikely to be due to non-specific effects associated with overexpression of the protein and that maintaining high levels of β-catenin able to interact with Tcf/Lef factors is sufficient to prevent both C/EBP-β induction and transdifferentiation into B-13/H cells.

**An additional β-catenin-dependent, Tcf/Lef-independent mechanism(s) is essential for DEX-dependent transdifferentiation**

To further examine the role of Tcf/Lef factors in transdifferentiation of B-13 cells, the effects of pharmacological modulators of Tcf/Lef were examined. Fig. 6A shows that WNT agonist abrogated DEX-dependent induction of C/EBP-β and transdifferentiation (i.e. blocked expression of mRNA encoding CYP2E protein), suggesting that changes in Tcf/Lef activity are crucial for B-13 cell transdifferentiation into B-13/H cells and confirming a role for Tcf/Lef in the expression of C/EBP-β. Quercetin is a Tcf/Lef inhibitor (Park et al., 2005) and Fig. 6 shows that quercetin treatment alone results in the induction of mRNA encoding C/EBP-β and C/EBP-β protein, again supporting a role for Tcf/Lef in the repression of C/EBP-β. However, quercetin treatment did not result in transdifferentiation of B-13 cells, as judged phenotypically (data not shown).

Fig. 4. Transdifferentiation of B-13 cells into B-13/H cells is stimulated by suppression of β-catenin expression. (A) B-13 cells in six-well plates were transfected for 48 hours with the indicated siRNA (3 μg/well of six-well plate) and treated with DEX or vehicle control (–) as outlined in the Materials and Methods. The cells were then fixed and examined by confocal microscopy (red fluorescence, siRNA-transfected cells; green fluorescence, β-catenin or CYP2E expression as indicated). No 1° antibody, siRNA-transfected cells immunostained without addition of anti-β-catenin or CYP2E primary antibody. Images were taken after subtraction of background fluorescence based on untransfected and unstained cells and under identical conditions. (B) As outlined for A, except that cells were subjected to western blotting for the indicated protein. All data typical of three separate experiments.
not shown) or from expression of CYP2E and albumin. Quercetin potentiated the effects of DEX on transdifferentiation (Fig. 6B), supporting a role for Tcf/Lef and C/EBP-β in transdifferentiation and confirming that quercetin was effective at inhibiting Tcf/Lef transcriptional activity (as indicated in Fig. 3A and Fig. 5B).

Interestingly, quercetin treatment also resulted in reduction in mRNA encoding β-catenin and β-catenin protein, suggesting that Tcf/Lef might regulate β-catenin levels at the transcriptional level.

Discussion
The data in this paper demonstrate that B-13 cells constitutively express WNT3a protein and have constitutively high WNT signalling activity. Treatment with glucocorticoid resulted in the transient loss of WNT3a, which is associated with a reduction in WNT signalling activity and transdifferentiation of B-13 cells into hepatocyte-like B-13/H cells. WNT3a was readily detected in normal hepatocytes and probably accounts for the re-expression of WNT3a in B-13/H cells. WNT signalling remains an active signalling pathway in adult liver tissue and is responsible for the variable ‘zonated’ expression of genes in the liver (Benhamouche et al., 2006). However, it remains to be established whether proteins such as WNT3a regulate the WNT signalling pathway differently in B-13 and B-13/H cells.

Data in this paper suggest that suppression of WNT signalling by glucocorticoid is a crucial mechanism controlling differentiation, because inhibition of Tcf/Lef transcriptional activity occurs prior to expression of hepatocyte genes and phenotypic changes. Furthermore, knock down of the intracellular WNT messenger protein β-catenin substituted for glucocorticoid and brought about rapid transdifferentiation. The proposed mechanism is schematically illustrated in supplementary material Fig. S2.

The B-13 cell was originally isolated from the AR42J pancreatic cancer acinar cell line by the Kojima laboratory (Mashima et al., 1996). It was first shown to express liver-specific genes, such as albumin, in response to glucocorticoid treatment several years later by Shen et al. (Shen et al., 2000). Subsequent investigations have demonstrated that the B-13 cell undergoes widespread coordinated differentiation into hepatocyte-like cells that are qualitatively and quantitatively functionally comparable to freshly isolated rat hepatocytes (Shen et al., 2000; Marek et al., 2003; Lardon et al., 2004; Wallace et al., 2009). An important question regarding the B-13 cell response to glucocorticoid is whether it is relevant to any real physiological response in the embryo, developing foetus or adult. Circulating levels of glucocorticoid are low in the embryo and foetus, because of placental enzymes that metabolise glucocorticoids to inactive products (Seckl and Holmes, 2007). As the foetal hypothalamus-pituitary-adrenal axis develops, foetal glucocorticoid levels rise and probably play a major role in cell differentiation and tissue maturation, most notably in the lung (Grier and Halliday, 2004).
We propose that B-13 cells could therefore be modelling a mature endodermal response to glucocorticoid, because the cells express SOX17 and respond to glucocorticoid but not to FGF-2 and BMP-4. Alternatively – or additionally – B-13 cells might be modelling the response of adult tissue progenitor cells to pathologically high glucocorticoid levels. The latter model is supported by our recent observation that hepatocytes appear within the rodent pancreas after a relatively short period of elevated glucocorticoid treatment (Wallace et al., 2009). Furthermore, in a transgenic mouse model of sustained elevated glucocorticoid levels, the exocrine pancreas transdifferentiates into hepatocyte-like tissue and mice begin to malabsorb and lose weight, an effect reversed by feeding mice with pancreatic porcine enzymes (Rao et al., 1988; Yeldandi et al., 2002). Hence, the rodent pancreas after a relatively short period of elevated glucocorticoid treatment is an effect reversed by feeding mice with pancreatic porcine enzymes (Rao et al., 1988; Yeldandi et al., 2002). Hence, the rodent pancreas after a relatively short period of elevated glucocorticoid treatment (Wallace et al., 2009). Furthermore, in a transgenic mouse model of sustained elevated glucocorticoid levels, the exocrine pancreas transdifferentiates into hepatocyte-like tissue and mice begin to malabsorb and lose weight, an effect reversed by feeding mice with pancreatic porcine enzymes (Rao et al., 1988; Yeldandi et al., 2002). Hence, the rodent pancreas after a relatively short period of elevated glucocorticoid treatment (Wallace et al., 2009). Furthermore, in a transgenic mouse model of sustained elevated glucocorticoid levels, the exocrine pancreas transdifferentiates into hepatocyte-like tissue and mice begin to malabsorb and lose weight, an effect reversed by feeding mice with pancreatic porcine enzymes (Rao et al., 1988; Yeldandi et al., 2002). Hence, the rodent pancreas after a relatively short period of elevated glucocorticoid treatment.
