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Mammary Epithelial Cells Are Not Able to Undergo Pregnancy-Dependent Differentiation in the Absence of the Helix-Loop-Helix Inhibitor Id2

KEIKO MIYOSHI, BARBARA MEYER, PETER GRUSS, YONGZHI CUI, JEAN-PIERRE RENOU, FANTA V. MORGAN, GILBERT H. SMITH, MOSHE REICHENSTEIN, MOSHE SHANI, LOTHAR HENNIGHAUSEN, AND GERTRAUD W. ROBINSON

Laboratory of Genetics and Physiology (K.M., Y.C., J.-P.R., F.V.M., M.S., L.H., G.W.R.), National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, and Basic Research Laboratory (G.H.S.), National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892; Department of Biochemistry (K.M.), School of Dentistry, The University of Tokushima, Tokushima 770-8504, Japan; Department of Molecular Cell Biology (B.M., P.G.), Max Planck Institute of Biophysical Chemistry, Goettingen 37077, Germany; Institut National de la Recherche Agronomique (J.-P.R.), Jouy-en-Josas Cedex 78280, France; and Agricultural Research Organization (M.R., M.S.), Volcani Center, Bet Dagan 50250, Israel

Mammary alveolar development during pregnancy is triggered by hormone signals. The prolactin receptor/Jak2/signal transducer and activator of transcription (Stat) 5 signal transduction pathway is the principal mediator of these cues and alveolar development is abrogated in its absence. The loss of the basic helix-loop-helix protein inhibitor of differentiation (Id)2 results in a similar defect. To investigate the role of Id2 in mammary epithelium, we performed structural and molecular analyses. Id2-null mammary epithelial cells were unable to form alveoli; the epithelial architecture was disorganized and dissimilar from early stages of alveologenesis in wild-type glands. The epithelial cells retained the ductal marker Na-K-Cl cotransporter (NKCC)1. Nuclear localization of Stat5a and down-regulation of NKCC1 was observed in some areas, indicating a limited response to pregnancy signals. The differentiation status of Id2-null tissue at term was further characterized with cDNA microarrays enriched in mammary specific sequences (mammochip). Some of the early differentiation markers for mammary epithelium were expressed in the Id2-null tissue, whereas genes that are expressed at later stages of pregnancy were not induced. From these results, we conclude that, in the absence of Id2, mammary epithelial development is arrested at an early stage of pregnancy. (*Molecular Endocrinology* 16: 2892–2901, 2002)

MAMMARY ALVEOLAR EPITHELIUM proliferates and differentiates during pregnancy through the combined action of growth factors and peptide and steroid hormones (1). Gene deletion studies in mice have identified a number of genes whose absence disrupts mammary gland development (2). Not surprisingly, members of the prolactin (PRL) signaling pathway are indispensable in this process. The PRL receptor (PrlR), Jak2 and signal transducer and activator of transcription (Stat)5 play a central role in the specification (3, 4), proliferation, and differentiation (5–8) of mammary alveolar epithelium. Mammary epithelial cells that lack one of the partners in this signaling pathway fail to develop into secretory alveolar cells and maintain ductal characteristics during pregnancy. Recently, it was found that the helix-loop-helix inhibitor of differentiation (Id2) is also required for mammary alveolar proliferation during pregnancy and formation of alveolar structures is attenuated in Id2-null mammary epithelium (9). The lack of expression of the whey acidic protein (WAP) and α-lactalbumin, and the reduced expression of β-casein and WDNM1 further suggested a role of Id2 in cell differentiation. Stat5 activity was reduced in Id2-null mammary tissue, indicating that the lack of complete lobulo-alveolar development was due to impaired PRL signaling, and it was speculated that Id2 indirectly communicates with the Jak2/Stat5 pathway (9).

Id proteins form heterodimers with members of the basic helix-loop-helix family of transcription factors and regulate cell growth and differentiation. They act as dominant-negative inhibitors of their binding partners, some of which are ubiquitously expressed or restricted to specific tissues. Depending on the binding partners, they have been found to inhibit tissue-specific transcription and differentiation or stimulate cell proliferation. These initial results on the dual role of Id proteins in cell regulation obtained in tissue culture cells have been corroborated more recently by gene deletion experiments in mice (10).

Abbreviations: Id, Inhibitor of differentiation, NKCC, Na-K-Cl cotransporter; PRL, prolactin; PrlR, PRL receptor; SREBP, sterol regulatory element-binding protein; Stat, signal transducer and activator of transcription; WAP, whey acidic protein; wtL, wild-type lactating; wtV, wild-type virgin.

The morphological similarity between the mammary gland phenotype of Id2-null mice with mice defective in PRL signaling prompted us to investigate the molecular lesions and differentiation status of Id2-null epithelium. We specifically investigated whether Id2 was required for the specification of the alveolar lineage within the mammary gland. Electron microscopy, histological staining for cell specific markers, and microarray-based expression analyses demonstrated that Id2 is required for functional differentiation of alveolar epithelial cells.

RESULTS AND DISCUSSION

Lack of Alveolar Development in the Absence of Id2

The lack of Id2 results in a lactation defect in mice and was ascribed to an intrinsic defect in cell proliferation and survival (9). To identify the cell population affected by the loss of Id2 and to understand the nature of the developmental disruption, we have analyzed Id2-null mammary epithelium on a histological level and have localized indicators, which identify distinct cell types in the mammary gland. To eliminate systemic effects of the Id2 mutation, which also affects the immune system and may create alterations in hormonal stimuli, we transplanted mammary epithelium from Id2-null and wild-type mice into contralateral cleared fat pads of wild-type recipients. Outgrowth and development were analyzed in virgin mice and after the first pregnancy. Whole mounts obtained from wild-type and Id2-null transplanted mammary epithelium from mature virgins did not reveal any significant differences and Id2-null epithelium completely filled the fat pad (data not shown). In hematoxylin and eosin-stained sections the epithelial cells of Id2-null virgin ducts appeared slightly disorganized compared with wild-type epithelium. The cells were irregularly shaped, and in some regions the cellular contact seemed disrupted (data not shown).

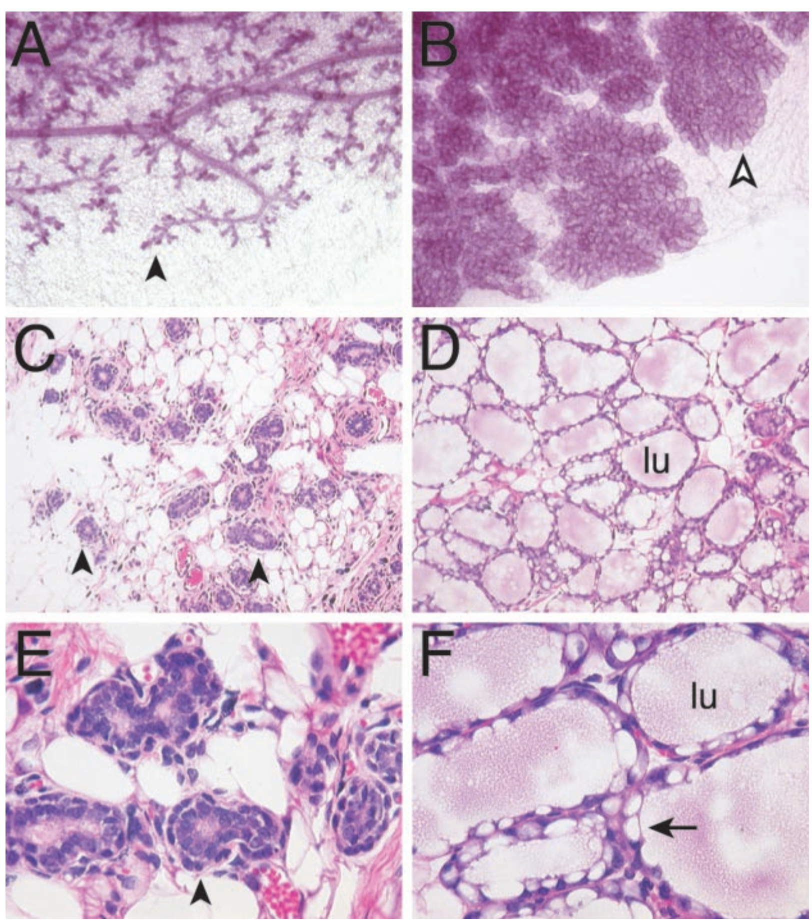

Fig. 1. Histological Analyses of Id2-Null and Wild-Type Transplanted Mammary Tissue at Parturition

Mammary tissue from wild-type and Id2-null mice was transplanted into wild-type hosts and tissues were isolated at term after the first pregnancy. A, Whole mount of Id2-null tissue. B, Whole mount of control tissue. C and E, Histological sections from the whole mount shown in A and B. D and F, Histological sections from the whole mount shown in A and B at higher magnification. *Black arrowhead*, Alveolar-like structures; *white arrowhead*, secretory alveoli; *arrow*, lipid droplet; lu, lumen.

Id2-null epithelium isolated after one pregnancy at parturition lacked identifiable functional alveoli (Fig. 1A) compared with wild-type transplants (Fig. 1B). The ductal tree was similar to that observed in virgin glands, but additional side branching had occurred. The structures decorating the ductal tree were reminiscent of immature alveoli (Fig. 1A). Histological sections of wild-type transplants at parturition revealed large alveoli with expanded lumina (Fig. 1, D and F). The alveolar cells were actively secreting milk as judged by the presence of large lipid droplets in the cytoplasm (Fig. 1F). In contrast, there was no histological evidence of lipid droplets in the Id2-null transplants (Fig. 1E). The number of ducts and small alveolar-like units was increased compared with virgin tissue. However, the alveoli were compact and not expanded as in tissue from wild-type transplants. In Id2-null tissue, small central lumina were present that frequently were filled with secreted material of unknown nature (Fig. 1C). The cells forming the lumina were irregularly shaped, had large nuclei, and the most conspicuous feature was an irregular basal surface due to abnormal appearance of myoepithelial cells (Fig. 1E). These cells, which in wild-type tissue are rather flat and closely attached to the secretory cell layer, appeared distant from the central cells and had a round shape. They were multilayered in some areas.

Evaluation of the ultrastructure of Id2-null epithelium in the electron microscope confirmed some of the features already evident on the level of light microscopy (Fig. 2A). Although a small central lumen was present, the architecture of the tissue was severely disorganized. In Id2-null epithelium harvested at term, the alveolar cells did not display the usual organized orientation toward the lumen, neighboring cells, and the basement membrane. The cells had large nuclei and a small cytoplasm, and they displayed limited secretory activity that was not directed toward the apical membrane and lumen. Most conspicuously, small lipid droplets were found in basal regions of secretory cells and also uncharacteristically in myoepithelial cells that contained actin filaments. It appeared that the cells had lost their polarity and were secreting in a nonpolarized way. Furthermore, cells identified as myoepithelial due to a prominent actin filament system were found interspersed with luminal cells away from the basal lamina, a situation not observed in normal developing tissue. Along most of the tissue interphase,

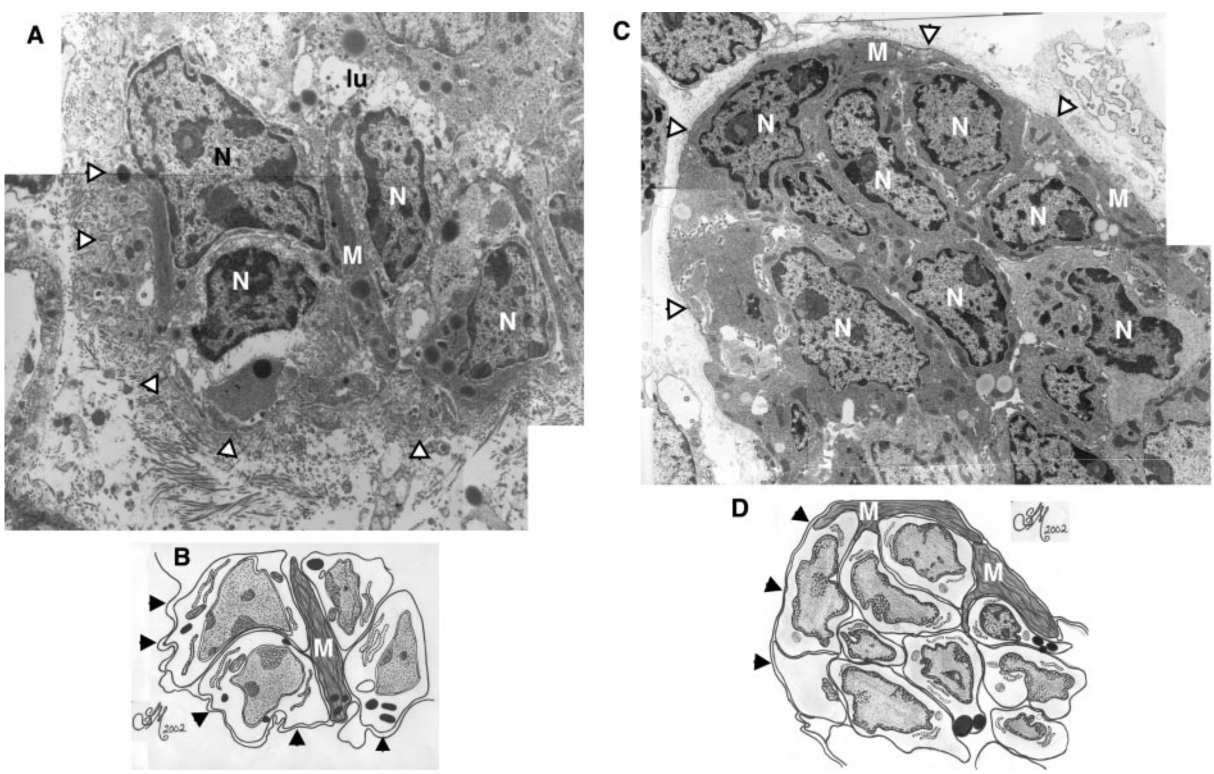

Fig. 2. Electron Microscopic Analysis of Id2-Null and Wild-Type Mammary Tissue

A, Micrograph of Id2-null term tissue. Note the multilayering of cells, aberrant position of myoepithelial cell and secretory vesicles, and lipid droplets at the basal aspect of epithelial cells and crenated basal lamina (*arrowheads*). B, Schematic presentation of mammary epithelium from Id2-null term tissue. One myoepithelal cell is interspersed between secretory cells, which are piled up. Lipid droplets (*dark circles*) are also found in the vicinity of the basal lamina (*arrowheads*). C, Micrograph of mammary tissue from wild-type mice at d 7 of pregnancy. Cells are densely packed. A distinct lumen has not yet formed, but they show signs of secretory differentiation with some lipid droplets. Myoepithelial cells are positioned at the periphery and are in contact with the basal lamina. D, Schematic presentation of micrograph. N, Nucleus; M, myoepithelial cell; lu, lumen.

the basal lamina was folded (Fig. 2, A and B). Based on the morphological similarity between Id2-null epithelium at term and developing alveolar structures in early pregnancy, we also investigated the ultrastructure of mammary tissue from wild-type mice on d 7 of pregnancy. At this stage, active alveologenesis and proliferation of alveolar cells take place and are associated with changes in the spatial arrangement of epithelial and myoepithelial cells. As shown in Fig. 2, C and D, the initiated alveoli were filled with densely packed secretory cells, and myoepithelial cells were located at the periphery closely associated with the basal lamina. The epithelial cells had large nuclei. Organelles indicative of active secretion were starting to form and in some cells lipid droplets were found in a basal position. Apoptotic cells were less frequent than in Id2-null epithelium. The cellular organization was regular and clearly distinct from Id2-null tissue. This indicates that the absence of Id2 causes perturbations in the establishment of normal tissue architecture and function and cannot simply be explained as an arrest of development at an early stage of pregnancy. Furthermore, we suggest that, in addition to inhibiting cell proliferation (9), Id2 is also involved in aspects of alveolar cell development and differentiation. Specifically, there appears to be a defect in the overall organization as evidenced by the disorientation of myoepithelial cells and the lack of proper polarization of organelles toward the lumen (Fig. 2, C and D). The mediators downstream of Id2 that control the establishment of an organized polarized epithelium and proper myoepithelium remain to be identified.

Expression of Ductal and Alveolar Cell Markers in Id2-Null Epithelium

The absence of Id2 resulted in a failure of the mammary epithelium to develop into organized secretory alveolar entities at parturition. However, it was not clear from the histological analyses whether the epithelial buds represented immature alveoli or merely ductal side branches. Furthermore, the abnormal cell organization in Id2-null epithelia suggested improper differentiation and expression of cell adhesion molecules. Immunohistochemical analysis with antismooth muscle actin antibodies demonstrated the presence of myoepithelium surrounding the ducts of wild-type mammary tissue in virgins (*green* fluorescence in Fig. 3B). The outer layer of myoepithelial cells in Id2-null ductal structures in the virgin was wider, thus confirming the difference observed in the electron microscope (Fig. 3A). A much broader ring of staining at the periphery of the ducts in Id2-null tissue at term indicated the presence of misshapen myoepithelial cells (Fig. 3C). In contrast, in lactating wild-type tissue, the myoepithelial cells were discontinuous and stretched around the expanding alveoli (Fig. 3D). Identical staining patterns for myoepithelial cells were observed with cytokeratin 5, which is expressed in all basal epithelial cells (*red* fluorescence in Fig. 3, E–H). To determine the cellular character of Id2-null epithelium, we examined the profile of NKCC1 protein. NKCC1 is a Na-K-Cl cotransporter, which is present at high levels on the basolateral membrane of ductal epithelial cells in the virgin (11). It is expressed at reduced levels during pregnancy and lactation and loss of expression serves as a marker for secretory alveolar differentiation (3, 4). Id2-null and wild-type epithelium isolated from virgin mice showed comparable levels of NKCC1 (*red* fluorescence in Fig. 3, A and B). Whereas no NKCC1 was detected in wild-type mammary epithelium at parturition (Fig. 3D), patches of expression were retained in Id2-null epithelium (Fig. 3C). These results suggest that Id2-null mammary epithelium failed to completely respond to pregnancy-mediated signals, and NKCC1 expression was only partially suppressed. Because the appearance of Id2-null tissue indicated a perturbation of cell-cell contacts, we analyzed the expression of β-catenin (*green* fluorescence in Fig. 3, E–H) and E-cadherin (*green* fluorescence in Fig. 4). No significant differences were observed between Id2-null and wild-type tissues from virgin and lactating stages. Furthermore, the morphology and frequency of organelles involved in cell adhesion such as junctional complexes and desmosomes were similar to wild-type tissue (data not shown).

Nuclear Localization of Stat5a in the Absence of Id2

Expression of Id2 is high during pregnancy and lower in virgin and lactating tissue (9), suggesting that it might be regulated by Stat5. Reduced activity of the transcription factor Stat5, as evidenced by band-shift assays, has been observed in Id2-null mammary tissue (9). The heterogeneity in alveolar cell differentiation revealed by NKCC1 staining prompted us to analyze Stat5a activity on a cellular level by immunofluorescence in Id2-null tissue at parturition. Stat5a was detected in the nuclei of most cells and clusters of cells in distinct regions displayed robust staining. These areas were located typically at the termini of ducts and at branch points (Fig. 4A). Similar strong nuclear Stat5a staining was observed in the majority of alveolar epithelial cells in control tissue at d 1 of lactation, a stage when they are fully stimulated by PRL (Fig. 4B). These results suggest that Id2-null epithelial cells, which are poised to form lobulo-alveolar structures, respond to pregnancy-mediated signals by activation of Stat5a. However, they fail to integrate these signals as evidenced by a lack of proliferation and functional differentiation.

The relationship between Stat5 and Id2 was further analyzed by semiquantitative RT-PCR using RNA from Stat5a/b-null and PrlR-null tissue (4). In both tissues, Id2 was expressed at similar levels as in wild-type tissue (data not shown), demonstrating that Id2 is not a downstream gene of the PrlR/Stat5 signaling pathway.

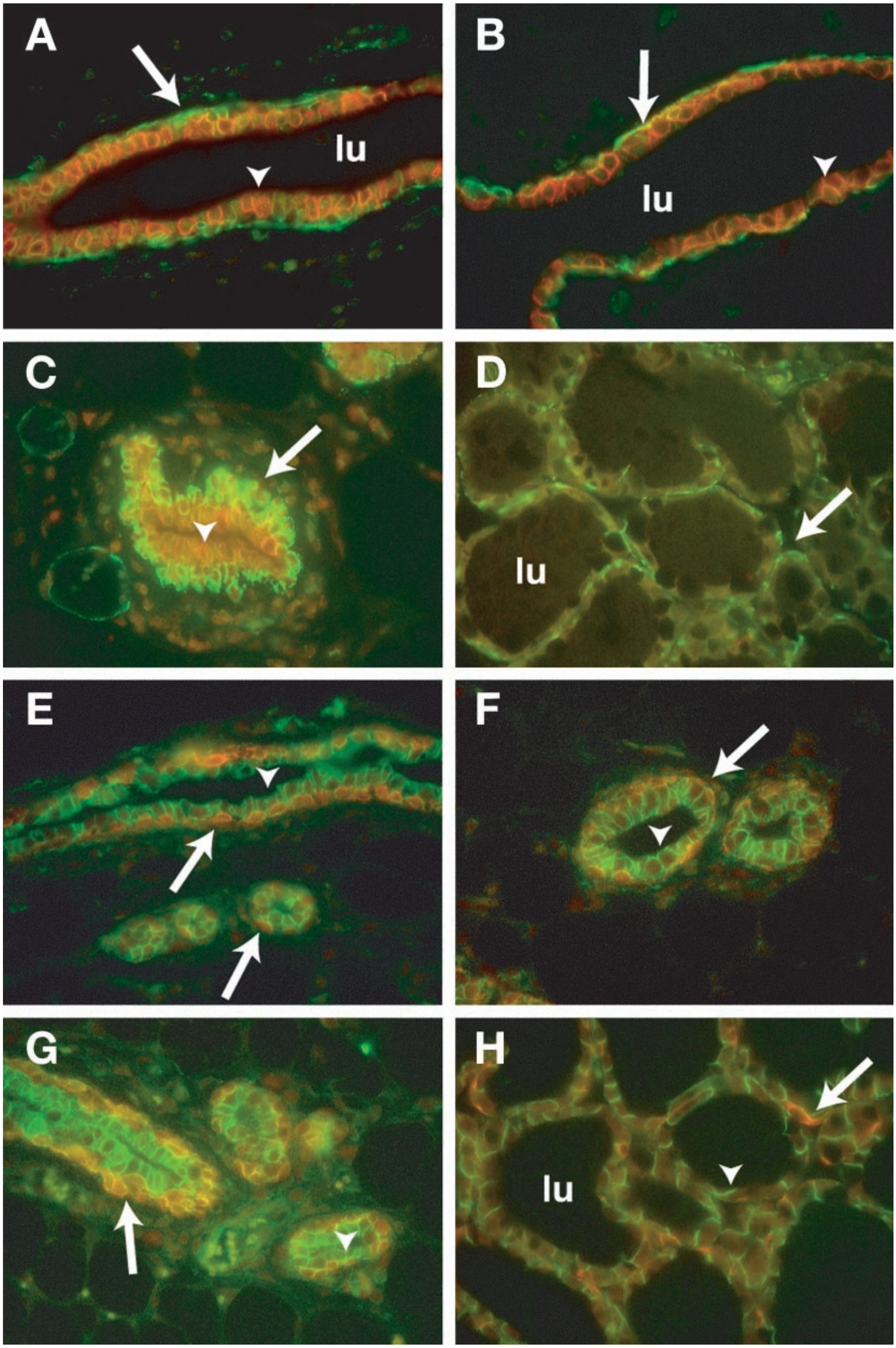

Fig. 3. Immunohistochemical Analyses of Id2-Null (A, C, E, G) and Wild-Type (B, D, F, H) Mammary Tissue

A–D, Antibodies against smooth muscle actin (*green*) and NKCC1 (*red*). The basal layer of myoepithelial cells (*arrow*) with smooth muscle actin immunoreactivity is more prominent in Id2-null tissue (A and C) than in wild-type tissue (B and D). NKCC1 staining of luminal cells (*arrowheads*) is similar in virgin tissues (A and B). At parturition, no NKCC1 staining is found in wild-type tissue (D), whereas patches of luminal cells maintain NKCC1 staining (*arrowhead*) (C). E–H, Antibodies against cytokeratin 5 (*red*) and β-catenin (*green*).

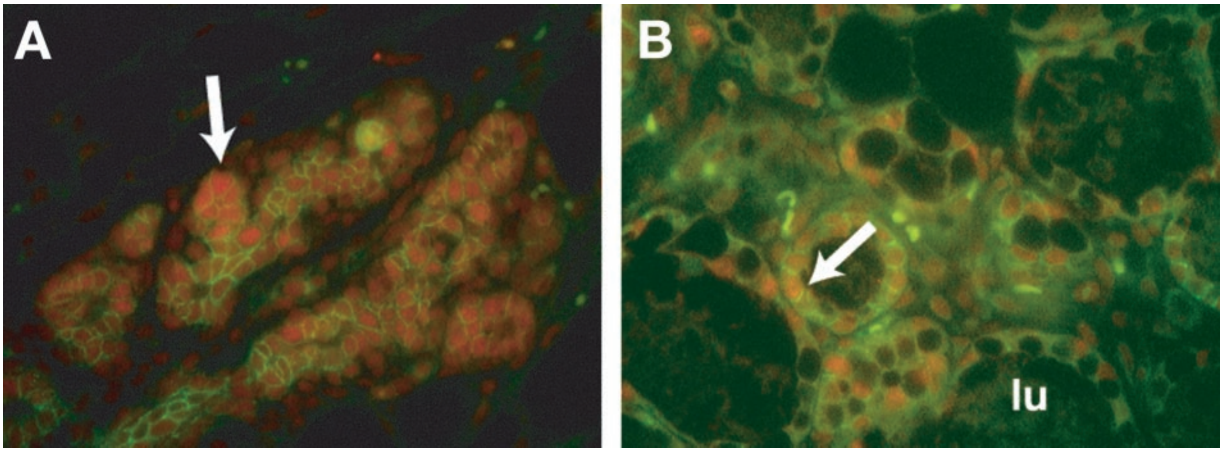

Fig. 4. Stat5a Expression in Id2-Null Mammary Epithelium at Parturition

Immunofluorescence analysis of Id2-null (A) and wild-type (B) mammary tissue harvested at parturition. Antibodies against Stat5a (*red*) and E-cadherin (*green*) were visualized. In Id2-null tissue, Stat5a (*arrows*) is abundant in alveolar like structures (A). Nuclear localization of Stat5a (*arrows*) is seen in the majority of alveolar cells in lactating wild-type tissue (B). E-cadherin is used to visualize cell membranes. lu, Lumen.

Id2-Null Epithelium Fails to Differentiate Fully during Pregnancy

In an attempt to gauge the differentiation status of Id2-null mammary tissue at parturition on a larger scale, we performed microarray analyses using a mammochip. The mammochip is a cDNA microarray enriched for genes expressed in mouse mammary tissues that was developed in our laboratory (see *Materials and Methods*). RNA prepared from wild-type virgin mammary tissue (wtV) was used as a common hybridization partner for RNA from wild-type lactating tissue (wtL) in one set of hybridizations and RNA from Id2-null (Id2-null) term tissue in the other. Differences in expression of more than 3-fold in at least one experiment were scored. A total of 217 of approximately 6000 cDNA clones fulfilled this criterion and were analyzed further. A total of 206 genes were differentially expressed between wtV and wtL mammary tissue, 199 genes were differentially expressed between wtL and Id2-null term transplants, and only 42 genes were differentially expressed between wtV tissue and Id2-null term transplants (Table 1). Furthermore, we identified a number of unknown genes (ESTs) that showed differential expression (Table 2). The full list of results including the signal intensities for both channels appears as supplemental data on The Endocrine Society's Journals Online web site (http://mend.endojournals.org).

Mori *et al.* (9) had observed reduced expression of genes encoding the milk proteins WDNM1, β-casein, WAP, and α-lactalbumin in mid-pregnant Id2-null tissue, but they did not evaluate term tissue. Similarly, reduction of Id2 expression in mammary epithelial tissue cultures was shown to inhibit β-casein expression (12). Table 1 shows four groups of known genes displaying the greatest differences in the microarray expression analysis organized according to their expression behavior. This allowed us to characterize the differentiation status of the Id2-null tissue at term. The results from the microarrays not only confirmed the data by Mori *et al.* (9) but extended the analysis to a greater number of milk proteins and new differentiation markers that are activated at the end of pregnancy. The first group contains genes whose transcription was induced in wild-type tissue at parturition and to a lower extent in Id2-null tissue. This group contains β- and κ-casein and WDNM1, mammary specific genes whose expression increases early in pregnancy (13, 14). The second group encompasses genes that exhibited high expression in wild-type term tissue compared with virgin tissue but were not upregulated in the Id2-null term tissue. The genes in this group represent differentiation specific molecules whose expression is not elevated in the absence of Id2. In contrast to β- and κ-casein and WDNM1, we determined that other milk protein genes (α-, δ-, γ-caseins, WAP) were highly expressed only in wtL tissue, but not in Id2-null tissue (group 2). The expression pattern of the different caseins serves as indicator for the differentiation status as these genes are activated in a distinct sequence during development (13). From this expression profile, we conclude that Id2-null tissue at term exhibits a differentiation status equivalent to wild-type tissue in early pregnancy. The expression

Staining of myoepithelial cells (*arrow*) with cytokeratin 5 demonstrates the irregular layering of these cells in Id2-null tissue (E and G) compared with wild-type tissue (F and H) in virgin (E and F) and lactating tissue (G and H). No differences in β-catenin immunoreactivity are seen between Id2-null (E and G) and wild-type (F and H) tissue (*arrowhead*). lu, Lumen.

Table 1. Known Genes with Greatest Expression Differences

		wtL/wtV	Id2-null/wtV
Group 1: Differentiation-Specific Genes Weakly Induced in Id2-Null			
IMAGE:1314775	*Casein β*	*133.46*	*11.43*
IMAGE:875031	*Casein κ*	*104.05*	*4.43*
IMAGE:832158	*Extracellular proteinase inhibitor (WDNM1)*	*21.33*	*2.28*
Group 2: Differentiation-Specific Genes Not Induced in Id2-Null			
IMAGE:851207	Arsenate resistance protein 2	133.38	No diff
IMAGE:1511486	*Whey acidic protein*	*128.46*	″
IMAGE:874488	*Casein α*	*117.02*	″
IMAGE:1054751	*Casein γ*	*99.48*	″
IMAGE:1400768	*RAB18, member RAS oncogene family*	*55.61*	″
IMAGE:1511509	PDZ protein interacting specifically with TC10	48.12	″
IMAGE:1511382	*Casein Δ*	*34.92*	″
IMAGE:1400441	E74-like factor 5	14.82	″
IMAGE:1510567	Serum amyloid A 3	13.93	″
IMAGE:891142	Lysosomal acid lipase 1	9.16	″
Group 3: Genes Expressed Preferentially in Virgin Mammary Tissue			
IMAGE:1247588	*Adipocyte complement related protein of 30 kDa*	*0.13*	″
IMAGE:862835	Short stature homeobox 2	0.13	″
IMAGE:862840	Interferon consensus sequence binding protein	0.13	″
IMAGE:851374	*Stearoyl-coenzyme A desaturase 1*	*0.14*	″
IMAGE:1314739	*Carbonic anhydrase 3*	*0.14*	″
IMAGE:832109	*Resistin*	*0.15*	″
IMAGE:874166	*Fat specific gene 27*	*0.16*	″
IMAGE:850835	Small inducible cytokine subfamily B, member 15	0.16	″
IMAGE:1448821	*Fatty acid binding protein 4, adipocyte*	*0.17*	″
IMAGE:1348345	Follistatin-like 3	0.19	″
IMAGE:1247911	Ewing sarcoma homolog	0.20	″
IMAGE:1247470	*Tnfa-induced adipose-related protein*	*0.20*	″
Group 4: Genes Specifically Regulated in Id2-Null Tissue			
IMAGE:1178689	Cartilage glycoprotein-39	No diff	4.38
IMAGE:831668	*Retinoid binding protein 7, cellular*	″	*5.83*
IMAGE:1398011	Guanine nucleotide binding protein (G protein), γ 10	″	0.17
IMAGE:1313822	Sterol regulatory element binding factor 1	No diff	0.29

The expression ratios for wild-type mammary tissue from lactating and virgin mice (wtL/wtV) and Id2-null mammary tissue at parturition and wild-type virgins (Id2-null/wtV) are shown. The results are the means of three hybridizations with duplicate spots on each array. For additional information, see the supplemental data for this article on The Endocrine Society's Journals Online web site (http://mend.endojournals.org). Genes in group 1 are highly expressed in wild-type lactating but not in wild-type virgin tissue, and they are induced in Id2-null term tissue. Group 2 contains genes highly expressed in wild-type lactating tissue but not in wild-type virgin or Id2-null term tissue. Group 3 contains genes highly expressed in wild-type virgin tissue and Id2-null tissue but not in wild-type lactating tissue. Group 4 contains genes potentially regulated by Id2. Genes whose function can be related to the differentiation status of the tissue or are discussed in the paper are *italicized.*

pattern of Rab18, a small guanosine triphosphatase, might explain our histological findings of a lack of directed secretion in Id2-null cells. Rab18 is involved in the regulation of vesicular transport and is preferentially expressed in polarized epithelia of renal proximal tubules and intestine (15). Interestingly, Rab18 is not expressed in wtV samples and Id2-null term tissue. The third group of genes was more highly expressed in wtV and Id2-null term tissue compared with wild-type term tissue. The majority of these genes, which include stearoyl-coenzyme A desaturase 1, carbonic anhydrase 3, and fatty acid binding protein 4, are associated with adipocyte differentiation. This expression pattern reflects the ratio between epithelium and stroma in virgin and Id2-null term tissue compared with wtL tissue. The expression of the fourth group of genes was altered in Id2-null term tissue but not in wild-type tissue, suggesting that they are directly or indirectly regulated by Id2. Activity of these genes was either induced (cartilage glycoprotein-39, retinoid binding protein 7) or suppressed [sterol regulatory element-binding protein (SREBP-1), malic enzyme, G protein]. Retinoid binding protein 7 was expressed strongly in Id2-null term tissue, but was low in wtV and wtL tissue. Its expression in mammary tissue is developmentally regulated. It is higher in male mammary tissue than in nonpregnant female mammary tissue but is up-regulated during pregnancy (16). This is yet another indicator that Id2-null tissue at term differentiates to some degree and expresses some of the

Table 2. Unknown Genes Differentially Expressed in Id2 Null Compared with Wild-Type Virgin Tissue

		wtL/wtV	Id2 KO/wtV
Group 1: Differentiation-Specific Genes Weakly Induced in Id2 Null			
IMAGE:1245783	ESTs, Weakly similar to ZF95_MOUSE ZINC FINGER PROTEIN 95 (ZFP-95) (*M. musculus*)	123.72	10.38
IMAGE:1384161	ESTs	45.42	4.58
Group 2: Genes Specifically Regulated in Id2 Null Tissue			
IMAGE:1383397	Unknown	No diff	3.48
IMAGE:1246189	ESTs	"	3.25
IMAGE:1349720	Expressed sequence AW554339	"	0.32
IMAGE:1430633	*Mus musculus*, similar to myosin regulatory light chain interacting protein	"	0.31
IMAGE:1396547	Unknown	"	0.30
IMAGE:1247491	RIKEN cDNA B430104H02 gene	"	0.30
IMAGE:820307	Expressed sequence AI158848	"	0.29
IMAGE:1248075	RIKEN cDNA 2700018N07 gene	"	0.29
IMAGE:947659	ESTs	"	0.25
IMAGE:863633	RIKEN cDNA 2310016K04 gene	"	0.24
IMAGE:948774	Unknown	"	0.19
IMAGE:1383196	Expressed sequence AI118577	"	0.19
IMAGE:1195295	ESTs	"	0.18
IMAGE:891096	Expressed sequence AW538652	"	0.17
IMAGE:964092	RIKEN cDNA 6330417C12 gene	"	0.13
IMAGE:873874	RIKEN cDNA 2810413P16 gene	"	0.11

List of gene sequences whose identity is unknown. These genes are ESTs selected from mammary gland libraries as described in *Materials and Methods.*

genes that increase during pregnancy. Furthermore, expression of the adipocyte determination and differentiation factor 1/SREBP-1 gene was reduced in Id2-null tissue. Id2 and Id3 have been shown to interact directly with adipocyte determination and differentiation factor 1/SREBP-1, which controls the expression of several genes involved in adipose metabolism, and inhibits its DNA binding and transcriptional activity in adipocytes (17). The low expression could be a consequence of the absence of the inhibitory Id2 partner.

Conclusions

Inactivation of the PrlR/Jak2/Stat5 pathway and Id2 leads to overtly similar defects in pregnancy-mediated mammary gland development, in particular the lack of functional alveoli. However, Id2 expression is not dependent on Stat5 and Id2 and Jak2/Stat5 may signal through parallel pathways, which are functionally dependent upon each other. Nuclear Stat5 is detected in Id2-null cells at ductal termini and branch points. This correlates with the expression of some genes that are characteristic for early pregnancy and indicates that Stat5 expression is not sufficient to execute alveolar development in Id2-null epithelia. Alternatively, Id2 may be required to execute signals downstream of other mammary differentiation factors such as CCAAT/enhancer-binding protein (C/EBP)β whose absence causes a similar defect in alveologenesis (18). Lack of these signals prevents the alveolar compartment to be formed, possibly because the cells never acquire the correct specification. Mori *et al.* (9) have demonstrated a requirement of Id2 in epithelial cell proliferation in early pregnancy, a process that precedes alveolar cell differentiation. Our data demonstrate an additional function for Id2 in alveolar cell specification during pregnancy similar to its role in cell fate decision making in the hematopoietic system, where it has been shown to commit the fate of bipotent progenitor cells to the natural killer (NK) cell lineage (10, 19, 20).

MATERIALS AND METHODS

Experimental Animals

Id2-null mice were in the mixed background (129/Sv x NMRI) and have been described previously (9, 21). Mice used for the recipients of transplants were 3-wk-old female athymic nudes (nu/nu). Twenty transplanted mice were used for the analysis. The animals were treated humanely and surgical procedures were performed according to the Animal Use and Care protocol.

Antibodies

The NKCC1 antibodies (22) were kind gifts from Dr. Jim Turner (NIDCR, NIH). The E-cadherin and β-catenin antibodies were purchased from Transduction Laboratories (Lexington, KY). Cytokeratin 5, smooth muscle actin, and Stat5a antibodies were obtained from Babco (Richmond, CA), Sigma (St. Louis, MO), and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively.

Transplantation

The transplantation technique has been described previously (23). In brief, small pieces (1 mm) of mammary tissue were excised from the donor mice (mature Id2-null or wtV). The recipient nude mice were anesthetized with avertin. The proximal part of the inguinal mammary gland containing the mammary epithelium was surgically removed and pieces of Id2-null or wild-type mammary tissue were inserted into a small pouch in the center of the epithelial-free fat pad. Id2-null and Id2 wild-type glands were contralaterally transplanted in the same mouse. Eight weeks after transplantation, the transplanted mammary tissue was harvested for virgin mammary tissue, or mice were mated and harvested the day after parturition.

Whole Mount Analysis and Histological Analysis

Mammary glands were fixed overnight at room temperature in Carnoy's fixative (6 parts 100% ethanol, 3 parts chloroform, 1 part glacial acetic acid) and stained in carmine alum (0.2% carmine alum, 0.5% aluminum potassium sulfate, 1 crystal of thymol). After dehydration and clearing in Histoclear (National Diagnostics, Atlanta, GA), the samples were permanently mounted (Permount, Fisher Scientific, Pittsburgh, PA). For histological analyses tissues were fixed in Tellyesniczky's fixative (7 parts 100% ethanol, 0.5 parts formaldehyde, 0.5 parts glacial acetic acid) for 4 h at room temperature. After fixation, tissues were placed in 70% ethanol, dehydrated, cleared in xylene, embedded in paraffin, and sectioned at 5 μm. Hematoxylin and eosin staining was performed by standard procedures.

Electron Microscopy

Electron microscopic analysis was described previously (4). Tissues were fixed in 3.8% paraformaldehyde and 0.025 glutaraldehyde in PBS. After postfixation in 2% osmium tetroxide in 0.5 M sodium cacodylate buffer, they were dehydrated and embedded in plastic. Ultrathin sections were contrasted with Karnovsky's lead hydrate and uranyl acetate and examined with a JEOL 1010 electron microscope.

Immunofluorescence

Paraffin-embedded sections were deparaffinized and rehydrated through an alcohol series to PBS. Antigen retrieval was performed by boiling in antigen unmasking solution (Vector Laboratories, Inc., Burlingame, CA) for 2 min. Sections were subsequently blocked in PBS containing 3% normal horse serum for 30 min. Primary antibodies were applied (cytokeratin 5, 1: 200; β-catenin, 1:100; smooth muscle actin, 1:1000; NKCC1, 1:1000; Stat5a 1:100; E-cadherin, 1:200), and the sections were incubated for 1 h at 37 C (cytokeratin 5 and β-catenin, smooth muscle actin and NKCC1) or overnight at 4 C (Stat5a and E-cadherin). After rinsing in PBS, the sections were incubated with fluorescence-conjugated secondary antibodies (Molecular Probes, Inc., Eugene, OR) for 30 min at room temperature in the dark. The samples were mounted in Vectashield (Vector Laboratories, Inc.) and visualized with a Carl Zeiss (Jena, Germany) Axioscop equipped with filters for fluorescein isothiocyanate, tetramethyl rhodamine isothiocyanate and fluorescein isothiocyanate:tetramethyl rhodamine isothiocyanate. Images were acquired using a Sony (Tokyo, Japan) DKC-5000 digital camera.

Microarrays Analyses

Around 6,000 unique sequences were selected as highly enriched in mammary libraries from two libraries comprising a total of 52,000 sequences: Soares NbMMG Genbank ID: 403 9 (virgin) and Soares NMLMG Genbank ID: 636 (lactating). Each sequence was spotted at least twice on glass slides to generate a 12.9-K cDNA array mammochip: Mm-M-v1p1 (Advanced Technology Center, NCI, NIH), which covers about 18% of known genes, and 82% of unknown genes. The list is accessible at http://nciarray.nci.nih.gov/gal_files/gal_custom.html.

RNA was prepared from mammary tissue from wtV and wtL d 1 and from Id2-null transplants at parturition using TRIzol extraction (Life Technologies, Inc., Gaithersburg, MD) followed by two ethanol precipitations. For fluorescence labeling, 20 μg of total RNA were reverse transcribed in the presence of 300 U of SS II (Life Technologies, Inc.) and labeled with either cy3-deoxyuridine triphosphate or cy5-deoxyuridine triphosphate (NEN Life Science Products, Boston, MA). Samples were combined, purified and concentrated with YM30 Microcon columns (Millipore Corp., Bedford, MA). Slides were prehybridized for 1 h and hybridized overnight at 42 C, in 25% formamide. They were washed as described (24) and scanned on a GenePix 4000A scanner (Axon Instruments, Union City, CA). The images were analyzed by GenePix Pro 3.0 (Axon Instruments). Each experiment was repeated three times, once with reversing the labeling nucleotide. The data were deposited in the NCI-CIT microarray database and normalized data were analyzed with the included mAdb tools with some modifications: the differentially expressed genes were selected by signal intensity of 5-fold over background in at least one channel and an expression ratio of 3 or higher in at least one experiment. These criteria had to be met also in the reversed-labeled experiments.

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Address all correspondence and requests for reprints to: Gertraud Robinson, Laboratory of Genetics and Physiology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Building 8, Room 101, 8 Center Drive, Bethesda, Maryland 20892-0822. E-mail: traudl@nih.gov.

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