Hepatocyte nuclear factor-4α promotes differentiation of intestinal epithelial cells in a coculture system

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Submitted 15 September 2007; accepted in final form 17 November 2007

Lussier CR, Babeu J-P, Auclair BA, Perreault N, Boudreau F. Hepatocyte nuclear factor-4α promotes differentiation of intestinal epithelial cells in a coculture system. Am J Physiol Gastrointest Liver Physiol 294: G418–G428, 2008. First published November 21, 2007; doi:10.1152/ajpgi.00418.2007.—Normal cellular models able to efficiently recapitulate intestinal epithelial cell differentiation in culture are not yet available. The aim of this work was to establish and genetically characterize a mesenchymal-epithelial coculture system to identify transcriptional regulators involved in this process. The deposition of rat intestinal epithelial cells on human intestinal mesenchymal cells led to the formation of clustered structures that expanded shortly after seeding. These structures were composed of polarized epithelial cells with brush borders and cell junction complexes. A rat GeneChip statistical analysis performed at different time points during this process identified hepatocyte nuclear factor-4α (HNF-4α) and hepatocyte nuclear factor-1α (HNF-1α) as being induced coincidentally with the apparition of polarized epithelial structures. Stable introduction of HNF-4α in undifferentiated epithelial cells alone led to the rapid induction of HNF-1α and several intestinal-specific markers and metabolism-related genes for which mRNA was identified to be upregulated during epithelial differentiation. HNF-4α was capable to transactivate the calbindin 3 gene promoter, a process that was synergistically increased in the presence of HNF-1α. When HNF-4α-expressing cells were plated on mesenchymal cells, an epithelial monolayer formed rapidly with the apparition of dome structures that are characteristics of vescicular ion transport. Forced expression of HNF-1α alone did not result in dome structures formation. In sum, this novel coculture system functionally identified for the first time HNF-4α as an important modulator of intestinal epithelial differentiation and offers an innovative opportunity to investigate molecular mechanisms involved in this process.

intestinal epithelial cells; mesenchymal cells; HNF-4α; HNF-1α; differentiation

The intestinal epithelium is organized in crypts mostly populated by proliferative cells as well as villi composed of differentiated cells [for a review, see Sancho et al. (47)]. Stem cells constantly produce precursor cells that migrate along the crypt-to-villus axis. These progenitor cells progressively undergo a succession of molecular changes that lead to an arrest of proliferation and a commitment into specific differentiation programs completed as the cells reach the crypt-villus junction (40). Two different lineages will result from this commitment: the columnar absorptive enterocytes (comprising 90% of all small intestinal epithelial cells) and the secretory cell lineages that include goblet, enteroendocrine, and Paneth cells.

Intestinal homeostasis is dependent on a cross talk between epithelial and underlying mesenchymal cells (18, 33). Several signaling pathways that involve mesenchymal secreted molecules orchestrate this cellular communication (10). One example is the Wnt/β-catenin pathway that controls proliferation as well as migration of enterocytes and Paneth cells (16). Moreover, extracellular matrix deposition between the epithelial and mesenchymal compartments influences specific cell signaling in a position-dependent manner along the crypt-to-villus axis (2, 51).

Interactions between intestinal epithelial cells and their environment affect intrinsic gene expression (41, 47). To date, numerous endodermal transcription factors were identified to participate in intestinal epithelial gene regulation (59). Cdx2, an intestinal epithelial specific transcription factor, regulates transcriptional activity of several intestinal epithelial-specific genes [for a review, see Guo et al. (17)]. Other transcriptional regulators such as GATA-4 or hepatocyte nuclear factor-1α (HNF-1α) cooperate with Cdx2 and regulate transcription of sucrase-isomaltase (SI) (6), lactase-phlorizin hydrolase (31, 56), calbindin 3 (60), liver fatty acid binding protein gene (52), and claudin 2 (13, 46). Restriction of apolipoprotein A-IV (ApoA-IV) expression to villus-differentiated enterocytes has been functionally linked to hepatocyte nuclear factor-4α (HNF-4α) (49). Computer-based analyses recently suggested that HNF-4α could act as a regulator of villus genes involved in lipid metabolism (53). However, no functional data have clearly demonstrated the role of HNF-4α in small intestinal epithelial functions.

Immortalized intestinal epithelial cell (IEC) lines derived from the ileum have been established from weaned rats (43). Morphological and immunological characterization classified these cells as undifferentiated small intestinal crypt cells (42, 43). The first demonstration that these cell lines could differentiate came from xenografts experiment that involved combination of IEC-17 with fetal rat gut mesoderm (24). This experimental approach confirmed that IEC-17 could generate a mature epithelium composed of the four major intestinal epithelial cell lineages (24). The potential of these cells to differentiate in culture was provided when IEC-6 cells were engineered to conditionally express Cdx2 (54). The induction of Cdx2 resulted in irregular cellular proliferation with the accumulation of multicellular layers covered with few differentiated cells (54). Since the natural contact between epithelium and mesenchyme supplies IEC with extracellular matrix and soluble molecules (33, 34), we hypothesized that intestinal

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epithelial crypt cells needed to interact with mesenchymal cells to efficiently reproduce villus epithelial differentiation in culture.

Here, we present evidence that immortalized IEC seeded on fetal intestinal mesenchymal cells can rapidly polarize and differentiate in culture. These cells display the morphological characteristics of an epithelium and support the expression of multiple intestinal functional markers. A GeneChip analysis identified HNF-4α and HNF-1α as being strongly induced during IEC differentiation. Forced expression of HNF-4α in undifferentiated IEC induced the expression of several intestinal-specific markers and metabolism-related genes identified to be upregulated during IEC differentiation in coculture, a consequence that was not observed with forced expression of HNF-1α. This places HNF-4α as a strong candidate to support IEC differentiation.

MATERIALS AND METHODS

Establishment of mesenchymal primary culture. Human intestine from fetuses ranging from 17 to 20 wk of age (postfertilization) were obtained after legal abortion. The project protocol was approved by the Institutional Human Research Review Committee for the use of human material. The small intestine was divided in three equal parts and the most distal one was considered as the ileum where 5- to 8-cm fragments were used for mesenchymal cell isolation. Smooth muscle layers were pulled out with forceps and the intestinal tube was opened and cut into 0.5-cm² pieces. Ileum fragments were then incubated for 2 h at room temperature in an EDTA solution (3 mM EDTA, 0.05 mM DTT, in PBS) to separate the epithelium from the mesenchymal compartment (62). After isolation, mesenchymal pieces were incubated 4 h at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) containing 0.2 U/ml of collagenase H (Roche Diagnostics). Cells were recovered by a short centrifugation and seeded in 100-mm petri dishes. Cells were maintained under an atmosphere of 5% CO₂ at 37°C in DMEM supplemented with 4.5 g/l d-glucose, 25 mM HEPES, and 10% fetal bovine serum. Mesenchymal primary cultures were used between passages 3 and 8.

Cell coculture. The IEC-6 cell line was obtained from ATCC (CRL-1592). The IEC-6/L1 cell line was a gift from Dr. P. G. Traber. The IEC-6/L1-HNF-4α/Babepuro construct. The IEC-6/L1-HNF-1α cell line was generated by lentiviral infection of a rat HNF-4α/Babepuro construct. The IEC-6/L1-HNF-1α cell line was generated by lentiviral infection of a rat HNF-1α/pLent6/V5 construct. These cell lines were maintained in DMEM supplemented with 4.5 g/l d-glucose, 25 mM HEPES, 5% fetal bovine serum, and 0.1 U/ml of insulin as originally described (43, 54). Cells were maintained under an atmosphere of 5% CO₂ at 37°C and subcultured for 5 to 10 passages preventing each time the reach of confluence. Human primary mesenchymal cells were seeded in their complete growth medium (DMEM 10%) in an appropriate number to result in 105 cells/cm². Cells were maintained in a humidified atmosphere of 5% CO₂ at 37°C and subcultured for 5 to 10 passages preventing each time the reach of confluence. Human primary mesenchymal cells were seeded in their complete growth medium (DMEM 10%) in an appropriate number to result in 105 cells/cm². Cells were maintained in a humidified atmosphere of 5% CO₂ at 37°C and subcultured for 5 to 10 passages preventing each time the reach of confluence.

RNA interference targeting. Short hairpin RNA (shRNA) oligonucleotides were designed by using the small interfering RNA sequences GCTGCGATGCTGATAAGA and TTCAAGAGA as the loop sequence as described previously (5). The oligonucleotide-annaled product was subcloned into the plent6-U6 between BamH I and Xho I sites, giving rise to pLenti-shHNF-4α. An irrelevant (control) pLenti-sheGFP negative control was used in parallel (5). Lentiviruses were produced and used for cell infection accordingly to Invitrogen recommendations (ViraPower Lentiviral Expression System, instructions manual).

Electron microscopy. Cell cultures were prepared exactly as reported before (5). Thin sections were prepared by using an ultramicrotome, contrasted with lead citrate and uranyl acetate, and observed on a Jeol 100 CX transmission electron microscope. All reagents were purchased from Electron Microscopy Sciences (Cedarlane).

RNA isolation and RT-PCR analysis. Total RNA was isolated from cultured cells and subjected to a DNase treatment according to the manufacturer’s instructions (Totally RNA kit). Reverse transcriptions were carried out at 42°C for 1 h in the presence of 2 μg RNA, 40 U of polyoligo(dT)12-18, and 40 U of Reverse Transcriptase (Roche Diagnostics). Semiquantitative PCR was performed in a total volume of 20 μl in the presence of 1 μl of RT reaction, 0.6 U of Taq DNA polymerase (New England Biolabs), 0.2 mM dNTPs, and 30 ng of each specific primer. Primer sequences are available upon request. Quantitative RT-PCR (qRT-PCR) was performed using a LightCycler apparatus (Roche Diagnostics). Experiments were run and analyzed with the LightCycler software 4.0 according to manufacturer’s recommendations (Roche Diagnostics). Synthesis of double stranded DNA during the PCR cycles was monitored with SYBR Green 1 (QuanttiTect SYBR Green PCR Kit; Qiagen) and PCR programs designed as detailed in the QuanttiTect SYBR Green PCR Handbook (Qiagen). Target expression was quantified relatively to porphobilinogen deaminase or TATA binding protein expression. For this purpose, a standard calibration curve was prepared for each gene by using serial dilutions of the calibrator sample and cross point values were plotted vs. the log of the relative concentration of each dilution. This standard curve was used to correct for differences in PCR efficiencies.

Microarray screening and data analysis. Probes for the microarray analysis were generated from isolated RNA obtained at the 12 generated probes via the microarray platform of McGill University and Génome Québec Innovation Center (http://genomequebec.mcgill) exactly as described previously (35). To test for statistically significant changes in signal intensity (P values of ≤0.05), compiled data (robust multichip averaging analysis) were screened by using the software available on the microarray platform website. Genes were then filtered for up- or downregulation of expression of a minimum of 2.5-fold and a minimum magnitude change of 200 fluorescence units between day 1 and 8. Graphical representations as well as gene ontology analyses were performed with the use of the GeneSifter software (VizX).

Western blot analysis. Cell total extracts preparation and Western blotting were performed exactly as described previously (5). The following antibodies from Zymed Laboratories (Invitrogen) were used: occludin affinity-purified rabbit polyclonal antibody (no. 71-1500) and claudin-4 affinity-purified rabbit polyclonal antibody (no. 38-4800). The following antibodies from Santa Cruz Biotechnology (Santa Cruz) were used: HNF-1α affinity-purified goat polyclonal antibody (no. SC-6547), HNF-4α affinity-purified goat polyclonal antibody (no. SC-6556), GATA-4 affinity-purified goat polyclonal antibody (no. SC-1237), and actin affinity-purified goat polyclonal antibody (no. SC-1615). A rabbit polyclonal antibody raised against CDX2/3 was kindly provided by Dr. Rivard (CDX2/3-NR) (8).

Indirect immunofluorescence. Cells were cocultured in Lab-Tek chamber slides (Nalge Nunc International) for 10 days, fixed in 2% paraformaldehyde for 30 min at 4°C, permeabilized in 0.1% Triton X-100 in PBS, and blocked for 20 min in 2% bovine serum albumin in PBS. Primary antibody was diluted in the blocking solution and incubated with the cells for 2 h at room temperature. The following antibodies were used: occludin mouse monoclonal antibody (no. 33-1500, Zymed Laboratories), β-catenin affinity-purified rabbit polyclonal antibody (no. 9587, Cell Signaling Technology). Claudin-4 antibody was the same as described above. For F-actin staining, fixed cells were incubated with 1 μg/ml phalloidin-fluorescein isothiocya-
nate conjugated in replacement of the primary antibody. Slides were then washed in PBS and incubated 1 h at room temperature with the appropriate secondary antibody (Vector Laboratories) diluted in the blocking buffer.

**Transient transfections and luciferase assays.** The rat calbindin 3 promoter was amplified by PCR from purified genomic DNA isolated from IEC-6 cells. The primers used for the amplification included positions -1004 and +43 relatively to the transcriptional initiation site. The PCR product was subcloned into the pGL3basic luciferase reporter vector (Promega). Integrity of the subcloned PCR product was confirmed by sequence analysis. HEK293T cells were transfected by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendations. Cells at 50% confluence were incubated with 0.2 µg of luciferase reporter, 0.025 ng of HNF-4α and/or HNF-1α expression vector, 0.6 ng of the pRL SV40 Renilla luciferase vector (Promega), and a constant total DNA amount of 0.8 µg per transfected well in the presence of 2 µl of Lipofectamine 2,000/600 µl of DMEM containing 10% fetal bovine serum. The medium was replaced by fresh DMEM after an incubation of 4 h. The luciferase and Renilla activities were determined 48 h after the transfection using the dual luciferase assay kit (Promega Biotech). Each experiment was repeated four times in triplicate.

**RESULTS**

**Coculture of IEC-6/L1 with intestinal mesenchymal cells results in morphological differentiation.** IEC-6/L1 cells are derivative of IEC-6 cells modified to express Cdx2 (54). These cells proliferate in multicellular layers and differentiate poorly in vitro. To stimulate IEC-6/L1 cells to efficiently differentiate into a monolayer that mimics the normal intestinal epithelium, a coculture system was engineered. IEC-6/L1 cells were cultured on top of a monolayer of primary mesenchymal cells isolated at day 10 and 21 relatively to the transcriptional initiation of IEC in coculture. The parental IEC-6 cell line (43), negative for Cdx2 expression (54), remained flat and undifferentiated when it was cocultured under the exact same experimental conditions (data not illustrated). In addition, the prevention of physical contact between IEC6/L1 and mesenchymal cells with the use of Transwell permeable supports was inefficient to promote formation of differentiated clusters. These observations indicated that expression of Cdx2 in IEC as well as direct mesenchymal contact was decisive to allow IEC to rapidly polarize in a monolayer and to differentiate under these conditions.

**A gene profiling analysis confirmed the functional differentiation of IEC in coculture.** To better characterize the nature of the molecular changes occurring during differentiation of IEC-6/L1 in the coculture system, a gene expression profiling was next performed. To provide a stringent characterization of the changes occurring during the rapid differentiation of IEC/L1 cells, the analysis was performed in triplicate with RNA isolated at days 1, 3, 5, and 8 after the seeding of IEC-6/L1 on mesenchymal cells. The GeneChip Rat Genome 230 2.0 Arrays (Affymetrix) that contains more than 30,000 transcripts from the rat genome was utilized to screen for mRNA expression variations that occurred specifically in the rat IEC-6/L1 cell line. Statistical analysis predicted more than 10,000 rat transcripts being significantly modulated between days 1 and 8 of the coculture (P value ≤0.05). When more stringent conditions

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**Fig. 1.** Morphological features of IEC-6/L1 cocultured with human fetal mesenchymal cells from the ileum. A: light morphology of IEC-6/L1 monolayer at coculture day 8. Bar = 100 µm. B and C: electron microscopic analysis of IEC-6/L1 cocultured for 10 days with mesenchymal cells. Bar = 2 µm (B). Bar = 500 nm (C).
were applied (differential ratio $\geq 2.5$ and relative expression difference $\geq 200$), the number of transcripts reached 1,220 (Supplementary Tables S1 and S2). A gene ontology analysis for biological processes among these modified genes revealed an important representation for cellular processes related to macromolecule metabolism, response to stress, lipid metabolism, nucleic acid metabolism, immune response, and cell adhesion. The expression pattern of well-known intestinal epithelial cell differentiation markers was assembled (Fig. 2A). In general, these genes were not expressed in IEC-6/L1 cell line at day 1 but were induced with the apparition and multiplication of polarized cells between days 3 and 8 (Fig. 2A). The expression of several intestinal apolipoproteins and molecules implicated in lipid uptake and transport was markedly induced between days 3 and 8 (Fig. 2A).

To further validate some targets identified in the GeneChip analysis, expression of SI and ApoA-IV was monitored by RT-PCR. As expected, expression of SI was undetectable in the coculture system from days 1 to 6 but became assessable at day 8 with increasing expression throughout day 12 (Fig. 2B). In parallel, SI mRNA expression was undetectable in IEC-6/L1 cultured on plastic or IEC-6 cocultured on mesenchymal cells (Fig. 2B). ApoA-IV mRNA expression was undetectable at days 1 and 3, became expressed at day 5, and was upregulated between days 5 and 8 as quantified by qRT-PCR (Fig. 2C). ApoA-IV modulation of expression was quantitatively equivalent to the GeneChip profiling data obtained during IEC differentiation (Fig. 2C). A similar pattern was observed for ApoA-I, ApoB, ApoC-III, and Apobec, validating the data in Fig. 2A (data not illustrated). These results confirmed that

**Fig. 2.** Intestinal epithelial functional gene markers are expressed in cocultured IEC-6/L1. A: expression profiles of intestinal epithelial cell (IEC) differentiation markers and molecules implicated in lipid uptake and transport. B: semiquantitative RT-PCR analysis of sucrase-isomaltase (SI) expression was performed in IEC-6/L1 cells harvested at different time points after seeding on mesenchymal (mes.) cells (left) as well as in IEC-6/L1 or IEC-6 cultured in the coculture medium on plastic or on mesenchymal cells for 12 days (right). Total RNA from rat ileum was used as a positive control, and $\beta_2$-microglobulin mRNA level was monitored as a housekeeping gene control. C: comparison of apolipoprotein (Apo) A-IV relative mRNA expression monitored by GeneChip and quantitative (Quant.) RT-PCR analysis.
IEC-6/L1 cells can be rapidly induced to express multiple markers of functional differentiated enterocytes when cocultured with mesenchymal cells.

Polarized IEC developed well-defined cell junction complexes in coculture. Physiological functions of the mature intestinal epithelium are largely dependent on the establishment and maintenance of functional cell junction complexes (30, 32). Cell junction complexes components were detectable in cocultured IEC cells with several of them showing an upregulated expression during the time course (Fig. 3A). Indeed, occludin mRNA and protein levels were upregulated during the course of the coculture (Fig. 3A and B). Claudin-4 mRNA expression began to be detectable between days 5 and 8 (Fig. 3A), a pattern that was confirmed at the protein level (Fig. 3B). To further determine whether IEC developed well-defined cell to cell junctions in the coculture conditions, localization of cell junction proteins was next investigated by immunofluorescence. The tight junction proteins occludin and claudin-4 as well as adherens junction molecule β-catenin were localized at the apical membrane of polarized cells (Fig. 3C). The cytoskeleton component F-actin was also colocalized at the apical membrane with β-catenin (Fig. 3C). These results supported that cocultured IEC can develop well-established cell junction complexes linked to the cellular cytoskeleton.

Specific transcription factors of endoderm-origin are sequentially modulated in the coculture system. A number of regulators crucial to sustain the transcriptional activity of intestinal specific gene promoters have been identified (6, 7, 11, 49, 58). Characterization of the expression profile of some of these transcriptional modulators during the progression of the cocultures was next undertaken. Data from the gene profiling analysis indicated a statistical increase in Cdx2, GATA-4, HNF-1α, and HNF-4α mRNA expression when day 8 was compared with day 1 (Fig. 4A). Total protein extracts were then sequentially isolated from several coculture kinetics and Western blot analyses performed. The protein expression profile of these factors correlated well with the variations in mRNA expression during the coculture kinetics. Cdx2 and GATA-4 expression was progressively increased during the kinetics (Fig. 4B). A prominent induction of both HNF-1α and HNF-4α was observed around day 6, a time when polarized IEC structures had just appeared (Fig. 4B). HNF-1α and
HNF-4α protein was not detected in IEC-6/L1 cultured on plastic or in IEC-6 cocultured on primary mesenchymal cells (Fig. 4C). This indicated that both Cdx2 and mesenchymal cells were essential for the induction of these regulators within these conditions.

**HNF-1α is a downstream target of HNF-4α in IEC and cooperates with HNF-4α in transcriptional modulation of intestinal specific genes.** HNF-1α was shown to participate in the transcriptional regulation of several intestinal specific genes (6, 11, 31, 46, 57, 60). HNF-4α was recently suggested to act as a regulator of villus specific epithelial genes (53). However, the specific functional role of these regulators on IEC differentiation has never been directly assessed. We thus undertook to verify whether reintroduction of HNF-4α or HNF-1α was potent to induce expression of intestinal epithelial genes normally associated with IEC differentiation state. IEC-6/L1-HNF-4α lentivirus was generated and stably introduced into various IEC-6/L1-HNF-4α established cell populations as represented in Fig. 5A. Interestingly, HNF-1α protein was detectable among multiple IEC-6/L1-HNF-4α stable cell populations as reported in hepatocytes (26, 36). The expression of several genes linked to differentiation and metabolism and identified to be upregulated during the coculture kinetics (Fig. 2 and Supplementary Table S1) was next monitored at the gene transcript levels in these populations of cells. HNF-4α led to the induction of expression of several intestinal epithelial genes whereas HNF-1α alone did not impact on these genes (Fig. 5C). An experiment to downmodulate HNF-4α was next designed by a knockdown approach. A lentivirus construct that contained a shRNA sequence under the control of an U6 promoter and predicted to target the rat HNF-4α gene transcript was subcloned into a luciferase reporter vector (Fig. 7A). Specific detection of actin was done to control for protein integrity. A Western blot analysis was performed on total cell lysates with specific antibodies for the detection of HNF-4α and HNF-1α. Specific detection of actin was done to control for protein integrity. B: IEC-6/L1 cells were infected with empty or HNF-4α expression vector, and total RNA was harvested at different hours following the infection. Semiquantitative RT-PCR detection of HNF-4α and HNF-1α was performed in parallel to β2-microglobulin (β2-mic). C: semiquantitative RT-PCR detection of mRNA for several intestinal functional gene markers. β2-microglobulin mRNA level was monitored as a housekeeping gene control.

Fig. 5. HNF-4α induces expression of HNF-1α and intestinal functional gene markers in IEC-6/L1 cultured on plastic. A: IEC-6/L1 cells were infected with empty vector (control), HNF-1α, or HNF-4α expression vectors. After selection, stable IEC-6/L1 cell populations were harvested. Western blot analysis was performed on total cell lysates with specific antibodies for the detection of HNF-4α and HNF-1α. Specific detection of actin was done to control for protein integrity. B: IEC-6/L1 cells were infected with empty or HNF-4α expression vector, and total RNA was harvested at different hours following the infection. Semiquantitative RT-PCR detection of HNF-4α and HNF-1α was performed in parallel to β2-microglobulin (β2-mic). C: semiquantitative RT-PCR detection of mRNA for several intestinal functional gene markers. β2-microglobulin mRNA level was monitored as a housekeeping gene control.
Importantly, specific genes associated with terminal differentiation (for example, SI) were not induced under these conditions. This correlated with the poor potential of IEC-6/L1-HNF-4/H9251 and IEC-6/L1-HNF-1/H9251 cells to polarize and differentiate in a short-term range on plastic (data not illustrated). This suggested that HNF-4/H9251 and/or HNF-1/H9251 presence was not sufficient to trigger IEC-6/L1 cell terminal differentiation and required signaling from mesenchymal cells during this process.

HNF-4/H9251 but not HNF-1/H9251 promotes the formation of a fully functional polarized epithelium in culture only in the presence of cdx2 and mesenchymal cells. One critical feature of the mature intestinal epithelium is to exert a selective barrier function in relation to apical-to-basal transport. To test whether HNF-4α or HNF-1α could be involved in such processes, IEC-6/L1-HNF-4α, IEC-6/L1-HNF-1α, and control cells were plated on mesenchymal cells accordingly to the protocol described above. IEC that overexpressed HNF-4α did not form scattered clusters as opposed to HNF-1α or control cells (compare Fig. 8, A and B). However, large dome structures appeared rapidly at the surface of HNF-4α cocultured cells (Fig. 8B). These large domes were individually filled with trapped liquid material as directly visualized by experimenter’s eye and also illustrated in a low-magnification field (Fig. 8C). Electron microscopy confirmed the dome structure with the

Fig. 6. Downmodulation of HNF-4α with RNA interference reduces expression of intestinal functional markers in IEC-6/L1-HNF-4α cultured on plastic. A: real-time PCR was performed with total RNA isolated from IEC-6/L1-HNF-4α populations of cells that had stably integrated HNF-4α or irrelevant (control) short hairpin RNA (sh) lentiviruses. The expression of fatty acid binding protein 2, apolipoprotein A-IV, and guanylate cyclase 2c was quantified relative to TATA binding protein (TBP) (means ± SD, n = 3). B: Western blot analysis was performed with HNF-4α, HNF-1α, and actin polyclonal antibodies on total cell lysates obtained from populations of cells that had stably integrated HNF-4α or irrelevant (control) sh lentiviruses.

HNF-4α and HNF-1α synergistically activate the calbindin 3 gene. A: quantitative RT-PCR detection of calbindin 3 mRNA in stable IEC-6/L1 cell populations infected with an empty vector (control), HNF-1α, and HNF-4α. TBP mRNA level was monitored as a housekeeping gene control. Results obtained in triplicate were reported as the fold difference (mean ± SD) from the control values. B: 293T cells were cotransfected with HNF-4α, HNF-1α, and actin polyclonal antibodies on total cell lysates obtained from populations of cells that had stably integrated HNF-4α or irrelevant (control) sh lentiviruses.
suggested this factor was able to promote apical-basal transport in the presence of mesenchymal cells, a feature strictly restricted to a fully functional intestinal epithelium.

**DISCUSSION**

Intestinal epithelial cell differentiation is tightly regulated to maintain intestinal homeostasis (27, 44). Coordination of gene transcription is recognized to be closely related to the regulatory processes that promote the maturation and maintenance of intestinal epithelial functions. Although molecular pathways involved in these processes were largely investigated (59), the precise and sequential characterization of IEC differentiation was hampered by the lack of adequate cellular systems that can recapitulate this process in vitro. Well-characterized culture models of normal cryptlike cells already exist (38, 43). However, these cellular models lack the capacity to differentiate in normal cell culture conditions. The overexpression of Cdx2 in the IEC-6 crypt cell line (IEC-6/L1) can lead to primitive cellular differentiation in vitro (54). Indeed, these cells grow in multicellular layers after reaching confluence, and few cells will present differentiation features after 30 days in culture. In addition, HNF-4α and HNF-1α protein expression remain beyond the limit of detection even after this extensive period of time (data not illustrated). The present study aimed to culture IEC-6/L1 cells on top of mesenchymal cells to reconstitute a microenvironment more representative of the normal small intestinal mucosa (33, 34, 41). The cocultured IEC-6/L1 monolayer rapidly developed clusters of cells that began polarization and displayed morphological features that resembled differentiated enterocytes. The complete gene profiling analysis performed during the course of these morphological modifications supported that IEC were in the process of differentiation as illustrated with the complete and progressive panel of genes modulated during the course of this process. This analysis identified HNF-4α and HNF-1α as being strongly induced coincidently with the beginning of the differentiation process. Molecular analyses confirmed that HNF-4α was potent to activate intestinal gene transcript expression and that HNF-1α could be synergized with HNF-4α for the transcriptional activation of the calbindin 3 gene normally restricted to differentiated IEC. Finally, reintroduction of HNF-4α into the coculture conditions demonstrated its crucial role into the promotion of intestinal epithelial barrier functions.

Multiple signaling pathways implicated in IEC differentiation emerge from the cross talk between mesenchymal and epithelial cells (47). Signaling between these compartments involves secreted soluble and insoluble factors from the epithelium or the mesenchyme that can transduce a signal to neighbor cells. Members of the bone morphogenetic proteins (19, 21) and hedgehog (29, 55) families are well-described modulators of such interactions. Indirect modulators of epithelial-mesenchymal interactions were also identified. For example, the specific mesenchymal transcription factor Foxl1 controls epithelial Wnt signaling via overproduction of epithelial proteoglycans (39). Since the presence of mesenchymal cells is crucial for IEC-6/L1 efficient differentiation in vitro, communication between the two cell compartments is obvious. This can implicate regulation at multiple levels including protein phosphorylation and molecule translocation from one compartment to another as well as mesenchymal specific modification.

**accumulation of substrate in between IEC and mesenchymal cells (Fig. 8D).** In addition, IEC-6/L1-HNF-4α cells presented greater polarization features compared with control cells (compare Fig. 8, D and E) and IEC-6/L1-HNF-1α cells (data not illustrated). Indeed, the brush border was much more developed and the nucleus was localized at the basal side of enterocytes (Fig. 8D, arrows). Overall, these observations confirmed that HNF-4α was potent to induce differentiation-related genes in IEC. In addition, the formation of large dome structures that were dependent on the presence of HNF-4α...
of gene expression (16, 22, 63). Our findings demonstrate that mesenchymal cells are crucial to instruct IEC to endogenously express HNF-4α. It will be of interest to investigate the specific mesenchymal-to-epithelial signaling cascade responsible for the activation of epithelial HNF-4α gene expression.

The concept that immortalized IEC cells can retain progenitor properties has been provided in xenograft experiments in the past (24). Our results support the notion that Cdx2 expression is required for IEC to initiate differentiation into mature enterocytes in the mesenchymal context. Cdx2 expression increases with polarization and differentiation of IEC clusters. This observation is consistent with the increasing gradient of Cdx2 expression along the intestinal crypt (8, 23, 45). Other epithelial cell lineages (goblet and enteroendocrine cells) were rarely but detected throughout the coculture system (data not illustrated). This agrees with the normal composition of the intestinal mucosa in which secretory cells represent only 5 to 10% of total epithelial cells.

Some transcription factors were identified to modulate intestinal gene expression (59). Cdx2 modulates cellular functions and intestine-specific gene expression (6, 17, 50, 54). GATA-4 and HNF-1α are expressed in the intestinal epithelium and cooperate in transcriptional regulation of intestinal epithelial specific genes such as SI (6). Specific induction of these factors in differentiating IEC (Fig. 4) emphasizes the initiation of a differentiation program within the coculture system. Some suggestions from the literature have linked HNF-4α transcriptional action with the process of epithelial cell polarization (1, 9, 48). HNF-4α can induce the expression of cell junction molecules such as occludin and Claudins and can modulate subcellular localization of proteins associated with cellular polarity (9, 48). Interestingly, HNF-4α expression was detectable at day 5 when occludin and Claudin family members were upregulated in IEC polarized cells. HNF-4α is considered to be the master regulator of the human hepatocyte where it controls the expression of molecules implicated in lipid uptake and transport (20, 28, 36, 61). HNF-4α is expressed along both the vertical and horizontal axis of the gut epithelium and regulates intestinal epithelial expression of alkaline phosphatase, ApoA-IV, and meprin-1α genes (37, 49, 53). Conditional knockout of HNF-4α demonstrated its crucial role into the embryonic development of the mouse colonic epithelium, but its role in the small intestinal context still remains to be investigated (14). Metabolome, transcriptome, and bioinformatic analyses indicated that HNF-4α could be implicated in intestinal lipid metabolism with the regulation of apolipoprotein synthesis (53). Our findings demonstrate that HNF-4α is a potent activator of several intestinal genes normally associated with mature enterocyte functions. In addition, HNF-4α is able to rapidly induce HNF-1α gene transcript and protein levels within the IEC context. This molecular interaction is probably due to a direct transcriptional action of HNF-4α on the HNF-1α promoter since it was previously reported that HNF-4α can directly control the transcription of HNF-1α in hepatocytes (26, 36). HNF-1α was also shown to control HNF-4α transcription specifically in pancreatic cells (3), a relationship that was not observed in our culture model system. Our observations lead us to suggest that HNF-4α is required for the activation of HNF-1α expression in IEC. Since HNF-4α and HNF-1α can also physically interact to modulate gene transcription (12, 25), these transcription factors probably form a multicomponent transcriptional loop to regulate intestinal gene transcription. In support of this affirmation, our results show that HNF-4α can strongly collaborate with HNF-1α with the transcriptional activation of the calbindin 3 gene. Since the calbindin 3 gene 5’-flanking region contains predicted interacting sites for HNF-4α and since HNF-1α was already reported to influence calbindin 3 transcription via sev-

Fig. 9. Schematic of the cascade of events that lead to IEC differentiation. A: Cdx2 is required to initiate IEC to polarize and differentiate when cocultured on mesenchymal cells. This coincides with the induction of HNF-4α and HNF-1α only in the presence of Cdx2 and mesenchymal cells. B: HNF-4α activates HNF-1α and several intestinal epithelial genes. Forced expression of HNF-4α promotes features associated with apical-to-basal transport, a feature limited to well-polarized and fully differentiated epithelial cells.
eral interacting sites (60), the exact nature of the molecular interactions between these transcription factor and the calbindin gene must be complex.

In conclusion, this study led to the generation and detailed molecular characterization of a functional in vitro system for IEC polarization and differentiation and functionally identified for the first time HNF-4α as an important contributor for these actions. Combinatory mechanisms between multiple transcriptional regulators are likely to occur during IEC differentiation, and our findings contribute to a better definition of the molecular cascade that led to the establishment of the intestinal epithelial transcriptome. On the basis of our findings, we propose the following schematic (Fig. 9): Cdx2 is crucial to initiate IEC to polarize and differentiate when cocultured on mesenchymal cells. These processes are coordinated with the rapid activation of HNF-4α and HNF-1α expression, which requires both mesenchymal signals and Cdx2 action (Fig. 9A). On the other hand, HNF-4α activates HNF-1α expression. HNF-4α is a potent activator of intestinal epithelial genes and collaborates with HNF-1α to regulate intestinal genes. Forced expression of HNF-1α in the coculture system does not improve differentiation, an observation that correlates well with the absence of morphological defect in the intestinal epithelium of HNF-1α knockout mice (4). However, forced expression of HNF-4α leads to complete polarization and features normally associated with apical-to-basal transport (15) (Fig. 9B). Genetic modifications of the cellular counterparts of this system will give novel opportunities to sequentially assess the function of any molecules of interest during intestinal differentiation and should allow a better definition of the crucial pathways that regulate maturation of intestinal epithelial cells.

ACKNOWLEDGMENTS

The authors thank Dr. Peter G. Traber (Baylor College of Medicine, Houston) for providing the IEC-6/L1 cell line, Denis Martel and Charles Bertrand for assistance in electron microscopy procedures, and the Microarray Platform Lab from McGill University and Genome Québec Innovation Centre for the generation of the CDNA arrays.

GRANTS

This work was supported by Canadian Institute of Health Research (to F. Boudreau) and the Natural Sciences and Engineering Research Council of Canada (NSERC; Grant no. 262094-03). C. R. Lussier and J. P. Babeu are supported by the NSERC. F. Boudreau and N. Perreault are scholars from the Fonds de la Recherche en Santé du Québec and members of the FRSQ-funded Centre de Recherche Clinique Étienne E. Lebel.

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